Title: Effect of a mutagenized acyl-ACP thioesterase FATA allele from sunflower with improved activity in tobacco leaves and Arabidopsis seeds.

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Running title: sunflower acyl-ACP thioesterase mutant allele and its expression in sink and green tissues

Abstract

Acyl-acyl carrier protein (ACP) thioesterases hydrolyze plastid-localised acyl-ACP intermediates and make possible the export of acyl moieties to the cytosol for their incorporation into glycerolipids. The substrate specificity of the acyl-ACP thioesterases importantly determine the type of fatty acids that are exported from plastids. Thus, designing acyl-ACP thioesterases with different substrate specificities or kinetic properties would be of interest for plant lipid biotechnology to produce oils enriched in specialty fatty acids. Although they have not been yet crystallized, their tertiary structures have been modelled by comparison with other enzymes catalyzing similar reactions, suggesting candidate amino acid residues involved in substrate specificity. In the present work, the FatA thioesterase from *Helianthus annuu*s was used to test the impact of changes in the amino acids present in the binding pocket on substrate specificity and catalytic efficiency. Amongst all the mutated enzymes studied, Q215W was especially interesting as it had higher specificity towards saturated acyl-ACP substrates and higher catalytic efficiency compared to wild type *H. annuu*s FatA. Null, wild type and high-efficiency alleles were transiently expressed in tobacco leaves to check their effect on lipid biosynthesis. Expression of active FatA thioesterases altered the composition of leaf triacylglycerols but did not altered total lipid content. However, the expression of the wild type and the high efficiency alleles in *Arabidopsis thaliana* transgenic seeds resulted in a strong reduction in oil content and an increase in total saturated fatty acid content. The role and influence of acyl-ACP thioesterases in plant metabolism and their possible applications in lipid biotechnology are discussed.

Keywords: Acyl-ACP thioesterase, *Arabidopsis thaliana*, fatty acids, *Helianthus annuus*, tobacco, transient expression, triacylglycerols.

Introduction

Plant *de novo* fatty acid biosynthesis takes place in chloroplasts and in non green plastids. This process demands carbon chains in the form of pyruvate or glycolytic intermediates imported from the cytosol, and reducing equivalents in the form of NADPH (Rawsthorne 2002). Fatty acids are synthesized by successive elongations of acyl-ACP derivatives by the action of the fatty acid synthase fully dissociated enzyme complexes (Ohlrogge and Jaworski 1997). In oilseeds accumulating triacylglycerols (TAGs), the process of acyl-acyl-carrier-protein (acyl-ACP) elongation is terminated by acyl-ACP thioesterases that hydrolyze the thioester bond of those derivatives releasing a free fatty acid and free holo-ACP moieties (Ohlrogge and Browse 1995). Fatty acids are then transported to the cytosol and esterified to coenzyme A (CoA) by the action of acyl-CoA synthetases present in the different organelles of the cell (Shockey et al. 2002) and incorporated into glycerolipids via the Kennedy pathway. In this regard, there is evidence indicating that the substrate specificity of acyl-ACP thioesterases broadly determines which fatty acids are exported from plastids for glycerolipid synthesis and so they have an important contribution to the fatty acid composition of the oil accumulated in triacylglycerols in seeds. The thioesterases most commonly found in plants are of the FatA and FatB1 types (Jones et al. 1995). FatA displays high specificity towards oleoyl-ACP, the precursor of most unsaturated C18 to C22 fatty acids found in oilseeds (Dörmann et al. 1994; Salas and Ohlrogge 2002). FatB1 displays high specificity for the hydrolysis of palmitoyl-ACP and contributes significantly to the accumulation of saturated fatty acids (Jones et al. 1995; Bonaventure et al. 2003). Furthermore, species accumulating oils with unusual fatty acids often express acyl-ACP thioesterases with altered specificity. Some tropical species that accumulate fats containing high levels of short and medium chained fatty acids (C8 to C14) possess thioesterases of the FatB2 type, which effectively intercept and hydrolyse the elongating acyl-ACP chain to produce short and medium chain fatty acids (Dehesh et al. 1996; Voelker et al. 1997). Thioesterase specificity is not limited to acyl chain length; the accumulation of the unusual monoene petroselinic acid in *Coriandrum sativum* seeds is supported by a specialised acyl-ACP thioesterase to maintain high rates of export of the fatty acid from the plastid (Dörmann et al. 1994). These observations have made acyl-ACP thioesterases of a great interest in the field of plant lipid biotechnology. For example, the expression of a thioesterase of the FatB2 type in canola (*Brassica napus*) gives place to high laurate canola (Voelker et al. 1992). Moreover expression of FatAs

modified to increase their activity towards saturated fatty acids increased the content of stearic acid in canola transformants (Facciotti et al. 1999).

Oilseed genetic engineering is an important tool for the production of modified food oils and speciality oils containing unusual fatty acids for oleochemical industries (Ohlrogge 1994). Thus, designing and engineering new enzymes with new substrate specificities is of a great interest in this field. The most detailed studied enzymes involved in acyl-ACP modification are the acyl-ACP desaturases. An acyl-ACP desaturase was cloned from castor seeds, the protein crystallized and its structure elucidated by X-ray crystallography (Lindqvist et al. 1996). The data indicated which amino acids were involved in the hydrophobic pocket determining specificity and so it was possible to produce new engineered forms with altered substrate specificity without losing catalytic efficiency (Cahoon et al. 1998, 2000). There are no crystallographic data for plant acyl-ACP thioesterases but it was possible to construct a model of the tertiary structure by homology to the *Escherichia coli* 4-hydroxybenzoyl-CoA thioesterase. This model was consistent with previous research on the specificity of these enzymes, which were altered by changes in different protein residues and domain swapping (Yuan et al. 1995, 1996; Salas and Ohlrogge 2002). It is hypothesized that the active site of these enzymes displays a hot-dog fold pattern, with a catalytic triad of amino acids similar to that in papain (Mayer and Shanklin 2005; Serrano-Vega et al. 2005). Furthermore, the regions interacting with the substrate were identified, including a hydrophobic pocket that was a clear candidate in determining substrate specificity (Serrano-Vega et al. 2005; Mayer and Shanklin 2007).

In the present work modified versions of the acyl-ACP thioesterase FatA from sunflower (*Ha*FatA) were used to evaluate the effect of amino acid changes in its hydrophobic pocket. FatA are dimeric enzymes, encoded by nuclear genes, which are posttranslationally imported into plastids, involving the removal of an N-terminal transit peptide. *Ha*FatA is expressed at high levels in sunflower seeds and has higher catalytic efficiencies than other FatAs from plant species previously described (Serrano-Vega et al. 2005). Alterations in the binding pocket were affected by the exchange of selected amino acid residues with tryptophan which is hypothesized to interfere with pocket size and shape. Among the different mutations assayed the change Q215W provoked a general increase in the catalytic efficiency of the enzyme and T182W the loss of activity. The effect of this enzyme on lipid metabolism was studied by transient expression of wild type (WT) *Ha*FatA, T182W null mutant and Q215W mutant in

tobacco leaves and in transgenic Arabidopsis plants. The impact of the expression of these thioesterases on lipid metabolism of tobacco leaves and Arabidopsis seeds is discussed.

Materials and Methods

Biological material

Helianthus annuus and *Nicotiana benthamiana* plants were cultivated in growth chambers at 25 ºC/15 ºC (day/night cycles), with a 16 h photoperiod and a photon flux density of 300 μmol m^{-2} s^{-1}. Surface-sterilized *Arabidopsis thaliana* seeds (ecotype Columbia (Col-0)) were cold-treated at 4ºC and imbibed in the dark for 4 days on 1% phytoagar plates containing 1% sucrose and 0.5 MS media. The seeds were then germinated and grown in soil at 20ºC under a 16h day/8h night photoperiod. The *E. coli* strain XL1-Blue (Stratagene) was used as host for the expression of *Ha*FatA alleles and the *Agrobacterium tumefaciens* strain GV3101 was used for agroinfiltration and production of transgenic plants. Bacteria were grown at 37ºC in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7) with the appropriate antibiotic addition. All liquid cultures were growth with vigorous shaking.

FatA site-directed mutagenesis

The *HaFatA* coding sequence (Accession number: AY078350; Serrano-Vega et al. 2005) without signal peptide, was cloned into pQE-80L expression vector (Quiagen) using primers with internal *Sac*I and *Kpn*I restriction sites (SacI-FatA: 5'-GAGCTCATGGCGGTGAAGGTTGATGAGCAGC-3'; KpnI-FatA: 5'-GGTACCTTTTTTTGCGGGTTTTTTCCTCCATTCAGTGCG-3'). The construct was designated pQE-FatA. Several point mutations in the *FatA* gene were carried out, pQE-FatA construct and the QuickChange Site-Directed Mutagenesis Kit (Stratagene) were used for this objective. The amino acids mutated were: L118, T172, T182, R184, M206 and Q215. All of them were mutated to tryptophan using the following primers in the mutagenesis: L118W, 5'-GAGACGATTGCGAATCTGTGGCAGCAGGAGGTAGGAGGAAATCATGC-3'; T172W, 5'-GCGATGTGGTGGAAATTGAGTGGTGGTGTCAAGGTGAAGGG-3'; T182W, 5'-GTTGAAGGGAGAATCGGGTGGAGACGTGATTGGATTATC-3';R184W, 5'-GGGAGAATCGGGACTAGATGGGATTGGATTATCAAAGATC-

3';M206W, 5'-GCTACAAGCAAGTGGGTGTGGATGAACTCAGAAACTAGAAGAC-3';Q215W, 5'-CTCAGAAACTAGAAGACTCTGGAAAGTCAATGACGATATAAGAG-3. The mutated nucleotides are underlined.

Protein expression and purification

Five hundred milliliter cultures of *E. coli* XL1-Blue cells harboring pQE-FatA were grown as described above. Expression of *Ha*FatA alleles were induced at OD600 0.6 by adding 1 mM IPTG and growth for 4 h. The cells were harvested by centrifugation for 10 min at 2500g, washed with distilled water and resuspended in 5 ml of Binding buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4). Cells were lysed adding 0.5% Triton X-100, 5 mM DTT, 1 mM $MgCl_2$, 1 mM PMSF, 20 µg/ml DNase I (Roche) and 0.2 mg/ml lysozyme (Roche) for 45 min at 4 ºC with shaking. The resulting lysed cell suspension was centrifuged at 25000g for 30 min. The supernatant was filtered through 0.22 µm filters and loaded onto a HisTrap FF 1 ml column (GE Healthcare) interfaced with an Äktaprime system (Amersham Bioscience). Histidine-tagged proteins were eluted from the column using an imidazole gradient ranging from 20 to 500 mM. Fractions containing *Ha*FatA were subsequently used for thioesterase activity assays.

Gel electrophoresis of proteins

Protein samples were combined with SDS-PAGE loading buffer and heated at 95 ºC for 5 min. The samples were separated by electrophoresis on 4–12% NuPAGE Novex Bis-Tris gels in MES buffer (Invitrogen), and the gels were fixed and stained with 0.1% Coomassie R-250 in 40% ethanol, 10% acetic acid. Molecular weight standards used were obtained from GE Healthcare.

Preparation of acyl-ACP substrates

Labeled acyl-ACP substrates were prepared using a recombinant acyl-ACP synthetase from *E. coli*. Acylation reactions contained 50 µg of recombinant ACP from *E. coli* (Sigma), 180 kBq (approx. 0.1 µmol) of [1-^{14}C] fatty acid ammonium salt ([^{3}H] fatty acid in the case of 16:1Δ9), 5 mM ATP, 2 mM DTT, 400 mM $LiCl_2$, 10 mM $MgCl_2$, 100 mM Tris-ClH pH 8.0 and 10 µg acyl-ACP synthetase, in a final volume of 0.5 ml. Reactions were carried out at room temperature for 3 h and the acyl-ACPs were purified

and concentrated by ion exchange chromatography on DEAE-sepharose as described by Rock and Garwin (1979).

Acyl-ACP thioesterase assays

Thioesterase activity was assayed in 0.1 ml reactions containing 50 mM Tris-HCl pH 8.0, 5 mM DTT, 0.2-12 µg of the protein preparation and the amount of acyl-ACP substrate ranging from 0.02 to 0.08 nmol (30-170 Bq approx.). Reactions were carried out at room temperature for 5 min and stopped by the addition of 0.25 ml of 1 M acetic acid in 2-propanol. Unesterified fatty acids were then extracted twice with 0.3 ml hexane, and the radioactivity in the pooled organic phase was determined in a calibrated liquid scintillation counter (Rackbeta II; LKB). The data from thioesterase assays was fitted to the Hill equation by nonlinear least-squares regression analysis using OriginPro 8 software, and correlated at $P<0.005$ as determined by Student's t-test. Both the V_{max} and K_m were derived from these curves.

RNA preparation and cDNA synthesis

Approximately 0.25 g of tobacco or *Arabidopsis* leaves were ground in liquid nitrogen using a precooled sterile mortar and pestle. Total RNA was isolated using the RNeasy Mini Kit (Quiagen). RNA samples were treated with DNAseI (Promega) and this DNA-free RNA (1µg) was retrotranscribed with oligo(dT) primer and SuperScript II RT (Invitrogen).

Transient expression in tobacco

For tobacco-transient expression, *HaFatA* complete cDNAs corresponding to wild type allele and both T182W and Q215W mutants were amplified by PCR and cloned into pENTR Gateway vector (Invitrogen). Then, were transferred into the Gateway-compatible binary vector pB2GW7 (*Ha*FatA wt, T182W and Q125W cDNAs) (Karimi et al. 2002). The 35S:p19 viral suppressor construct and the agroinfiltration procedure were carried out according to Voinnet et al. (2003) omitting the acetosyringone treatment. Disk were punched from tobacco leaves 4 days after *Agrobacterium* infiltration and analyzed.

Arabidopsis thaliana transformation

pB2GW7-*Ha*FatAwt, pB2GW7-T182W or pB2GW7-Q125W constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed into *Arabidopsis* Col-0 plants by floral dip (Clough and Bent 1998). Seeds from the transformed plants were selected with 25 mg L^{-1} BASTA (glufosinate ammonium). Genomic DNA and cDNA from third-generation plants were extracted and used for genetic and expression analysis. PCR-positive plants were selected and their seeds were used for lipid analysis

Lipid analysis

Tobacco leaves or *Arabidopsis* seeds were harvested and ground for lipid analysis. Fatty acid composition was determined via acid-catalysed transmethylation and gas-chromatography with flame ionization detection (GC8000 Top, Thermoquest Separation Products, Manchester, UK), fitted with a 30 m long 0.25 mm ID SGE BPX70 column (SGE, Milton Keynes, UK). Helium was used as a carrier gas at 1 ml min^{-1} with a 30:1 split ratio. The oven was run isothermally at 110ºC for 1 min, then ramped to 180ºC at 20ºC min^{-1} then to 221ºC at 2.5ºC min^{-1} (Larson and Graham 2001). The pool of acyl-CoAs was determined by HPLC using a modified version of the method of Larson and Graham (2001; Larson et al. 2002), and neutral lipids were analyzed by LC/MS/MS according to Burgal et al. (2008).

Results and discussion

HaFatA site-directed mutagenesis

Sunflower acyl-ACP thioesterase cDNA (*HaFatA*) has been previously cloned and characterized and is probably the dominant form measurable in crude extracts from sunflower seeds (Serrano-Vega et al. 2005). Using the acyl-ACP thioesterase structural models that have been previously developed (Mayer and Shanklin 2005; Serrano-Vega et al. 2005) and bioinformatic tools was possible to identify amino acid residues candidates to be involved in substrate specificity of these proteins. In the case of *Ha*FatA, we identified a region from amino acid residue 80 to 228 that generates a hydrophobic pocket and includes residues that have been demonstrated to be involved in determining substrate specificity of thioesterase FatA1 from *Garcinia mangostana* (Facciotti et al. 1999), FatB from *Umbellaria californica* (Yuan et al. 1995) and in the FatB from *Arabidopsis thaliana* (Mayer and Shanklin 2007). Moreover, this region has also been identified in experiments with chimeric constructs as being involved in substrate specificity (Facciotti and Yuan 1998; Salas and Olhrogge 2002). The position and orientation into the hydrophobic pocket were the criteria used to select the amino acids Leu 118 (L118), Thr 172 (T172), Thr 182 (T182), Arg 184 (R184), Met 206 (M206) and Gln 215 (Q215). All of them have the lateral residues orientated towards the inner face of the pocket. T182 and R184 are situated in the deepest region; L118, T172 and M206 are in the intermediate region; and Q215 is in the most exterior zone of the pocket. These amino acids were mutated to tryptophan which is an aromatic amino acid with potential to modify the interaction of the enzyme with the acyl-ACP substrates. Figure 1 shows the structural model of the hydrophobic pocket region described above indicating those residues subjected to mutagenesis.

Heterologous expression in E. coli and biochemical characterization

*Ha*FatAs are expressed at a high level in the soluble fraction of recombinant *Escherichia coli*, can be efficiently recovered in the supernatant from lysed cells and subsequently purified by immobilized metal affinity chromatography (IMAC) (Serrano-Vega et al. 2005). When the WT *Ha*FatA was modified by site-directed mutagenesis and expressed in *E. coli,* only selected alleles gave recoverable protein. Only the mutant alleles L118W, T182W, M206W and Q215W were purified using the above mentioned protocol. The others produced toxic effects or were targeted to inclusion bodies (data not shown). In SDS-PAGE gel analysis, a single-protein band of about 37

kDa was observed, corresponding to the mature protein without the signal peptide (His-FatA). These data were in agreement with predicted mass and previous purification data (Serrano-Vega et al. 2005).

Kinetic studies were carried out using purified proteins and substrate specificity was assayed with the 16:0-ACP, 18:0-ACP, 16:1-ACP and 18:1-ACP substrates (Table 1). The modified *Ha*FatA forms displayed higher catalytic efficiencies (k_{cat}/K_m) towards unsaturated substrates than for saturated ones, similar to WT *Ha*FatA. In absolute terms all mutants displayed lower catalytic activities compared to WT *Ha*FatA except for Q215W, which had equivalent or improved activities towards all substrates with marked 2-fold and 2.6-fold increased activities for palmitoleoyl-ACP and palmitoyl-ACP, respectively, compared to WT *Ha*FatA. In contrast, the T182W mutant displayed catalytic activity values that were three orders of magnitude lower than those of WT *Ha*FatA. As a general rule the K_m values of the mutants assayed remained similar to those of WT form, except for the lower affinity on 18:1 shown by mutant M206W, but with generally lower V_{max}. Q215W and T182W were selected for further studies as improved and deficient alleles of *Ha*FatA, respectively.

Transient expression in tobacco leaves

Methods for stable transformation of plants are time-consuming and expensive, although they are necessary for the production of transgenic crops. However, transient expression is a useful way to obtain rapid information about the functionality of genes *in planta*. The transient expression of transgenes in leaves was first introduced by Kapila et al. (1997). With this technique, nuclei of permissive cells (Zipfel et al. 2006) are transformed via infiltration of abaxial air-spaces with *Agrobacterium* cultures harbouring expression constructs within T-DNA borders. Expression of transgenes in leaves is significantly enhanced by the co-infiltration of the viral suppression proteins P19 which prevents host transgene silencing (Voinnet et al. 2003). WT *Ha*FatA, T182W and Q125W complete cDNAs were transferred to Gateway-compatible binary vector pB2GW7 for their overexpression. Single T-DNA inserts will then usually result in the overexpression of the transgene (Karimi et al. 2002). These constructs were agroinfiltrated into *Nicotiana benthamiana* leaves in the presence of the P19 viral suppressor protein and the *HaFatA* expression analysis was carried out three days later (Figure 2). A single band was observed corresponding to *HaFatA* in the treated leaves, pointing to the overexpression was successful.

Nicotiana benthamiana leaves lipid analysis

Nicotiana benthamiana leaves are very active at synthesizing lipids, so they are a good model to test alterations in fatty acid metabolism. The expression of sunflower functional FatAs (*Ha*FatA and Q215W) in tobacco leaves did not increase the amount of total lipids with respect to the controls (leaves transformed with the empty vector or T182W) (Figure 3A). However, leaf TAGs were increased 2-fold and 4-fold when WT FatA and Q215W, respectively, were expressed (Figure 3B). TAGs typically represent < 2% of total leaf lipids, as seen in the null control. The first logical consequence of overexpression of a functional thioesterase in plant cells might be an increase in the flux of acyl moieties out of the plastid to the cytosol pathway, which is a necessity for TAG synthesis. These changes in metabolic flux did not appear to affect the prokaryotic pathway as the galactolipids components of the cell membrane and photosynthetic machinery suffer only a small reduction in their content, around 5%. This is unsurprising as the content of functional and structural lipids is strongly regulated in leaves and that there are mechanisms that control the amount of these lipids in plant cells. For example, when FatB from Arabidopsis was knocked-out there was an increase of both the rates of synthesis and degradation of lipids in order to equilibrate the deficiency of saturated fatty acids in the eukaryotic lipid pathway (Bonaventure et al. 2004). Nevertheless it seemed that leaf TAGs were not under this regulatory mechanism, so their amount on a fresh weight basis was affected by the expression of exogenous thioesterases and by their activity level. In this regard, the increase in TAG content was almost doubled when leaves were transformed with the construct Q215W. This result is in agreement with the kinetic characterization of Q215W thioesterase, which displayed the higher catalytic efficiency for most substrates (Table 1). The transient expression of other enzymes involved in TAG biosynthesis produced an effect similar to that described in these results. Thus, transient expression of acyl-CoA-diacylglycerol acyltransferase 1 gene from Arabidopsis also increased the tobacco leaf TAG fraction (Bouvier-Navé et al. 2000).

An increase in the flux of fatty acids mediated by an overexpressed thioesterase to cytosolic glycerolipids synthesis might also be expected to alter the pool size or composition of the cytosolic acyl CoA intermediates, which effectively connect plastidic fatty acid synthesis to ER-mediated glycerolipid synthesis. The amount of the acyl-CoA pool was measured in control and transformed plants and significant

differences were observed in their absolute amount, showing the higher average values leaves expressing WT *Ha*FatA and Q215W (Figure 3C). The composition of the acyl-CoA pools was also investigated, resulting in a significant increase of 18:0-CoA in lines expressing functional thioesterases (Figure 4). Increases in 16:0-CoA were also consistently observed but were not statistically significant. So our results indicate that the recombinant thioesterases increased the export of saturated fatty acids from the plastid. However, these results are maybe somewhat difficult to interpret because acyl-CoA is a very dynamic pool involved with simultaneous varied synthetic and degradative pathways. The increase in TAGs in the recombinant thioesterase infused leaves suggests that any increased acyl CoA pool may have been channelled efficiently into TAG. The total fatty acid composition of the leaf tissue was determined and no significant differences were found in the total amount (Figure 3A) nor in the relative amount of each fatty acid (Figure 5A). However, tissues agroinfiltrated with WT *Ha*FatA or Q215W displayed an increase in the absolute amount of TAGs (Figure 3B), being highest in plants transformed with Q215W. The unchanged total fatty acid composition in the agroinfiltrated leaves showing higher amounts of TAGs can be explained because TAGs only represent the 7% of the total lipid content. The TAG-specific fatty acid composition of recombinant lines showed an increase in saturated and a decrease in unsaturated acyl moieties relative to WT (Figure 5B), which was consistent with the acyl-CoA pool composition. This increase in saturated fatty acids suggests that the overexpressed thioesterase activities in the transformed plants induced a premature hydrolysis of 16:0 and 18:0 acyl-ACP plastidial intermediates prior to 18:1, which is the main plastidial precursor for polyunsaturated fatty acids typically found in chloroplastic lipids. Correspondingly, the increase in the cytosolic pools of saturated acyl-CoA derivatives would lead to a higher accumulation of saturated fatty acids in the leaf TAGs. Saturated acyl-ACPs are also exported in a high proportion by thioesterases of the B type (Bonaventure et al. 2003) and their export was curtailed when extra functional thioesterases were introduced.

Figure 6 shows the relative TAG species composition. TAG species containing saturated fatty acids increased in tissues agroinfiltrated with WT *Ha*FatA and Q215W at the expenses of species containing unsaturated acyl moieties, in good agreement with the results in Figure 5B. The diacylglycerol (DAG) pool was also analyzed (Figure 7) and no significant differences were found. This pool is also very variable due to it depends on the equilibrium between anabolic and catabolic reactions.

Lipids changes in transgenic Arabidopsis seeds

To check the effect of the expression of these *Ha*FatA alleles on oil accumulation in seeds as opposed to leaf tissues, we transformed the genes into *Arabidopsis thaliana* wild type line Col-0. The transgenic lines were not compromised in seed weight, but showed differences both in the seed oil content and in their fatty composition when compared to the wild type. Seeds from WT and *Ha*FatA T182 (i.e. null activity allele) plants were similar in oil content, around 6.02±0.3 μg FAs/seed, and composition (Figure 8). In contrast, *Ha*FatA and *Ha*FatA Q215W lines had yield decreases of 31 and 40%, respectively, relative to WT. As shown in Figure 8, the expression of *Ha*FatA and *Ha*FatA Q215W produced an increase in the content of saturated fatty acids, palmitic and stearic acids, with a concomitant decrease in oleic acid derivatives (Table 2A). This reduction in oleic acid derivatives was not homogeneous, being higher in those coming from the oleic and linoleic acids desaturation than those originated by elongation due to Fatty Acid Elongase activity (Table 2B).

The different behavior of the expression in seeds of these sunflower thioesterase FatA alleles compared with tobacco leaves can be explained assuming that in the Arabidopsis seed, during the active phase of lipid biosynthesis, all the produced oleoyl-ACP is already being hydrolyzed by the existing thioesterase activity, the artificial enrichment of this activity with enzymes of different substrates profiles and specific activities (Table 1) will produce the hydrolysis of substrates in previous steps of the biosynthetic pathway, in this case palmitoyl- and stearoyl-ACP (Figure 8).

The massive output of these saturated fatty acids from the plastid could produce a temporary starvation/lack of ACBPs in the cytoplasm, the accumulation of free acyl-CoAs, the inhibition of glucose 6-P input in the plastid (Johnson et al. 2000) and, consequently, a reduction in the synthesis of fatty acids, explaining the reduction of oil content observed in transgenic Arabidopsis lines expressing sunflower wild and Q215 FatA thioesterase alleles. In addition, the greater affinity of the Fatty Acid Elongase complex for saturated fatty acids will produce an increase of their derivatives.

Conclusions

Non-photosynthetic oilseed tissues contain enzymatic machinery with very high efficiencies for the synthesis of fatty acids that are accumulated into TAGs. Thioesterases from seeds including *Macadamia tetraphylla* (Moreno-Pérez et al 2011),

castor (Sánchez-García et al. 2010) and sunflower (Serrano-Vega et al. 2005) all have measured catalytic efficiencies one to two orders of magnitude higher than that reported for Arabidopsis (Salas and Ohlrogge 2002). Results in Table 1 demonstrated that by manipulating amino acid residues in *Ha*FatA it is still possible to obtain enzymes with even higher K_{cat} values and improved catalytic efficiencies. The ability of this enzyme to alter leaf lipid metabolism was demonstrated by transient expression in tobacco. Expression of this gene induced an increase in both the total TAG content and proportion of saturated fatty acids in leaves, and this increase was consistent with the measured catalytic efficiencies of the enzymes. However, the expression of these active thioesterase alleles in *Arabidopsis thaliana* seeds reduced seed oil content, although there was a relative increase in saturated fatty acids. Modified thioesterase alleles could thus be a useful tool in altering the plastidial export of fatty acids of interest, such as palmitoleic or stearic acids. Moreover, improved acyl-ACP thioesterases could play an important role at producing new leaf biomass crops. Results indicated that increased thioesterase levels in leaf tissue promoted the export of the de novo synthesized fatty acids from chloroplasts, which are later incorporated into TAGs. So, this could be an interesting method to produce biomass enriched in TAGs, which increased its energetic value and have the potential of being a future source of biofuels that not interacts with food production.

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Tables

Table 1. Kinetic parameters of purified recombinant *Ha*FatA proteins on different acyl-ACP substrates.

	Substrate	K_m	V_{max}	k_{cat}	k_{cat}/K_m
		(μM)	(nkat/mg prot)	(s^{-1})	(s^{-1} μM^{-1})
FatA wt	**18:1-ACP**	0.9	1008.6	35.6	39.1
	16:1-ACP	0.3	225.9	8.0	23.4
	18:0-ACP	0.7	22.7	0.8	1.2
	16:0-ACP	1.0	30.0	1.1	1.1
L118W	**18:1-ACP**	0.6	61.3	2.2	3.7
	16:1-ACP	0.3	16.3	0.6	1.8
	18:0-ACP	1.0	5.6	0.2	0.2
	16:0-ACP	0.7	4.8	0.2	0.3
T182W	**18:1-ACP**	0.6	0.9	0.032	0.050
	16:1-ACP	0.6	0.5	0.016	0.028
	18:0-ACP	1.5	0.1	0.003	0.002
	16:0-ACP	0.9	0.1	0.002	0.003
M206W	**18:1-ACP**	21.2	3567.0	125.8	5.9
	16:1-ACP	0.2	44.6	1.6	6.6
	18:0-ACP	0.4	5.4	0.2	0.5
	16:0-ACP	1.2	13.6	0.5	0.4
Q215W	**18:1-ACP**	2.2	2420.0	85.4	39.3
	16:1-ACP	0.1	177.0	6.2	48.0
	18:0-ACP	2.5	115.2	4.1	1.6
	16:0-ACP	0.9	67.1	2.4	2.8

Table 2. Estimation of the fatty acids released by the plastid (A) and the enzyme activities (B) from the total fatty acid composition in control (Col-0) and transgenic (Col-0 *Ha*FatA T182, Col-0 *Ha*FatA wt and Col-0 *Ha*FatA Q215) mature seeds. Data are expressed as the sum of percentages of the fatty acids in each tissue. Activities: TE, acyl-ACP thioesterase; KASII, β-ketoacyl-ACP synthase II; SAD, acyl-ACP desaturase; ODS, oleate desaturase; LDS, linoleate desaturase; and FAE, Fatty acid elongase.

	A					B				
	Relative fatty acid output (TE)					KASII	SAD	ODS	LDS	FAE
	16:0	∑16:1	∑18:0	∑18:1n9	∑18:1n7					
Col-0	15.5	n.d.	23.8	58.2	2.5	84.4	60.7	33.4	10.4	68.4
Col-0 *Ha*FatA T182	17.2	n.d.	23.5	56.8	2.5	82.8	59.3	33.7	12.8	60.5
Col-0 *Ha*FatA wt	29.7	n.d.	29.8	37.7	2.8	70.3	40.5	25.6	8.8	63.5
Col-0 *Ha*FatA Q215	33.0	n.d.	37.5	27.9	1.7	67.0	29.6	20.4	8.0	70.0

TE =∑16:1=16:1n7; ∑18:0=18:0+20:0+22:0+24:0;
∑18:1n9=18:1n9+18:2n6+18:3n6+18:3n3+20:1n9+22:1n9+24:1n9+20:2n6+20:3n3+22:2n6; ∑18:1n7=18:1n7+20:1n7.

KASII=18:0+20:0+22:0+24:0+18:1n9+18:2n6+18:3n6+18:3n3+20:1n9+22:1n9+24:1n9+20:2n6+20:3n3+22:2n6+18:1n7+20:1n7.

SAD=16:1n7+18:1n7+18:1n9+ +20:1n7+18:2n6+18:3n3+22:2n6+18:3n6+20:1n9+22:1n9+24:1n9+20:2n6+20:3n3.

ODS=18:2n6+18:3n3+20:2n6+20:3n3+22:2n6+18:3n6.

LDS=18:3n3+18:3n6+20:3n3.

FAE=(20:0+20:1n9+20:1n7+20:2n6+20:3n3)+2*(22:0+22:1n9+22:2n6)+3*(24:0+24:1n9)

Figure legends

Figure 1. Ribbon diagrams of the proposed structural model for sunflower FatA acyl-ACP thioesterase hydrophobic pocket. Lateral (a) and front (b) view of the hydrophobic pocket region showing in blue the residues that have been previously subjected to direct mutagenesis (Serrano et al. 2005) and in green the residues mutagenized in this work (L118, T172, T182, R184, M206 and Q215).

Figure 2. Expression analysis of *Ha*FatA alleles on tobacco leaves by RT-PCR. RuBisCo gen was used as positive control.

Figure 3. Total amount of fatty acids (a), triacylglycerols (b) and acyl-CoA esters (c) from agroinfiltrated tobacco leaves. Data are the average of five independent samples ± SD.

Figure 4. Agroinfiltrated tobacco leaves acyl-CoA esters composition (mol %). Data are the average of five independent samples.

Figure 5. Agroinfiltrated tobacco leaves total (a) and TAG (b) fatty acid compositions (mol %). Data are the average of five independent samples.

Figure 6. Agroinfiltrated tobacco leaves triacylglycerol species composition (mol%). Data are the average of five independent samples. Triacylglycerols species lower than 0.25% are not shown in the figure.

Figure 7. Agroinfiltrated tobacco leaves diacylglycerol species composition (mol %). Data are the average of five independent samples.

Figure 8. Fatty acid composition (mol%) from *A. thaliana* transgenic seeds. Data are the average of five independent samples ±SD.

Fig.1

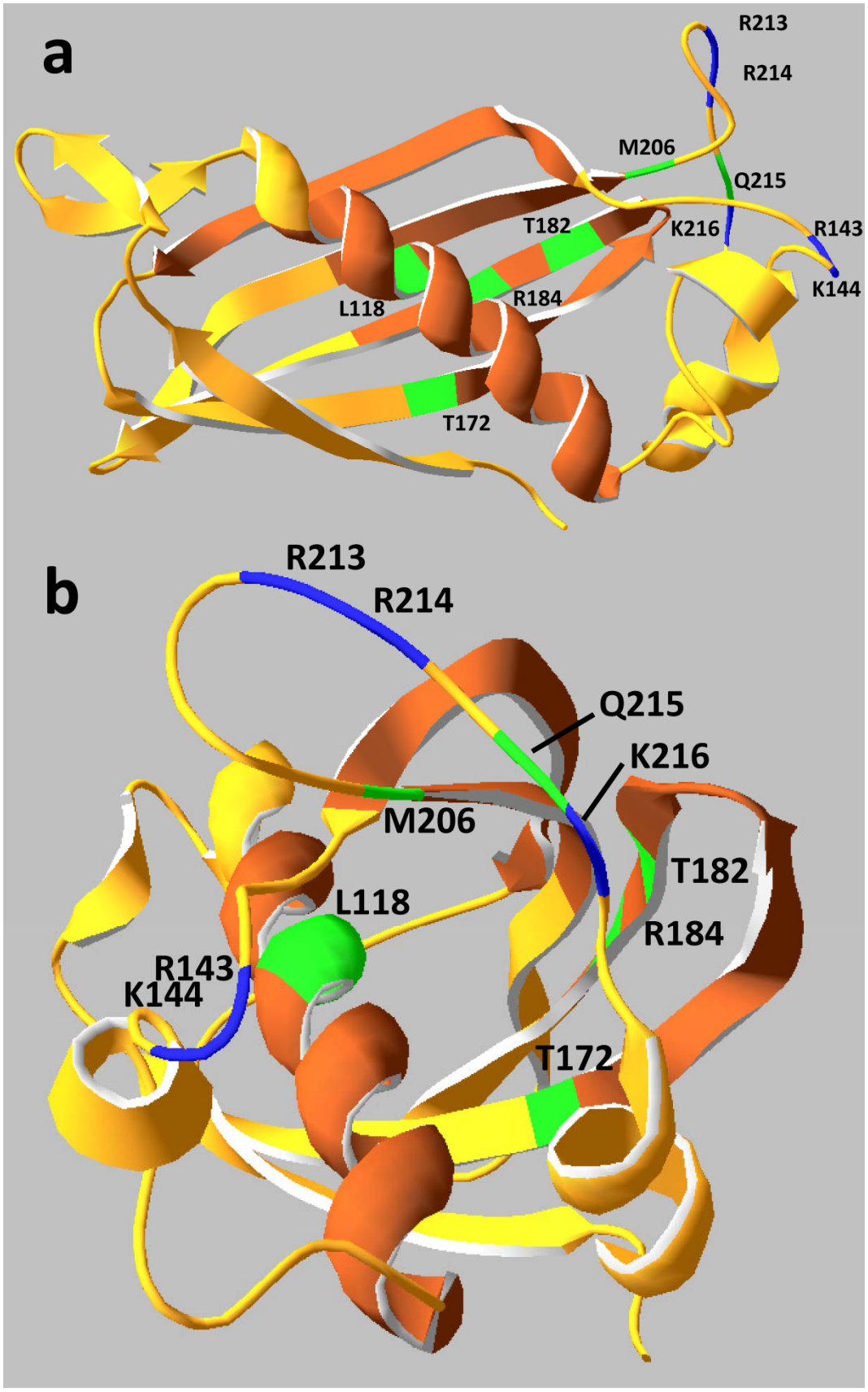

a
R213
R214
M206
Q215
T182
K216
R143
K144
L118
R184
T172
b
R213
R214
Q215
K216
M206
T182
L118
R184
R143
K144
T172

Fig. 2

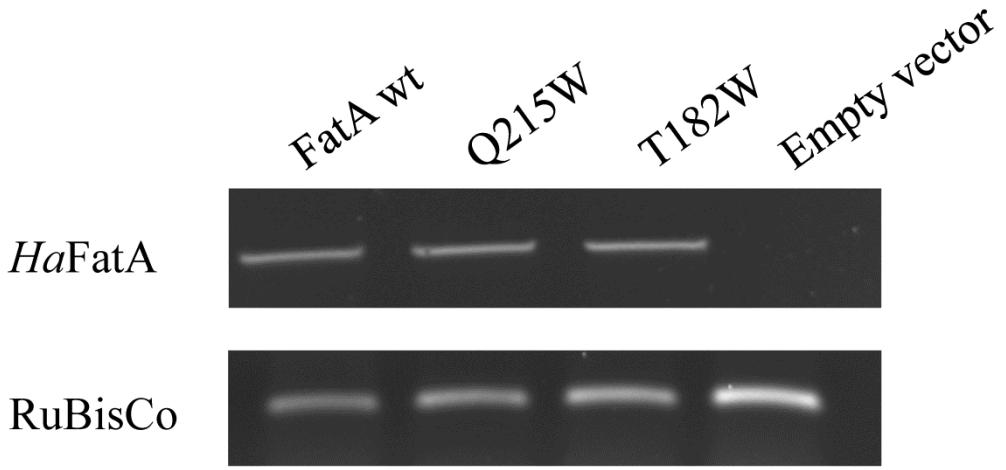


Fig. 3

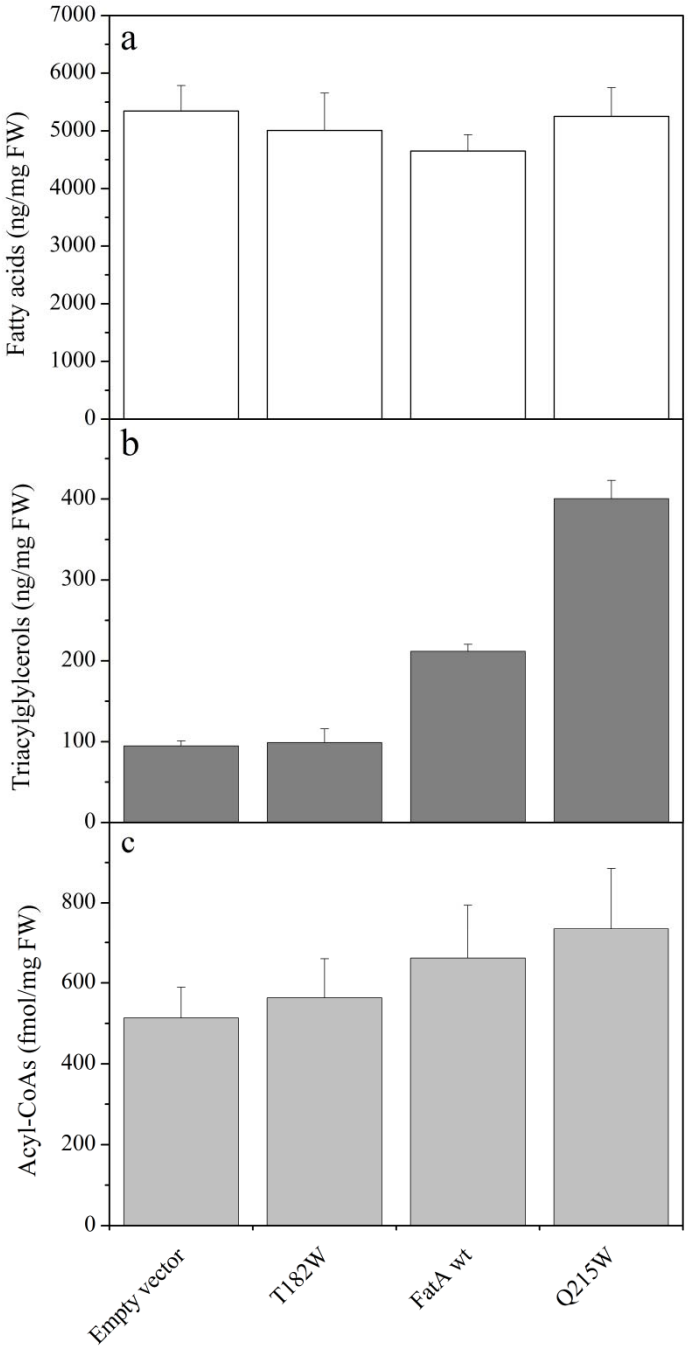

a
b
c
Fatty acids (ng/mg FW)
Triacylglylcerols (ng/mg FW)
Acyl-CoAs (fmol/mg FW)
0
1000
2000
3000
4000
5000
6000
7000
100
200
300
400
600
800
Empty vector
T182W
FatA wt
Q215W

Fig. 4

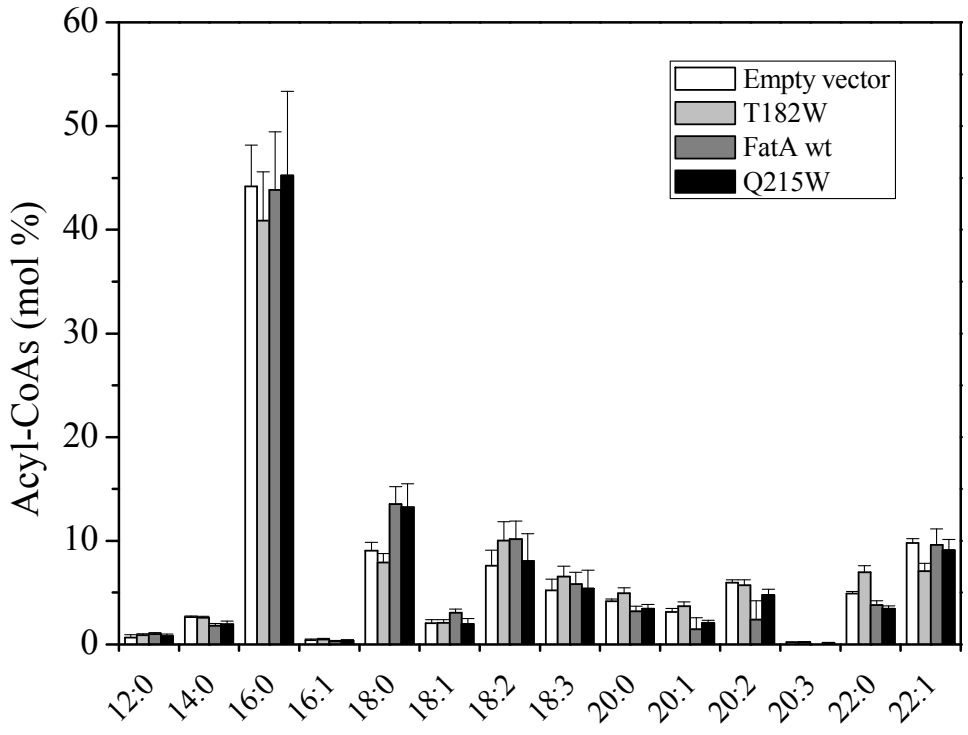

Acyl-CoAs (mol %)
60
50
40
30
20
10
0
Empty vector
T182W
FatA wt
Q215W
12:0
14:0
16:0
16:1
18:0
18:1
18:2
18:3
20:0
20:1
20:2
20:3
22:0
22:1

Fig. 5

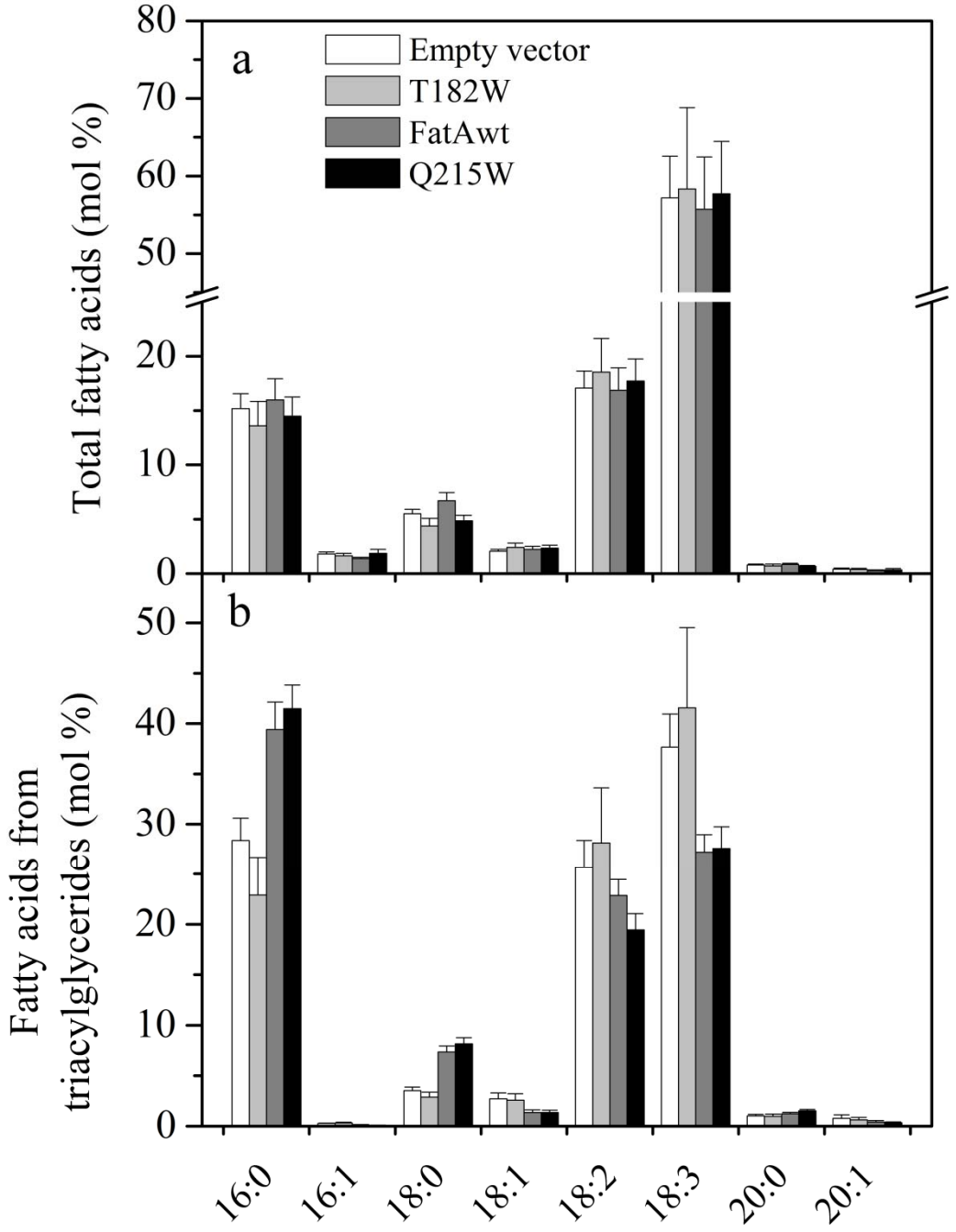
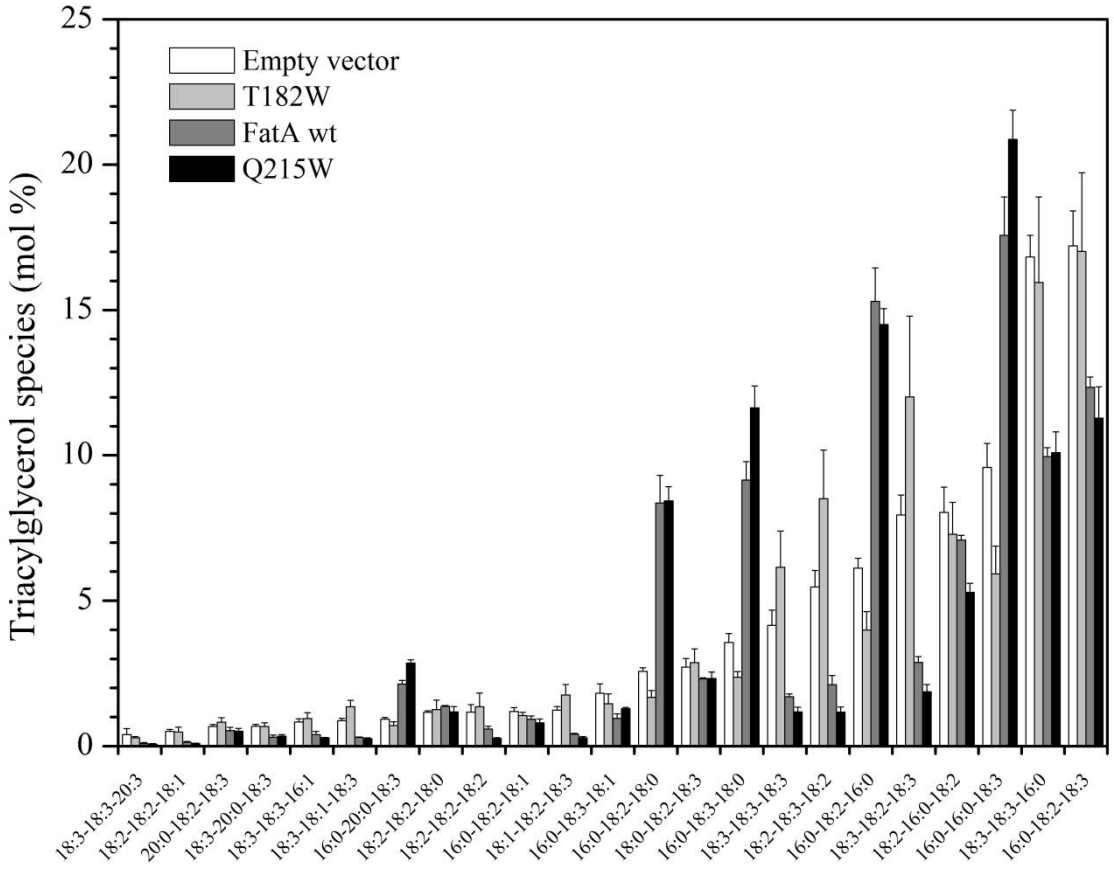

a
Empty vector
T182W
FatAwt
Q215W
Total fatty acids (mol %)
80
70
60
50
20
10
0
b
Fatty acids from triacylglycerides (mol %)
50
40
30
20
10
0
16:0
16:1
18:0
18:1
18:2
18:3
20:0
20:1

Fig. 6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FatA wt
Q215W
Triacylglycerol species (mol %)
0
5
10
15
20
25
18:3-18:3-20:3
18:2-18:2-18:1
20:0-18:2-18:3
18:3-20:0-18:3
18:3-18:3-16:1
18:3-18:1-18:3
16:0-20:0-18:3
18:2-18:2-18:0
18:2-18:2-18:2
16:0-18:2-18:1
18:1-18:2-18:3
16:0-18:3-18:1
16:0-18:2-18:0
18:0-18:2-18:3
16:0-18:3-18:0
18:3-18:3-18:3
18:2-18:3-18:2
16:0-18:2-16:0
18:3-18:2-18:3
18:2-16:0-18:2
16:0-16:0-18:3
18:3-18:3-16:0
16:0-18:2-18:3

Fig. 7

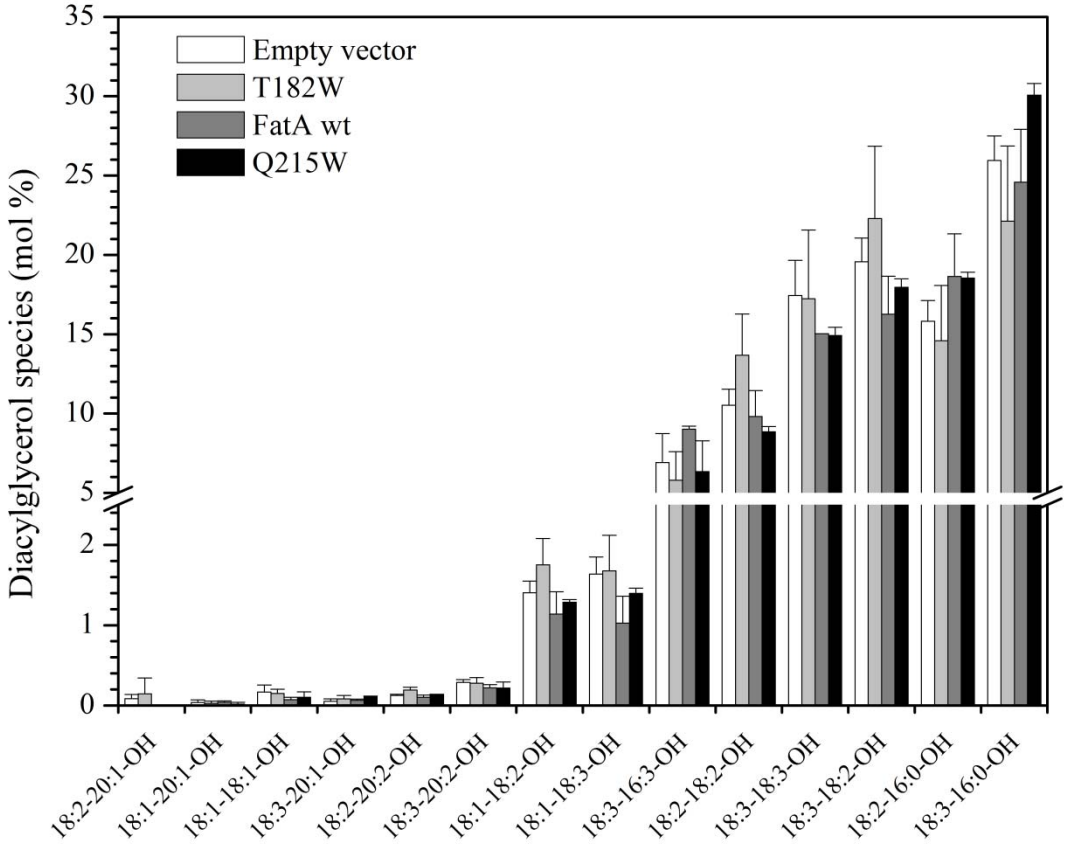
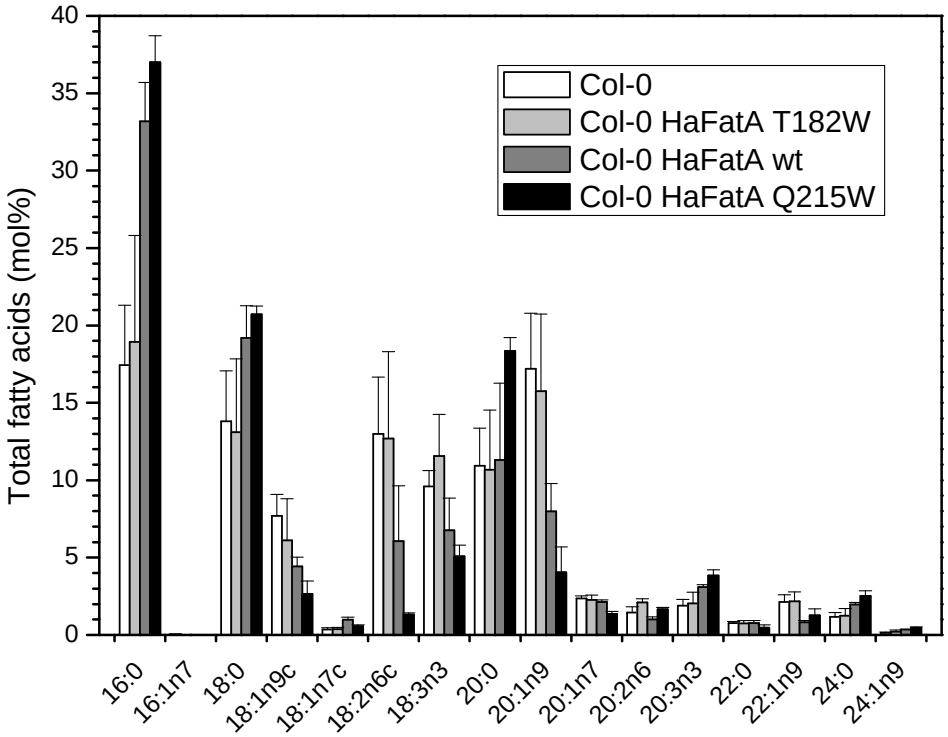

Empty vector
T182W
FatA wt
Q215W
Diacylglycerol species (mol %)
35
30
25
20
15
10
5
2
1
0
18:2-20:1-OH
18:1-20:1-OH
18:1-18:1-OH
18:3-20:1-OH
18:2-20:2-OH
18:3-20:2-OH
18:1-18:2-OH
18:1-18:3-OH
18:3-16:3-OH
18:2-18:2-OH
18:3-18:3-OH
18:3-18:2-OH
18:2-16:0-OH
18:3-16:0-OH

Fig. 8


Total fatty acids (mol%)
40
35
30
25
20
15
10
5
0
Col-0
Col-0 HaFatA T182W
Col-0 HaFatA wt
Col-0 HaFatA Q215W
16:0
16:1n7
18:0
18:1n9c
18:1n7c
18:2n6c
18:3n3
20:0
20:1n9
20:1n7
20:2n6
20:3n3
22:0
22:1n9
24:0
24:1n9