

Regioselectivity mechanism of the *Thunbergia alata* Δ^6 2 -16:0-acyl carrier protein desaturase

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1 Short Title: Mechanism of desaturase regioselectivity

2 Title: **Regioselectivity mechanism of the *Thunbergia alata* Δ^6 -16:0-acyl carrier protein**
3 **desaturase**

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75 **One Sentence Summary:** Predictions regarding the mechanism of Δ^6 regioselectivity of the
76 *Thunbergia alata* desaturase based on x-ray crystallography and molecular dynamics simulations
77 are confirmed by experiment .

78 **Key words:** Fatty acid modification, regioselectivity, enzyme mechanism, molecular dynamics,
79 x-ray crystallography.

80 **Abbreviations:** ACP, acyl carrier protein; FA, fatty acid; Δ^x -Y:Z- desaturase, a desaturase
81 enzyme that recognizes a FA of Y carbons in length, with Z double bonds that inserts a double
82 bond between carbons X and X+1 relative to the carboxyl group. MD, molecular dynamics
83 simulation.

84 **Author contributions:** Work was conceived and designed by JEG, YC, MDB, YL, SR and JS;
85 Experimental work was performed by JEG, YC, MDB, EW, JC, and X-HY. JEG, MDB and JS
86 wrote the paper with contributions from all other authors.

87

Abstract

Plant plastidial acyl-acyl carrier protein (ACP) desaturases are a soluble class of diiron-containing enzymes that are distinct from the diiron-containing integral membrane desaturases found in plants and other organisms. The archetype of this class is the stearoyl-ACP desaturase which converts stearoyl-ACP into oleoyl (18:1 Δ^9 *cis*)-ACP. Several variants expressing distinct regioselectivity have been described including a Δ^6 -16:0-ACP desaturase from black-eyed Susan vine (*Thunbergia alata*). We solved a crystal structure of the *T. alata* desaturase at 2.05 Å resolution. Using molecular dynamics (MD) simulations, we identified a low-energy complex between 16:0-ACP and the desaturase that would position C6 and C7 of the acyl chain adjacent to the diiron active site. The model complex was used to identify mutant variants that could convert the *T. alata* Δ^6 desaturase to Δ^9 regioselectivity. Additional modelling between ACP and the mutant variants confirmed the predicted regioselectivity. To validate the in-silico predictions, we synthesized two variants of the *T. alata* desaturase and analyzed their reaction products using gas chromatography-coupled mass spectrometry. Assay results confirmed that mutants designed to convert *T. alata* Δ^6 to Δ^9 selectivity exhibited the predicted changes. In complementary experiments, variants of the castor desaturase designed to convert Δ^9 to Δ^6 selectivity lost some of their Δ^9 desaturation ability and gained the ability to desaturate at the Δ^6 position. The computational workflow for revealing the mechanistic understanding of regioselectivity presented herein lays a foundation for designing acyl-ACP desaturases with novel selectivities to increase the diversity of monoenes available for bioproduct applications.

Introduction.

The plant acyl-acyl carrier protein (ACP) desaturases are a family of soluble plastid-localized enzymes that convert saturated fatty-acyl ACPs into their monounsaturated equivalents in an oxygen-dependent reaction (Shanklin and Cahoon, 1998). These enzymes play a key role in biosynthesis of mono-unsaturated fatty acids in plants, acting as the primary determinant for the composition of unsaturated fatty acids in cell membranes and seed storage oils (Thompson et al., 1991; Shanklin et al., 2009). The archetype of this group is the Δ^9 -18:0-ACP desaturase that introduces a *cis* double bond between carbons 9 and 10 to produce oleoyl-ACP (Shanklin and Somerville, 1991). The acyl-ACP desaturase family of Arabidopsis (*Arabidopsis thaliana*) consists of six homologs that are partially redundant in function (Kachroo et al., 2007) and

differentially activated during development (Kazaz et al., 2020). Acyl-ACP desaturases display remarkable regiospecific fidelity, indeed their discoverer, Nobel Prize winner Konrad Bloch noted that “The stereospecific removal of hydrogen in the formation of oleate, although predictable on principle grounds would seem to approach the limits of the discriminatory power of enzymes” (Bloch, 1969). Introduction of the double bond at the midpoint of the acyl chain maximizes the increase in melting temperature and hence its physiological effects on membrane fluidity (Browse and Xin, 2001). Plant acyl-ACP desaturases have also been shown to be involved in defense against biotic stresses (Kazaz et al., 2021) and in speciation in orchids (Schluter et al., 2011).

In animals and bakers’ yeast desaturation of stearic acid occurs between carbons 9 and 10, albeit by an evolutionarily distinct class of desaturase enzymes of the endoplasmic reticulum (Stukey et al., 1990; Sperling et al., 2003; Wang et al., 2015). In contrast, the acyl-acyl carrier protein desaturases of vascular plants are soluble proteins localized within the plastids (Nagai and Bloch, 1968; Lindqvist, 2001). In addition to the housekeeping Δ^9 -18:0-ACP desaturase, several variant desaturases have been characterized that recognize different acyl chain lengths or introduce the double bond at positions other than C9. Examples include the Δ^4 -16:0-desaturases from coriander (*Coriandrum sativum*) (Cahoon et al., 1992) and English ivy (*Hedera helix*) (Whittle et al., 2005), the Δ^9 -16:0-ACP desaturase from cat’s claw (*Doxantha unguis-cati*) (Cahoon et al., 1998), and the Δ^6 -16:0-desaturase from black-eyed Susan vine (*Thunbergia alata*) (Cahoon et al., 1994).

The castor 18:0 Δ^9 -desaturase is a diiron protein in which the metal center is coordinated with a central four helix bundle (Lindqvist et al., 1996). A boomerang shaped cavity extends from the surface of the desaturase past the diiron center, terminating in a blind ending. The cavity is long enough to accommodate an extended 18-carbon acyl chain that places carbons 9 and 10 adjacent to the diiron center. Comparison of the structures of the castor Δ^9 -18:0-ACP and ivy Δ^4 -16:0-ACP desaturases were performed to investigate the basis for regioselectivity (Guy et al., 2007). Even though they have enough similarity for their C- α traces to be closely superimposed, the comparison does not provide useful information regarding the determinants of their regioselectivity. However, solution of the structure of the castor Δ^9 -18:0- desaturase in complex with stearoyl-ACP led to the identification of a key Lys in the ivy desaturase at a position

corresponding to Asp280 of the castor 18:0 Δ^9 - desaturase (Guy et al., 2011). This Lys was hypothesized to make an ionic interaction with the phosphate of the phosphopantetheine group, stabilizing a binding mode for ACP that is rotated 60 degrees relative to that seen for the castor complex crystal structure. Rotation of the ACP enables deeper insertion of the 16-carbon acyl chain into the cavity to place carbons 4 and 5 adjacent to the activated oxygen in the active site. Experimental support for the hypothesis was obtained when it was shown that the castor Asp280Lys mutant was capable of desaturating short-chain substrates at the Δ^4 position (Guy et al., 2011).

Major oilcrops have very similar compositions of fatty acids, i.e., much fewer than the more than 450 naturally occurring variants fatty acids (Ohlrogge et al., 2018). The identification of genes encoding enzymes responsible for the production of the diverse fatty acid species has driven interest in creating crops that accumulate high levels of specialized unusual fatty acids (Thelen and Ohlrogge, 2002; Napier, 2007; Baud, 2018). While the molecular basis for the production of hydroxy (van de Loo et al., 1995), epoxy, acetylenic (Lee et al., 1998) and conjugated fatty acids (Cahoon et al., 1999) have been discovered, there are few examples of crops accumulating commercially viable levels of these specialty fatty acids. Likewise, efforts to express naturally occurring variant acyl-ACP desaturases with diverse specificities resulted in the accumulation of only a few percent of product (Cahoon et al., 1992; Whittle et al., 2005; Bondaruk et al., 2007). This is partially due to poor catalytic turnover of the variant enzymes and to the presence of specialized isoforms of ferredoxins (Schultz et al., 2000) and ACP (Suh et al., 1999) in the species of origin. However, at least one study has employed a Δ^9 -18:0-ACP desaturase engineered (Whittle and Shanklin, 2001) to efficiently desaturate 16:0-ACP to accumulate approximately 70% of ω -7 fatty acids, first in Arabidopsis (Nguyen et al., 2010) and subsequently in camelina (Nguyen et al., 2015). To expand the use of engineered enzymes, a major goal of the present work is to generate a general mechanistic understanding of regioselectivity of acyl-ACP

desaturases to enable their redesign to introduce double bonds in any desired locations on acyl chains of varying lengths. Towards this goal, we solved the crystal structure of the *T. alata* Δ^6 -16:0-ACP desaturase and used that structure to interpret existing biochemical data regarding the specificity of mutants. Molecular dynamics (MD) simulations of the 16:0-ACP-*T. alata* desaturase, together with 16:0-ACP docking simulations were used to investigate potential binding orientation of the ACP with respect to the desaturase and candidate residues that contribute to Δ^6 desaturation. The resulting hypotheses were tested by creating variants of castor and *T. alata* desaturases to reciprocally exchange their regioselectivities. Both docking simulations of the variants and enzyme assays of expressed variant enzymes were consistent with proposed substrate-determining residues identified via MD simulations.

Results

Cloning, protein purification and crystallization

The native *T. alata* Δ^6 -16:0-desaturase sequence lacking its transit peptide, and the N-11 truncation were cloned by PCR/overlap extension PCR from the naturally isolated cDNA (Cahoon et al., 1994). Both forms of the *T. alata* desaturase were expressed in pET9d in *E. coli* and purified using strong cation exchange chromatography in contrast to the purification of castor and ivy desaturases which used weak cation exchange (Whittle and Shanklin, 2001; Whittle et al., 2005). The *T. alata* desaturase and its variants showed a tendency to precipitate after cation exchange chromatography which was minimized by rapid dilution into four volumes of loading buffer containing 10% glycerol. Fractions enriched to near homogeneity (>95%) were pooled and concentrated with minimal losses. See Figure S1. for analysis of a representative purification of the N-11 *T. alata* desaturase. Crystallization trials performed using the full-length *T. alata* desaturase were unsuccessful. Because the first approx. 24 residues of the mature *T. alata*

desaturase were predicted to be disordered, we created a series of N-terminal truncations to evaluate for increased stability. The N-11 construct yielded protein with increased solubility, and crystallization trials produced initial crystals in a single condition with polyethylene glycol 6000 (as described in materials and methods). Data collection and refinement statistics are shown in Table 1. In the final structure, the asymmetric unit contains a single protein chain, which interacts with a neighboring symmetry-related molecule to form the biological dimer as shown in Figure 1A. While the crystallized N-11 construct begins from residue 12, no density was observed for residues 12-29, and the model therefore begins at Glu30. The quality of the electron density in the remainder of the sequence is very high, except for two flexible loop regions, residues 172-176 and 262-272, which did not have sufficient density to enable model-building and were therefore omitted from the final structure.

Overall structure

The overall fold of the *T. alata* desaturase monomer is very similar to those of castor (Lindqvist et al., 1996) (Figure 1B) and ivy (Guy et al., 2007) (Figure S2) with which it shares 69% and 64% sequence identity, respectively. Their monomer structures superimposed with RMSDs of 1.24 Å for 284 C α atoms and 1.10 Å over 274 C α atoms, respectively. The *T. alata* desaturase differs most notably from that of the previous desaturase structures in three regions. The most striking is the region comprising residues 111-123 of *T. alata* (116-128 in castor), which connect two helices of the core four-helix bundle; in the castor and ivy proteins these residues superimpose very well and form a loop, which packs relatively tightly against the body of the monomer. In the *T. alata* enzyme the equivalent residues protrude markedly from the structure, making crystal packing interactions with a symmetry-related chain, and include the β -hairpin formed from residues 114-118 and 121-124 (Figure 1B). Residues 257-261 also diverge from the positions of the equivalent residues in the castor and ivy desaturases, and are immediately followed by the disordered residues 262-272, although this region is well-ordered in the other structures. Finally,

each protein contains a long helix close to the C-terminus, residues 313 to 342 in *T. alata*, and in the castor and ivy enzymes this is followed by a β -hairpin structure. In the *T. alata* desaturase the helix is slightly longer than in the homologues and is tilted closer to the body of the protein at its C-terminus, ending in a loop structure instead of the β -hairpin seen in the castor and ivy desaturase structures. The quaternary structure of the *T. alata* desaturase is a highly symmetrical dimer (Figure 1A). The iron coordination in the *T. alata* desaturase is highly symmetrical and superimposes very well on that of the reduced active site in the castor desaturase. Two carboxylates (Glu136 and Glu224) bridge the two iron ions, and there is no evidence of a μ -oxo bridge. However, the distance between the two iron ions in the refined structure of the *T. alata* desaturase is 3.6 Å, which lies between those previously seen in the oxidized and reduced forms.

The desaturase substrate-binding cavity is a long tunnel that reaches from the surface of the protein to the active site, where it bends sharply adjacent to the two irons forcing the fatty acid into a semi-eclipsed conformation that presents two *Pro-R* hydrogens towards the active site oxidant thereby enabling *cis* desaturation. Beyond the active site the cavity continues through the core of the enzyme to accommodate the methyl end of the acyl chain. The base of the cavity is blind ending, and the residues lining it are important determinants of chain-length specificity. Figure 2 shows a comparison of the bottom of the substrate-binding cavity in the castor and *T. alata* desaturases, revealing that many of them are conserved in both identity and position. Notable exceptions are Tyr184 in *T. alata*, which replaces Phe189 in castor with a sidechain that is both bulkier and more hydrophilic, and Met109, which is conserved, but has a very different sidechain position in the *T. alata* enzyme that obstructs the cavity. It is very likely that the flexible Met109 sidechain would simply move aside to make space for the substrate. The importance of the Tyr184/Phe189 difference is however confirmed by the fact that an A188G/Y189F double mutation introduced into the castor desaturase produced an enzyme

capable of desaturating 16:0-ACP at a rate comparable to that observed with 18:0-ACP (Cahoon et al., 1997).

Putative ACP-binding site

ACPs interact with enzymes such as the desaturases primarily via its recognition helix (helix II) (Zhang et al., 2001), which is positioned just after phospho-Ser 38, that carries the pantetheine linker to the fatty acid substrate. The surface of ACP helix II is very negatively charged, and the ACP-enzyme interaction is therefore predominantly mediated by positively charged residues on the surface of the desaturase as shown in Figure S3. In the castor desaturase, 7 residues contribute to the ACP-interaction surface; Arg260 and Lys262 from the C-terminus of $\alpha 8$, and Arg333, Arg336, Leu337 and Arg340 from the long helix $\alpha 11$, and Arg344 from the following loop (Guy et al., 2011). In the *T. alata* desaturase structure, many of these residues are conserved, or are conservative mutations relative to other desaturases. For example, Lys255 is equivalent to Arg260 of the castor enzyme, and the position of the sidechain is relatively similar in both structures. Castor's Arg336 and Leu337 are replaced by Lys331 and Val332, respectively, in the *T. alata* desaturase all have similar positions in the two structures so it is conceivable that they could still be part of the ACP-interface. After Leu337/Val332, however, the two structures diverge because in the *T. alata* desaturase the helix curves at this point, causing it to tilt towards the main body of the desaturase and away from the ACP-binding site. Arg340 is conserved as Arg335, but the mainchain position is shifted by 5 Å, although the sidechain is still pointing outwards and would thus be able to interact. Of the seven residues that make up the ACP-interaction surface in the castor desaturase, only the properties of Arg344 are substantially changed in the *T. alata* protein, being replaced by Trp339. The bulky tryptophan sidechain is positioned close to the entrance to the substrate-binding cavity potentially influencing the position of the pantetheine group. It seems likely that these shape and surface charge distribution differences between the ACP-binding surface in the castor desaturase and the equivalent region of

the *T. alata* desaturase result in differences in the position and/or orientation of ACP for the 16:0- Δ^6 *T. alata* desaturase. This would be consistent with our previous findings that the mode of ACP interaction, and different position of the pantetheine group, was responsible for the different regioselectivities of the castor and ivy desaturases (Guy et al., 2011). To perform Δ^9 desaturation, the castor desaturase positions C9 and C10 of the substrate adjacent to the diiron active site, which requires enough distance for the pantetheine arm and carbons 1-8 to stretch from phospho-Ser38 of the ACP to the active site. The ivy desaturase, which preferentially performs Δ^4 desaturation, must position C4 and C5 adjacent to the active site, so has only the pantetheine group and C1-3 to bridge between this position to the ACP. Our previously published model of the ivy desaturase-ACP complex shows that this is achieved by a distinct ACP binding mode that results in deeper insertion of phosphoserine38, the pantetheine group and acyl chain into the cavity. That the *T. alata* enzyme, with its Δ^6 regioselectivity, has an ACP binding mode between those of castor Δ^9 and ivy Δ^4 desaturases seemed likely, and that the undefined 262-272 loop in the x-ray crystal structure does not influence regioselectivity (Figure S4).

To address this hypothesis, we use a combination of computational docking and MD simulation to probe possible ACP binding modes to the surface of the *T. alata* desaturase. We first constrained the position of the acyl chain so that C6 and C7 were adjacent to the diiron active site and performed MD simulations to assess potential ACP binding modes using both castor/ACP and ivy/ACP relative orientation as starting point. In both cases, ACP reorients in a few tens of nanoseconds (Figure S5). The simulation started from the Ivy/ACP relative orientation stabilizes to a structure with an average of 4 ionic close contacts (Figure S6) at the protein/protein interface, while the other starting orientation yields only 1 ionic contact (Figure S7). Additionally, the ivy/ACP forms a persistent hydrogen bond between the phosphate and Gln276 (Figure S6), no such interaction is observed for the castor/ACP. Under the reasonable assumption that the stability of the protein/protein complex is proportional to the number of ionic contacts, the

structure obtained from the Ivy/ACP orientation was adopted for subsequent analysis. This structure is intermediate between that of the ivy/ACP and the castor/ACP complexes (as shown in Figure 3). The same orientation is obtained using the ROSETTA docking protocol described recently (Baer et al., 2021). The recognition helix II of ACP interacts as in the castor and ivy complexes through multiple ionic interactions with a triad of positively charged residues, i.e., Arg328, Lys331 and Arg335 (see Figure 3). This interaction acts as a pivot point around which the ACP helix II can rotate.

Specifically, the orientation of the ACP is determined by the formation of an ionic interaction between the substrate phosphate oxygens and Arg263, which is occupied by a His in the castor Δ^9 enzyme. Additionally, stabilizing hydrogen bonding interactions are seen between the phosphate and Gln276, i.e., the position occupied by Lys in ivy and Asp in castor enzymes, which further stabilize the ACP orientation favoring Δ^6 desaturation. The pantetheine linker is maximally elongated in this mode, as reported for the binding for the ivy and castor enzymes. In agreement with the experimental findings in which replacement of the flexible loop from the castor Δ^9 desaturase was used to replace the undefined loop in the *T. alata* Δ^6 structure (see Supplemental Information and Figure S4), docking simulations predict binding orientations of the ACP consistent with Δ^6 desaturation stabilized by Arg263 and His266 for the *T. alata* desaturase.

In silico mutagenesis

In silico mutation studies of castor desaturase residues His267Arg and Asp280Gln were introduced into our experimental workflow (see Figure 4) to determine whether changing the key ACP binding mode-determining residues alone could convert Δ^9 to Δ^6 regioselectivity. The experiment was unsuccessful (Tables S1-4) because structural difference in the tilt of the helix beyond Leu337/Val332 in the castor desaturase (Figure S2) results in steric hindrance preventing the ACP from achieving an orientation that would allow it to perform Δ^6 desaturation. To address

this issue, we designed two variants of the castor desaturase in which His267Arg and Asp280Gln mutations were combined into a chimeric enzyme comprising the N-terminus of the castor enzyme fused to either shorter (34-residue) or longer (95-residue) sections of the C-terminus of the *T. alata* enzyme (see Figure 5). A reciprocal experiment was conducted in which Arg263His and Gln276Asp were introduced into the *T. alata* enzyme along with the substitution of equivalent shorter or longer sections of the C-terminus of the castor enzyme. We performed in silico docking simulations to assess the potential viability of these additional enzyme variants (Table S5). Inspection of the root mean square deviation of the position of the backbone of the recognition helix (residue 38-53) of the ACP with respect to ACP in the ivy (D4), castor (D9) and *T. alata* (D6) allowed us to identify binding orientations consistent with the desired changes in regioselectivity. The analysis of the docking simulations is described in detail in the Supporting Information.

Enzyme assays validate the predicted in silico regioselectivities of the variants

To test the in-silico predictions, we synthesized DNA constructs encoding the amino acid sequences for the four mutant variants. They were expressed in *E. coli* under the control of the T7 promoter and purified as described for the wildtype enzyme. The regioselectivities of purified *T. alata* and castor desaturases, along with their four mutant chimeric derivatives were assessed by in vitro desaturase assays in which the products were separated by gas chromatography-coupled mass spectrometry. Mass labeled tetra-deuterated, 7,7, 8,8 palmitoyl-ACP substrate was synthesized and enabled us to distinguish between enzymatic 16:1 products and 16:1 originating from assay components. Second, while different 16:1 *cis* isomers have characteristic GC retention times, e.g., the Δ^6 isomer elutes ahead of the Δ^9 isomer, dehydrogenation at C6 and C7 results in the loss of a hydrogen from C6 and a deuterium at C7, whereas dehydrogenation at C9 and C10 results in the loss of two hydrogens. Thus, the product mass ion resulting from Δ^6 desaturation is 271 AMU, whereas Δ^9 desaturation produces a product of 272 AMU. The combination of

retention times and product masses provide unambiguous product identification. The in vitro assays shown in Figure 5, along with single ion monitoring (SIM) mass spectrometry analysis shown in Figure S8 provide experimental validation of the in-silico predictions. The *T. alata* wild type enzyme (TD6) exclusively converts 16:0-ACP to the 16:1 Δ^6 -ACP product. Substitution of Arg263 for His and Gln276 for Asp along with additional substitution of 34 C-terminal residues from the castor enzyme (TV1) results in the production of low levels of 16:1 Δ^9 that was enhanced by the substitution of 95 residues of the castor C-terminus (TV2). Conversely, the castor wild type enzyme (CD9) exclusively converts the 16:0-ACP substrate into 16:1 Δ^9 product. Substituting His267 for Arg and Asp280 for Gln into the castor enzyme along with substituting a short C-terminal portion of the *T. alata* enzyme (CV1) resulted in a substantial reduction in Δ^9 desaturation and the gain of detectable Δ^6 desaturation that was further enhanced by increasing the length of the C-terminal portion of the *T. alata* enzyme in the CV2 construct.

Discussion

The ability to manipulate the substrate specificity of desaturases, and the regioselectivity of double-bond insertion, is highly desirable for biotechnological applications. Previous attempts to manipulate these properties relied on a combination of sequence comparison, crystallography, modelling and mutagenesis to investigate the mechanisms of desaturase specificity (Cahoon et al., 1997; Whittle et al., 2008; Zhang et al., 2008; Guy et al., 2011). Mutagenesis studies performed based on the castor desaturase crystal structure (Lindqvist et al., 1996; Cahoon et al., 1997) showed that chain-length specificity is primarily controlled by residues lining the base of the substrate-binding cavity (Cahoon et al., 1998). An amino acid position was identified at the entrance to the substrate-binding cavity, represented by Asp280 in the castor desaturase and Lys275 in the ivy enzyme, that play key roles in controlling regioselectivity in Δ^9 and Δ^4

370 desaturases, respectively (Guy et al., 2011). However, uncovering the determinants of Δ^6
371 regioselectivity was only partially successful in that previous efforts were unable to separate
372 control of regioselectivity from that of chain length specificity (Cahoon et al., 1997). This was in
373 part attributable to the lack of crystallographic data for the Δ^6 desaturases, and to difficulties in
374 modelling their structures due to sequence differences in key regions. Determination of the 2.05
375 Å crystal structure of the *T. alata* desaturase presented here overcame this obstacle.

376 The previous identification of Asp280 of the castor desaturase as a “toggle switch” activated by
377 its substitution for Lys to favor Δ^4 desaturation provided a specific example of regiospecific
378 control. In the present study, our identification of Arg263 and Gln276 of the *T. alata* desaturase
379 along with factors affecting the tilt of the C-terminal helix provide a more generalized view of
380 regiospecificity. That regioselectivities other than Δ^4 , Δ^6 and Δ^9 have not been described suggests
381 either the sampling of natural sources has been too sparse or that the FA products with double
382 bond positions other than these did not provide a selective advantage.

383 In contrast to previous substrate docking studies on the ivy desaturase (Guy et al., 2011), we were
384 unsuccessful in using the *T. alata* crystal structure to identify candidate ACP binding modes to
385 understand its regioselectivity in the present work, in part because of the structurally unresolved
386 residues in a 12 amino acid loop adjacent to residues of the ACP-desaturase interface. We
387 therefore undertook MD simulations and additional docking calculations, to obtain candidate
388 ACP binding modes consistent with Δ^6 regioselectivity. We extended our computational studies
389 to screen mutants of the castor 18:0- Δ^9 desaturase and the *T. alata* 16:0- Δ^6 desaturase to identify
390 stable ACP binding modes that could reciprocally switch their regioselectivities. This
391 computational screening successfully identified modes that were experimentally confirmed by in
392 vitro assays.

One interesting finding from the in vitro validation experiments was that the variants of the castor desaturase did not fully eliminate Δ^9 desaturation. This likely results from intrinsic flexibility of either the interaction region of the desaturase, or other ACP-interacting residues that partially stabilized an acyl-ACP binding mode that favors Δ^9 desaturation. Analysis of docked ACP-desaturase complexes show suitable ACP orientation for Δ^9 desaturation for mutants CV1 and CV2 that identified interactions between the ACP and Lys262 of the castor desaturase. Even though this residue is unchanged as Lys257 in *T. alata*, the difference in mainchain position means the sidechain of this residue is pointing away from the ACP-interface. Thus, the lack of this stabilizing interaction likely contributes to the exclusive Δ^6 desaturation of TD6.

As described above, previous work identified Asp280 in the castor Δ^9 desaturase as a single key regioselectivity-determining residue. Its mutation to Lys resulted in the formation of an ionic interaction between its positive charge and a negative charge on a phosphate oxygen of the phosphopantetheine group of ACP. In turn, this enables ACP to bind in a low-energy mode enabling Δ^4 desaturation, simulating the regioselectivity of the ivy Δ^4 desaturase. The resulting altered binding orientation of ACP on the desaturase involves an approximately 65-degree rotation relative to that on the castor enzyme, enabling deeper penetration of the phosphopantetheine group and acyl chain into the substrate binding channel. For the *T. alata* desaturase, the basis for Δ^6 regioselectivity primarily involves desaturase residues Arg263 and Gln276, along with factors affecting the tilt of the C-terminal helix that result in a rotation of ACP of approximately 8 degrees with respect to the desaturase helix resulting in a relative orientation of ACP that is intermediate between those of the castor and ivy enzymes as shown in Figure 6.

In addition to acyl-ACP desaturases, ACP interacts with multiple enzymes of fatty acid synthesis (Chan and Vogel, 2010). The nature of the interaction between ACP and its various enzyme

partners is key to its multiple protein-protein interactions. ACP is an acidic protein and its interactions are predominantly through negatively charged residues in its recognition helix (Zhang et al., 2001) pairing with positive patches (composed of Arg and Lys) on its interacting proteins. Having a predominantly charge-based interaction of this type allows for changes in binding orientations mediated by small changes to positively charged patches on the interacting protein. Its diversity of binding would be less likely to occur if the interaction was based on a mixture of interaction properties such as charge, side chain size and/or hydrophobic interactions, which would be more resistant to fine tuning.

The methodology described herein (Figure 4) represents an advance in our ability to identify the basis of desaturase selectivity and specificity. Earlier, and in the absence of crystal structures amino acid sequence comparisons were used to design chimeric enzymes to map specificity-determining residues (Cahoon et al., 1997; Broun et al., 1998). The process was partially automated using the conserved property difference locator algorithm (Mayer et al., 2005). Solution of the crystal structures of several acyl-ACP desaturases with different regioselectivities i.e., the castor Δ^9 -18:0- and Δ^4 -16:0-desaturases and a substrate complex of the castor Δ^9 -18:0-ACP desaturase with its 18:0-ACP substrate gave the first clues as to the mechanism of remote control of regioselectivity alluded to by Bloch. In this approach, modeling of the ivy Δ^4 -16:0-ACP desaturase with its ACP substrate was used to identify distinct ACP-desaturase binding modes that permitted the large changes in the register of fatty acid moiety in the substrate-binding cavity required to enable changes in specificity. This approach was not generally useful because it failed to identify any potential ACP binding for the *T. alata* Δ^6 -16:0-desaturase, frustrating efforts to build a more general model of regioselectivity. This hurdle was overcome with the introduction of MD simulations into the workflow presented here that enabled the identification of the ACP binding on the *T. alata* Δ^6 desaturase. Furthermore, it permitted in silico evaluation of mutants designed to change regioselectivity in desired ways. This computational prescreening reduced the

number of promising candidates for which DNA was synthesized for expression and biochemical characterization. Similar approaches have been used in pharmacological applications as recently reviewed (Temml and Kutil, 2021), but to our knowledge this is the first application of design using MD simulation of diiron enzymes. While MD simulation-aided design has until recently been restricted to cases for which detailed x-ray or NMR structures are available, the availability of high fidelity AlphaFold-generated structural models may greatly increase the use of approaches such as that described herein (Jumper et al., 2021).

In addition to regioselectivity, several cases of altered chemoselectivity have been reported for acyl ACP desaturases including the ability of the castor Δ^9 desaturase and several mutants thereof to perform a second round of oxidation after the initial conversion of stearoyl-ACP to oleoyl-ACP. In one example, oleoyl-ACP was converted to erythro 9,10-dihydroxystearate by an unprecedented dioxygenase reaction (Whittle et al., 2020). In a separate example a triple mutant of the castor Δ^9 -18:0-desaturase converted oleoyl-ACP to the allylic alcohol trans-isomer (E)-10-18:1-9-OH (Whittle et al., 2008). The application of computational approaches to understanding these reaction trajectories may provide future insights upon which to base the design of additional functionality for this versatile class of diiron enzymes.

Conclusions

A structure-based approach in combination with *in silico* modeling was used to identify two key *T. alata* desaturase residues, Arg263 and Gln276, that enable stable binding of ACP in an orientation that favors Δ^6 desaturation. Additionally, factors in the C-terminal portion of the *T. alata* desaturase control the tilt of the C-terminal helix which prevents steric clashes between ACP and the C-terminal helix, thereby permitting an ACP binding orientation with respect to the desaturase enzyme that predisposes the positioning of C6 and C7 of the acyl chain adjacent to the

diiron center to enable Δ^6 desaturation. The mechanistic knowledge gained from these studies and the methods used herein lay the foundation for the redesign of desaturase regioselectivities at desired locations on the acyl chain to enable the synthesis of novel monoene products.

Materials and Methods

Cloning of the N-11 and N-11CLS variants of the T. alata Δ^6 -16:0-ACP desaturase.

Cloning and expression of the *Thunbergia alata* Δ^6 desaturase was previously described (Cahoon et al., 1994). Several variants were also cloned and characterized in this work. One such variant, designated N-11 was created in which 11AA were deleted from the N-terminus of the mature peptide by PCR amplification employing the following oligonucleotides:

T Δ 6TDVntrmDelNcoIF:

CTTTAAGAAGGAGATATACCATGGATAGAAAAATATCGTCTACTCGA and T Δ 6BgIIIR: ACTTGCATAGCAAGATCTACACTACACTCTTCTCCCAAAGAT. In this construct, K11 of the mature open reading frame was replaced by Met, and the open reading frame therefore started with the amino acid sequence MDRKISSTRR and ended at the natural c-terminus, i.e., ...PFSWIFGRRV. The oligonucleotide primers introduced flanking NcoI and BglII restriction sites into the amplified ORF that were used to clone the fragment into the corresponding sites of the pET9d expression vector (Novagen, Madison WI).

Both N-11 and N-11CLS *Thunbergia* Δ^6 desaturase variants were fully sequenced before expression and purification of the corresponding polypeptides.

Expression and Purification of the T. alata Δ 6 desaturase and its N-11 and N-11CLS variants.

pET9d constructs expressing N-11 were cultured in TB to an OD₆₀₀ of 0.5, at which time the media was supplemented with IPTG to 0.4 mM. Four hours after IPTG supplementation, cells

were collected by centrifugation at 15,000 x g. Approximately 2 grams of cells were resuspended in 5 ml 20 mM CAT buffer (30 mM MES, 33 mM HEPES, 33 mM NaOAc) pH 7.5, 50 mM MgCl₂ supplemented with 1 mg of lyophilized DNase. The suspension was passed twice through the French Pressed at 10,000 PSIG to lyse the cells and clarified by centrifugation at 25,000 x g for 30 mins at 4°C. The clarified extract was loaded onto 1.6 ml Poros 20HS sulphopropyl cation exchange column at 4 ml/min (Perceptive Biosystems, Framingham MA), washed with 10 column volumes of CAT buffer and developed using a linear gradient of CAT buffer to CAT buffer containing 1.2 M NaCl over a 4 min period (10 column volumes). Fractions were analyzed by 11% SDS-PAGE visualized by Coomassie Brilliant Blue stain. Desaturase-containing fractions were pooled and diluted 5-fold with 20 mM CAT, containing 0.24 M NaCl and 10% (w/v) glycerol to minimize protein precipitation. Desaturase-containing fractions were pooled and concentrated using a Millipore Amicon Ultra Centrifuge concentrator with a 30 kDa molecular weight cutoff (EMD-Millipore, Danvers, MA). In some instances, the desaturase was further purified by high performance size exclusion chromatography employing a Tosoh Bioscience (King-of-Prussia, PA) G3000SW column. Peak fractions were identified as concentrated as before. The resulting desaturase pool was divided into aliquots that were flash frozen on liquid nitrogen and stored at -80°C until analysis. The same procedure was used to purify *T. alata* mutants. The castor Δ^9 -18:0-desaturase and its variants were purified using a 20CM cation exchange column in place of the 20HS sulphopropyl used for the *T. alata*.

Crystallization, data collection and structure determination

Initial crystallization screening was performed by the sitting-drop vapor-diffusion method in 96-well plates using commercially available sparse matrix crystal screens (Qiagen). The *T. alata* desaturase N-11 construct produced initial crystals in a single condition, which was then optimized in 96-well plates. Diffraction-quality crystals of *T. alata* desaturase N-11 were ultimately grown at 277 K in sitting drops consisting of 0.75 μ l of protein solution (6 mg ml⁻¹)

mixed with 0.75 μ l of reservoir solution (0.25M CaCl₂, 0.1M Tris pH 8.0 and 16% PEG 6000) equilibrated against 60 μ l of reservoir solution. Prior to data collection, crystals were cryo-protected with 20% glycerol and flash-frozen in liquid nitrogen. X-ray diffraction data were collected at the European Synchrotron Radiation Facility (ESRF), Grenoble, France. Native data were collected to 2.05 Å resolution from a single cryo-cooled crystal on beamline ID23-2, which is equipped with a Pilatus 2M detector, at a wavelength of 0.8726 Å. The data were integrated in XDS (Kabsch, 2010), and scaled using AIMLESS (Evans and Murshudov, 2013) from the CCP4 suite (Winn et al., 2011), which determined the space group to be I2 with unit cell parameters of $a = 51.8$, $b = 76.6$, $c = 83.3$ Å, $\beta = 101.9^\circ$. Data collection and processing statistics are presented in Table 1. The structure of the *T. alata* desaturase N-11 construct was determined by molecular replacement in MOLREP (Vagin and Teplyakov, 1997) using a monomer of the castor desaturase structure (pdb id: 1AFR) as the model, and located one chain in the asymmetric unit. The structure was refined with alternating cycles of restrained refinement in REFMAC5 (Murshudov et al., 1997) and manual inspection and model-building in COOT (Emsley et al., 2010), with 5% of randomly selected reflections used to monitor the R_{free}. Towards the end of refinement, water molecules were added automatically in Coot before being checked manually and refined. Annealed composite omit maps, produced in PHENIX (Adams et al., 2010), were used periodically to ensure accuracy of the model. The geometry of the model was checked using the validation functions of COOT in addition to a final analysis in PROCHECK (Laskowski et al., 1993), and the fit between the data and model was assessed in SFCHECK (Vaguine et al., 1999). Figures for the manuscript were produced using VMD (Humphrey et al., 1996). Coordinates and structure factors for the high-resolution model have been deposited in the Protein Data Bank (PDB), with accession code 7PP7.

Molecular dynamics simulations

An all-atom simulation approach was used to find the optimal orientation of the ACP-*T. alata* desaturase complex that would permit Δ^6 desaturation on a C16 substrate. All simulations used the all-atom OPLS force field (Jorgensen and Tirado-Rives, 1988) in conjunction with the TIP3P water model (Jorgensen et al., 1983). Parameterization of the phosphopantetheine group was taken from our previous study (Baer et al., 2021). The iron atoms of the active site are modelled with the default Lennard-Jones parameters and a point charge of 2+. The system was neutralized by additional 40 sodium ions. Simulations were performed using the GROMACS 4.6.2 package (Hess et al., 2008) in the NVT ensemble at 300 K temperature using a velocity rescaling thermostat ($\tau = 0.1$ ps) (Bussi et al., 2007) and a time step of 2 fs. Hydrogen bonds were constrained using the LINCS (Hess, 2008) and SETTLE (Miyamoto and Kollman, 1992) algorithms and van der Waals interactions were shifted to 0 at 1.1 nm. Electrostatic interactions are treated using particle-mesh Ewald with a real space cut-off of 1.2 nm (Essmann et al., 1995).

The simulation consisted of a *T. alata* dimer with two ACPs and the substrate bound in their respective cavities. The substrate was modelled such that its acyl chain was oriented optimally with respect to the active site to facilitate Δ^6 desaturation, and the position of the acyl-chain as well as the desaturase backbone and iron atoms were restrained through the simulation using a force constant of 1,000 kJ/mol⁻¹ nm². The *T. alata* desaturase structure was aligned with those of the ivy and castor desaturases. Two previously identified starting configurations for the ACP were chosen, one corresponding to that of the ACP bound to the castor desaturase, and the other to that of modelled ivy desaturase orientation. The ACP was maintained as fully free to explore possible binding orientations over a period of 600 ns.

Model building for the simulations

The crystal structure revealed that residues 257-261 diverge from the positions of the equivalent residues in the castor and ivy desaturases, and are immediately followed by disordered residues 262-272, for which no density is observed. The missing residues were modelled by superimposing the castor structure and mutating the sidechains into the *T. alata* sequence. The loop structure was first relaxed and optimized using ROSETTA followed by running 100 ns of MD simulation of the solvated protein with restraints on the backbone C α and iron atoms.

ACP and Desaturase Docking

Two different docking protocols were used in this study. The first used ROSETTA to identify favorable ACP-*T. alata* desaturase complexes based on their interfacial interaction energy following the same protocol as in (Baer et al., 2021). Simulations were performed within the docking module of ROSETTA, (Davis and Baker, 2009; Leaver-Fay et al., 2011) using the two-stage protocol. The ACP model did not contain the substrate and serine 38 was replaced by a glutamate to preserve its overall charge distribution. A total of 6,000 trial structures were obtained using 3 different starting orientations for the ACP with respect to the *T. alata* desaturase: one taken from the castor complex, one from the modeled ivy complex and a third randomly generated control.

The modeling of mutated *T. alata* or castor desaturases with ACP were performed using the Haddock server (<http://haddock.chim.uu.nl/>). As HADDOCK uses information from identified or predicted protein interfaces to drive the docking process, with different starting configurations for interacting residues, different solutions can be obtained. The resulting complexes are clustered and scored based on the weighted sum of various energy terms (e.g., buried surface area, electrostatic energy, van der Waals energy). The phospho-serine residue was modeled as a glutamic acid in the Haddock procedure following the same protocol as we previously used (Guy et al., 2011).

Mutagenesis and in vitro enzymatic activity assay

Four variants were constructed based on castor and *T. alata* desaturase sequences. Representations of these variants as linear sequences are shown in Figure 6. Briefly, the castor variant (CV)1 contains residues 1-328 from the castor and 325-356 from the *T. alata* desaturase with mutations His268Arg and Asp280Gln; CV2 contains residues 1-267 from the castor and 263-356 from the *T. alata* desaturase; similarly, TV1 contains residues 1-324 from *T. alata* and 329-362 from the castor desaturase with mutations Arg263His and Gln276Asp; TV2 contains *T. alata* desaturase residues 1-262 and 268-362 from the castor desaturase. All four mutants were first cloned into a donor vector and subsequently introduced into a pHis9 vector for expression and purification of target proteins. A Tobacco Etch Virus (TEV) protease cleavage site was included between the His-tags and the N-termini of all four mutants to facilitate removal of the affinity tag after purification.

In vitro FA desaturase assay was performed as previously described (Cahoon et al., 1997). Methyl-esters of the products were analyzed by GC-MS. Deuterium-labelled 7,7,8,8-d₄-Palmitoyl-ACP was used as the substrate to distinguish between Δ^6 and Δ^9 desaturation: Δ^6 desaturation would give rise to a product with a mass ion -3 AMU relative to the substrate, i.e., 271 AMU for Δ^6 desaturation, whereas Δ^9 desaturation would yield a product with mass ion -2, i.e., 272 AMU.

Accession numbers: Coordinates for the crystal structure of the *Thunbergia alata* Δ^6 -16:0-acyl carrier protein desaturase was deposited in the Protein Data Bank as 7PP7.

609 **Supplemental Text.**

610 **Supplemental Figure S1. Purification of *T. alata* N-11 Δ^6 -16:0-ACP desaturase.**

611

612 **Figure S2. Overlay of *T. alata* desaturase monomer, in orange with mauve iron ions,**
613 **superimposed on the ivy desaturase monomer, depicted in green.**

614 **Figure S3. An electrostatic surface representation of the face of the Ivy, *T. alata*, and castor**
615 **desaturases that interact with ACP**

616 **Figure S4: The poorly defined *T. alata* 262-272 loop does not influence Δ^6 regioselectivity.**

617

618 **Figure S5: Time evolution of the root mean square deviation (RMSD) of ACP backbone**
619 **atoms with respect to the starting structure.**

620

621 **Figure S6: Time evolution of key distances between residues of the ACP and desaturase**
622 **starting from the ivy orientation.**

623

624 **Figure S7: Time evolution of key distances between residues of the ACP and desaturase**
625 **starting from the castor orientation.**

626

627 **Figure S8. Single ion monitoring (SIM) results for the fatty acid methyl ester products of**
628 **desaturase variants.**

629

Table S1: Docking results for TD6 and various mutants comparing the 10 highest ranked structures.

Table S2: Docking results for TD6 mutants comparing the 10 highest ranked structures.

Table S3: Docking results for CD9 and various mutants comparing the 10 highest ranked structures.

Table S4: Docking results for CD9 mutants comparing the 10 highest ranked structures.

Table S5: Docking results for TD6, TV1, TV2, CD9, CV1, and CV2 comparing the top 10 structures.

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653 Table

654 Table 1: Data collection and refinement statistics for the *T. alata* desaturase structure.

655 Data in parentheses correspond to the highest resolution shell.

<u>Data collection</u>		656
<u>Beamline</u>	<u>ESRF ID23-2</u>	657
<u>Wavelength (Å)</u>	<u>0.8726</u>	
<u>Space group</u>	<u>I2</u>	658
<u>Cell axes (Å)</u>	<u>51.51, 76.17, 83.17</u>	
<u>Cell angles (°)</u>	<u>90, 102.1, 90</u>	659
<u>Resolution (Å)</u>	<u>47.51-2.05 (2.11-2.05)</u>	
<u>R_{merge}</u>	<u>0.135 (0.696)</u>	660
<u>Mean I/σI</u>	<u>7.9 (2.0)</u>	
<u>Mean CC_{1/2}</u>	<u>0.992 (0.622)</u>	661
<u>Completeness (%)</u>	<u>99.6 (99.8)</u>	
<u>Number of reflections</u>	<u>70245 (5689)</u>	662
<u>Number of unique reflections</u>	<u>19684 (1509)</u>	
<u>Wilson B-factor (Å²)</u>	<u>10.2</u>	663
<u>Refinement</u>		
<u>Resolution (Å)</u>	<u>47.51-2.05</u>	664
<u>R/R_{free} (%)</u>	<u>19.8/26.9</u>	
<u>Number of non-hydrogen atoms</u>	<u>2700</u>	665
<u>Mean B value (Å²)</u>	<u>26.3</u>	
<u>Number of waters</u>	<u>150</u>	666
<u>rmsd bond lengths (Å)</u>	<u>0.015</u>	
<u>rmsd bond angles (°)</u>	<u>1.702</u>	667
<u>Ramachandran plot</u>		
<u>Residues in favored regions (%)</u>	<u>97.3</u>	668
<u>Residues in allowed regions (%)</u>	<u>2.7</u>	
<u>Residues in disallowed regions (%)</u>	<u>0</u>	669

Figure Legends

Figure 1: The structure of the *T. alata* desaturase dimer. Panel A, protomers of the dimer are shown in orange and grey. Cylinders represent the core four helix bundle that coordinates the diiron center, shown as magenta spheres. The blind-ending substrate binding channel that extends from the surface of the protein adjacent to the diiron center and beyond, is shown in green. Panel B, Ribbon representation of the superposition of the C α traces of the *T. alata* desaturase, gold and the castor desaturase in cyan. Key differences discussed in the text between the two structures are circled.

Figure 2 The base of the substrate-binding cavity. The *T. alata* desaturase structure (orange) is shown superimposed on the structure of the castor desaturase (cyan) with modelled 18C substrate (cyan). Residues lining the base of the substrate-binding cavity are represented as sticks.

Figure 3. The *T. alata*–ACP interface from simulations, with the desaturase (orange) and its residues labeled in black, and the ACP (blue) and its residue labeled in blue. Residues participating in the desaturase–ACP interaction are shown in stick representation, including the phospho-serine38. The C16 substrate is also shown as cyan.

Figure 4. Scheme to illustrate the integrated structural, computational and experimental workflow used to determine the basis for regioselectivity of the *T. alata* Δ^6 -16:0-desaturase.

Figure 5. In vitro enzyme activities of TD6, CD9 and variants using palmitoyl-ACP (7,7,8,8-d4) as a substrate. A, TD6; B, TV1; C, TV2; D, CD9; E, CV1; F, CV2. Schematics on the right show the sequences, with orange and blue bars derived from TD6 and CD9 sequences,

respectively. The identities of residues at the two key locations discussed in the text are indicated (note equivalent castor numbering is *T. alata* +5).

Figure 6. Comparison of the model of the *T. alata* Δ^6 desaturase-ACP complex determined herein with those of the castor Δ^9 desaturase-ACP and ivy Δ^4 desaturases. Alignment was based on superposition of the C α -fold of the central four helix bundle of the desaturases. The angle between helix X of the desaturase and the helix II of the ACP is shown. Highlighted in blue are the identified key positively charged residues along helix X for the desaturase, as well as residues directly interacting with the pantetheine linker.

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SUPPLEMENTAL INFORMATION: Guy et. al.

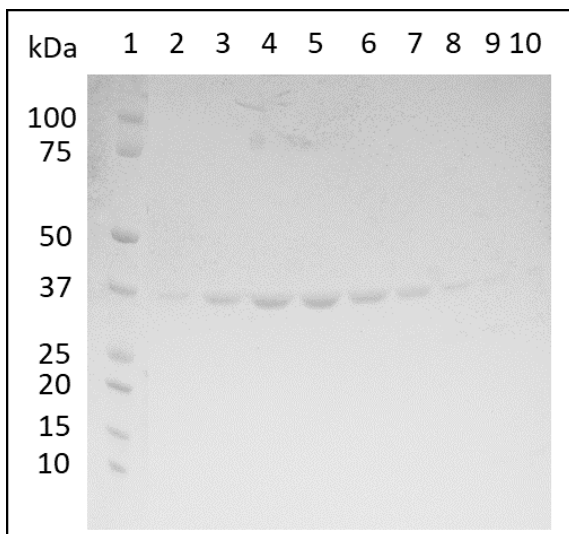
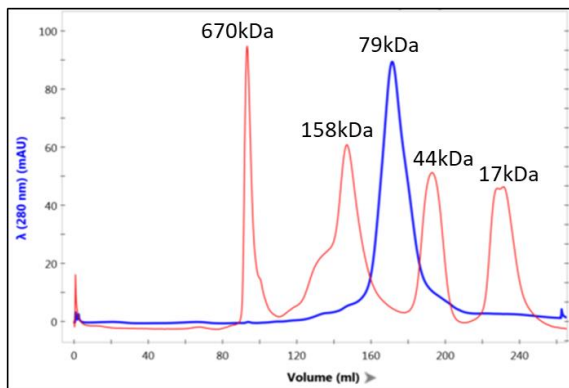
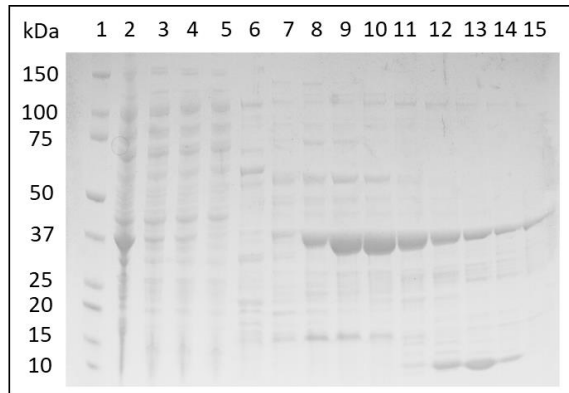


Figure S1. Purification of *T. alata* N-11 Δ^6 -16:0-ACP desaturase.

Upper panel: Fractions from *T. alata* N-11 expression and purification separated on SDS gel. Lane 1, molecular weight marker; lane 2, cell lysate; lane 3, clarified extract; lane 4, clarified extract passed 0.2um filter; lane 5, 20HS column flow through; lane 6-15, Poros 20HS sulphopropyl ion exchange chromatography fractions. Lane 9-11 were pooled for further purification by size exclusion chromatography (SEC) using preparative G3000 SW column.

Middle panel: SEC profile. Red trace, molecular weight standards; blue trace, *T. alata* N-11 dimer.

Lower panel: *T. alata* N-11 fractions eluted from SEC column. Lane 1, molecular weight marker; lane 2-10, SEC fractions. Lane 4-6 were pooled for crystallization.

Structural comparison of *T. alata* and ivy desaturases

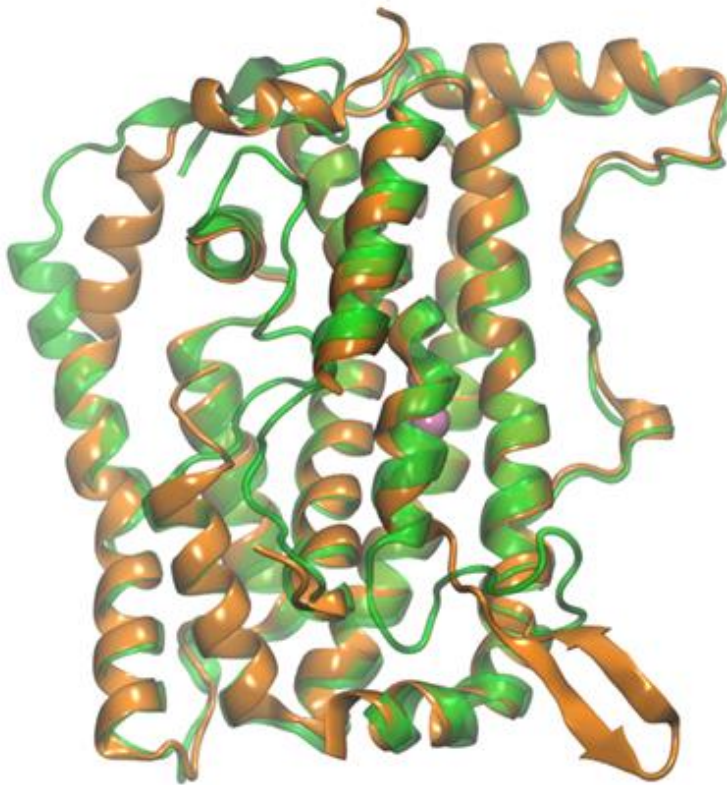


Figure S2. Overlay of *T. alata* desaturase monomer, in orange with mauve iron ions, superimposed on the ivy desaturase monomer, depicted in green.

Electrostatic surface potential of acyl-ACP desaturases from different sources.

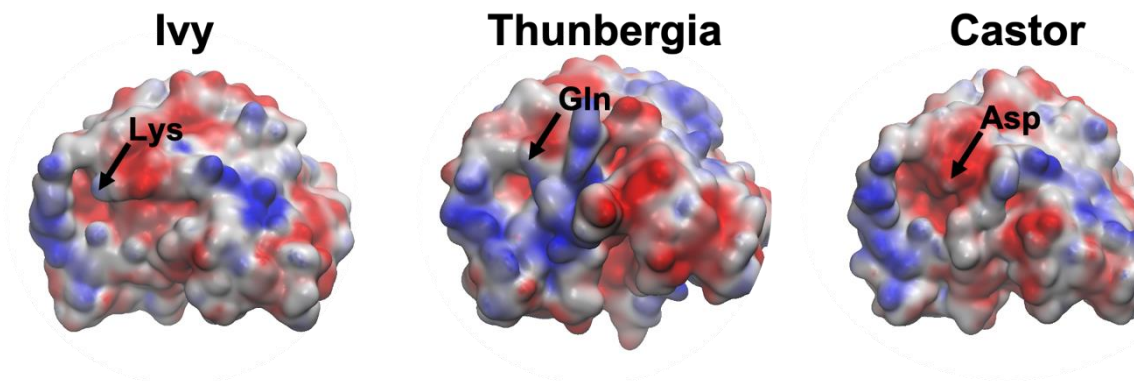


Figure S3. An electrostatic surface representation of the face of the Ivy, *T. alata*, and castor desaturases that interact with ACP. The identity of residues corresponding to the position of Asp280 of the castor desaturase are indicated in the ivy and *T. alata* desaturases.

Residues 262-272 that are undefined in the crystal structure are not involved in specifying regioselectivity.

To test whether residues 262-272 play a role in determining Δ^6 regioselectivity, we created a chimeric mutant N-11CLS in which the *T. alata* residues were replaced by the equivalent sequence from the Δ^9 castor desaturase. The mutant *T. alata* enzyme contained an 11 amino acid deletion along with a substitution of residues 262-272 for their equivalents in the castor desaturase. We compared the activity of the native *T. alata* desaturase with the N-11 and the N-11CLS. Each enzyme was expressed in *E. coli*, purified to near homogeneity and assayed for activity. To discriminate between desaturation reaction products and naturally occurring fatty acid contaminants that occur in substantial abundance in the reaction components, we used the tetra-deuterated, 7,7, 8,8 palmitoyl-ACP substrate as described in the main text. Figure S4, Panel A shows a reaction mixture lacking desaturase enzyme in which tetra-deuterated and endogenous 16:0, labeled as 1 + 2, in addition to endogenous 16:1 Δ^9 as peak 4 can be seen. Addition of native *T. alata* desaturase (Panel B) shows the accumulation of a unique additional peak 3 corresponding to 16:1 Δ^6 product that is unambiguously identified from the fragmentation of its dimethyl disilyl products (Panel E) of 175 and 190 AMU. Similarly, Panels C and D show the products formed when N-11 and the N-11CLS were added to the reaction, respectively. In all cases the fragmentation was indistinguishable from that shown in Panel E. Single ion monitoring for other 16:1 species such as 16:1 Δ^9 , failed to identify any additional products. Because exclusively 16:1 Δ^6 product is formed with *T. alata* desaturase containing both its native 262-272 loop sequence and *T. alata* N-11CLS desaturase, we conclude that this poorly defined loop does not contribute to regioselectivity at the Δ^6 position.

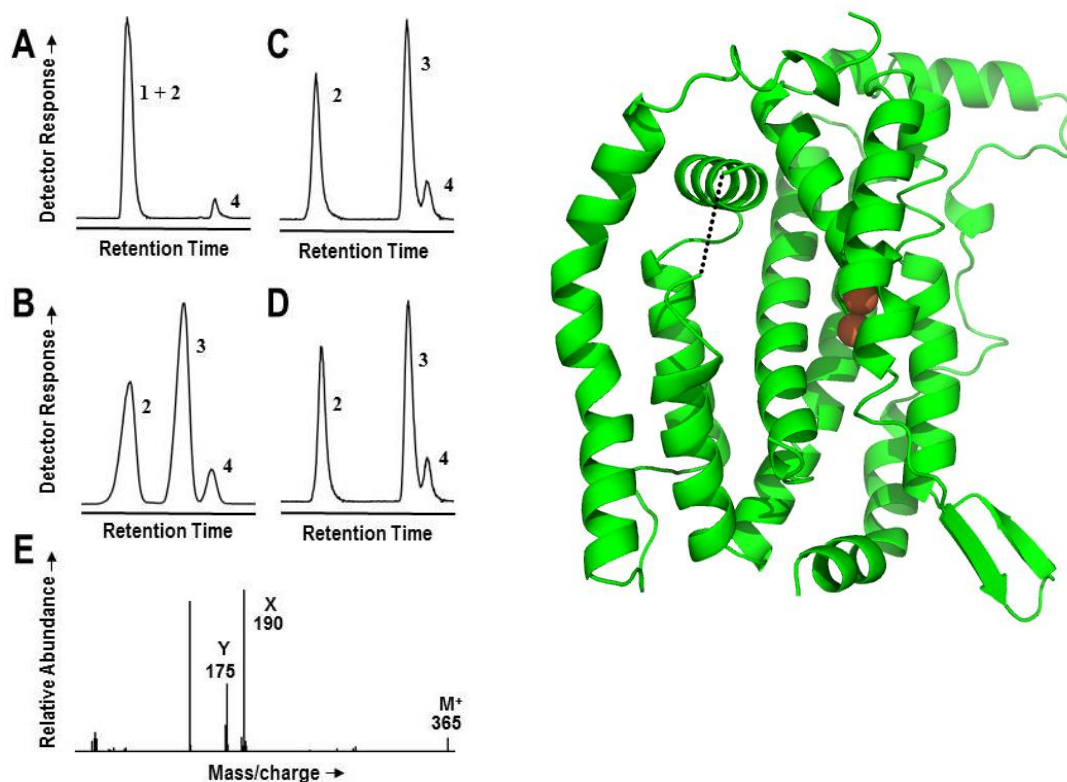


Figure S4: The poorly defined *T. alata* 262-272 loop does not influence Δ^6 regioselectivity. Left panel shows GC traces of methyl esters of reaction mixture without desaturase A, or with the native *T. alata* desaturase, B; *T. alata* N-11 C; and *T. alata* N-11CLS constructs D. Peak assignments are: 1, d4 16:0; 2, d0 16:0; 3, 16:1 Δ^6 ; 4, 16:1 Δ^9 . Panel E shows the diagnostic fragmentation pattern of the dimethyl disulfide derivative of 16:1 Δ^6 . Right panel shows the structure of the *T. alata* desaturase monomer, with the position of missing residues 262-271 indicated by a dashed line and the diiron center shown as brown spheres.

Molecular Dynamics Simulation Data

The time evolution and stability of the ACP-desaturase interface is analyzed along the MD simulations by monitoring ionic interactions and the root mean square deviation (RMSD) of the backbone of the ACP with respect to the starting structure. In all cases the RMSD indicates that ACP reorient in the first 20 ns of simulation (Figure S5). The structure obtained starting from the castor/ACP orientation shows larger deviations than that obtained starting from the ivy/ACP orientation. The RMSD of the backbone atoms of the recognition helix (residues 38-53) parallels the global RMSF.

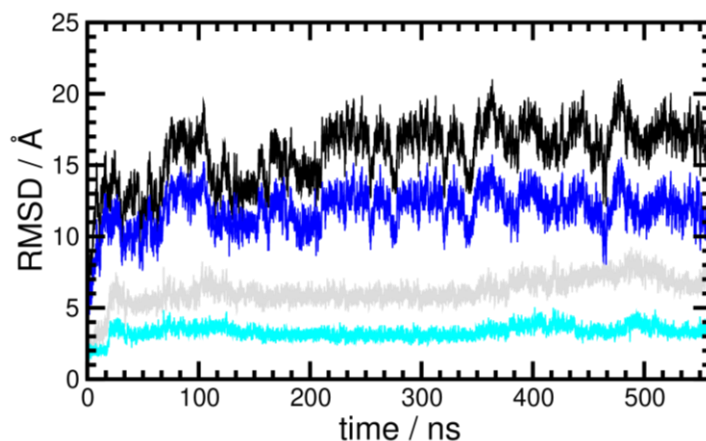


Figure S5: Time evolution of the root mean square deviation (RMSD) of ACP backbone atoms with respect to the starting structure. The RMSD of ACP for the simulation started from the ivy/ACP and castor/ACP starting orientation are reported as blue and black traces, respectively. The RMSD of the backbone atoms of recognition helix II (residues 38-53) are shown in cyan and gray for the ivy/ACP and castor/ACP starting orientations, respectively.

The main interactions between the ACP and desaturase are ionic in nature due to the high acidic residues of the ACP and basic residues of the desaturase at the protein/protein interface. The ionic interactions are analyzed by calculating all ionic contacts between charged residues of the ACP and desaturase. The contact distance between two residues is defined as the closest side chain/side chain distance between heavy atoms. A contact is defined if this distance is less than 4 Å. Different choices for the cutoff distance give consistent results. Additionally, the closest distance between the phosphate oxygens of the pantetheine linker and the surrounding polar residues are also shown in Figure S6 and S7. The time evolution of all the distances between

pairs of residues that have at least one contact at any time are shown in Figure S6 (ivy/ACP starting configuration) and S7 (castor/ACP starting configuration). The average number of contacts as a function of time is also reported at the bottom of Figure S6 and S7. Starting from the ivy/ACP orientation an average of 4 ionic contacts are formed, which include phosphate/GLN276 and phosphate/ARG263 interactions. In contrast, starting from the castor/ACP orientation only 1 contact is formed.

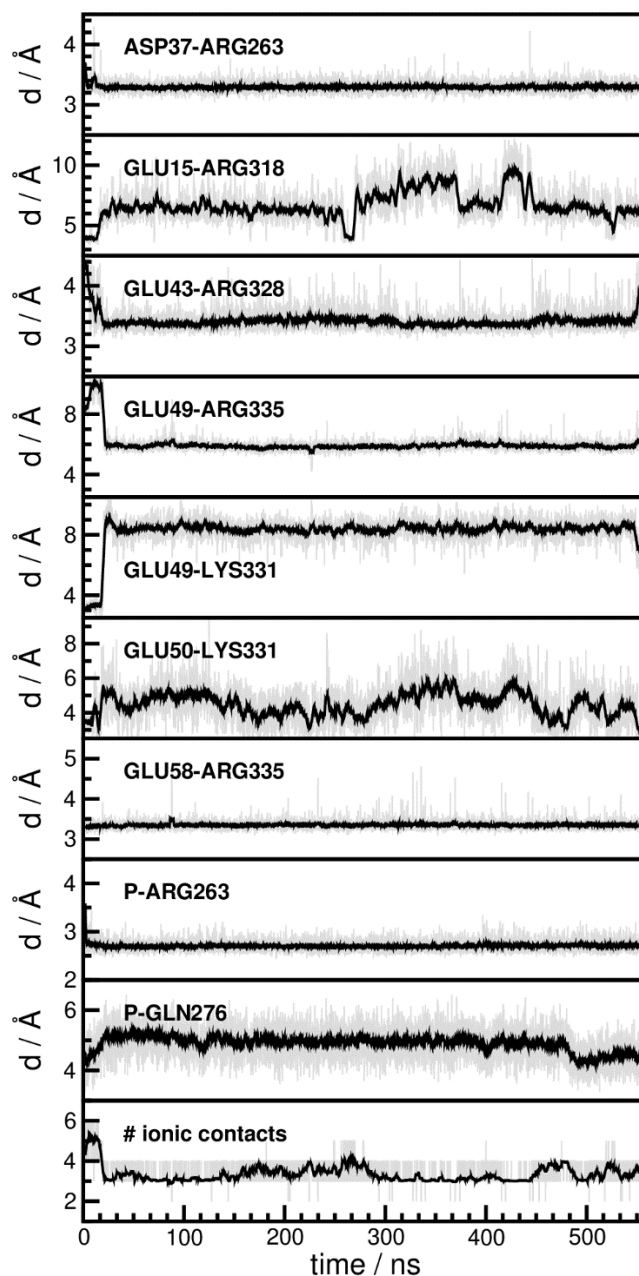


Figure S6: Time evolution of key distances between residues of the ACP and desaturase starting from the ivy orientation. Shown are all residues that form ionic contacts at least once during the simulation. Ionic contacts are defined using the distance between any sidechain heavy atom of charged residues between ACP and desaturase. In second and third to the last panels, P stands for the phosphate group. A cut-off distance of 4 Å was adopted to calculate the average number of contacts.

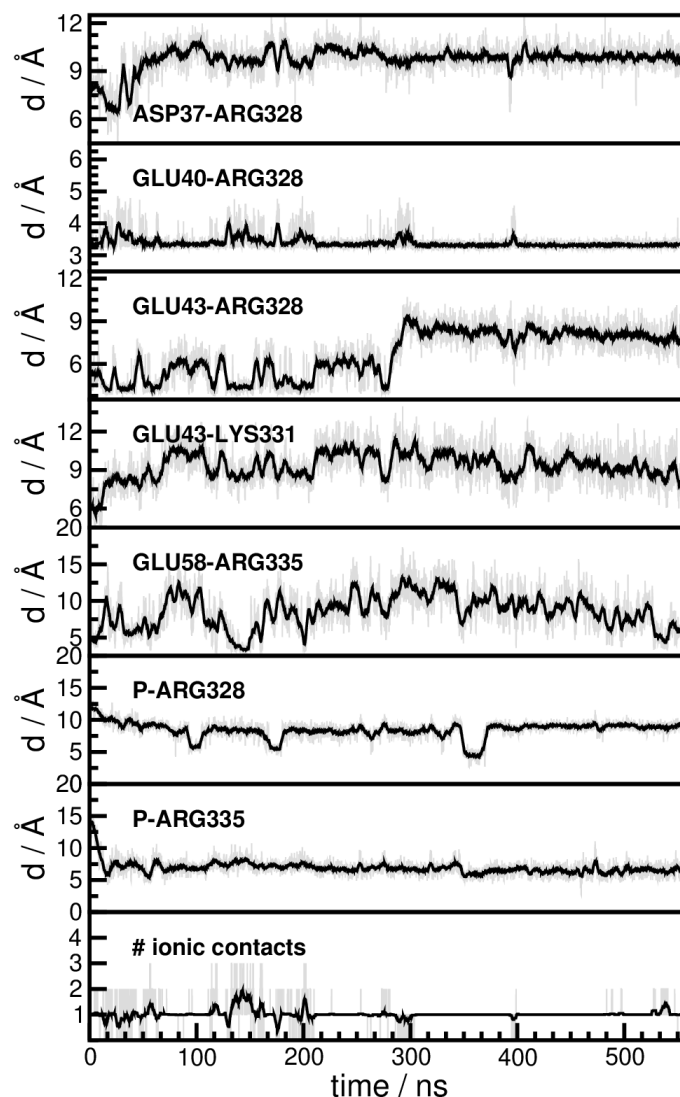


Figure S7: Time evolution of key distances between residues of the ACP and desaturase starting from the castor orientation. Shown are all residues that form ionic contacts at least once during the simulation. Ionic contacts are defined using the distance between any sidechain heavy atom of charged residues between ACP and desaturase. In second and third to the last panels, P stands for the phosphate group. A cut-off distance of 4 Å was adopted to calculate the average number of contacts.

Docking Analysis

Docking simulations are quantitatively analyzed to identify how mutations change the orientation of the ACP with respect to the desaturase. Only the top 10 structures predicted by the Haddock2.4 workflow were considered. To assess which mutations are optimally orienting ACP for the desired regioselective desaturation, the top 10 leads were further ranked according to the RMSD of the backbone atoms of the recognition helix (residue 38-53) of the ACP calculated with respect to the ivy (Δ^4), castor (Δ^9) and *T. alata* (Δ^6) after superimposing each desaturase with the castor reference structure (PDB ID 1AFR). Structures with RMSD smaller than 5 Å are considered favorably aligned to yield the desired regioselectivity. Tables S1 and S2 summarize the mutation studies for the TD6, showing that most mutations conserve the D6 orientation, but mutations that mimic the CD9 (R263H and Q276D) or ivy (R263H and Q276K) do not lead to any preferred orientation. Similarly, mutations of CD9 (Tables S3 and S4) show a markedly favorable Δ^9 orientation and do not result in any favorable Δ^6 orientation. Analysis of ACP docked to the designed desaturases (Table S5) TV1, TV2, CV1, and CV2 show great agreement with the major products observed experimentally.

TD6										
score	-81.5±6.3	-74.6±7.1	-73.4±9.2	-71.2±14.3	-68.5±6.6	-66.3±3.3	-61.6±7.9	-60.4±3.3	-53.2±8.3	-51.9±2.7
rmsd(d4)	9.8±0.4	7.6±0.4	14.7±0.2	15.3±1	9.4±0.7	13.9±0.1	12.7±0.6	17.2±0.5	10.7±1.2	6.8±0.3
rmsd(d6)	12.3±0.8	3.1±0.3	18.6±0.5	9.6±0.9	13.1±0.6	13.8±0.9	11.6±0.5	15.1±0.4	13.3±1.6	6.9±1.8
rmsd(d9)	16±0.5	9.5±0.7	19±0.3	13.3±1.2	13.5±0.8	12.6±0.4	14.4±0.2	20.8±0.5	16.3±1.1	6.6±1.5
TD6-R263H										
score	-79.7±4.9	-79.5±6.9	-57.2±5.3	-55.9±9	-47.8±9.3	-43.5±9.5	-42.8±6.4	-41±2.1	-39.5±2.5	-19.8±3
rmsd(d4)	15.5±0.6	13.5±0.7	16.3±1.1	17.1±1.6	15.2±0.8	15.1±0.6	13.2±1.4	16.5±0.3	14.9±0.7	17.4±1.5
rmsd(d6)	19.3±0.5	13.1±1.8	14.6±0.5	11.5±1.1	15.9±3.4	15±1.9	17.6±1.2	17.6±0.6	14.8±1.3	12.7±2.5
rmsd(d9)	19.9±0.7	14.5±2.1	19.9±1	16.3±2.9	13.9±1.4	16.5±2	13.8±0.8	10.2±0.3	18.3±0.6	16.9±2.8
TD6-R263H, K255R, M256K										
score	-72.6±11.2	-69.5±3.8	-67.5±2.6	-63.4±8.1	-56.6±5.1	-55±0.9	-52.1±3.3	-44.6±12.4	-43.7±6.6	-42.1±1.8
rmsd(d4)	9.7±0.3	14.3±0.2	14.7±0.5	15.2±0.8	13±1.4	8±1.1	14.7±0.6	14.4±1.2	13.4±0.9	15.5±0.6
rmsd(d6)	12.7±0.7	13.1±0.3	18.1±0.6	9.5±1	17.1±1.6	2.6±1.1	11.4±0.1	15±0.4	11.5±0.7	10.9±0.2
rmsd(d9)	12.9±0.9	13.6±0.6	18.7±0.4	14.4±1.3	12.7±2.3	9.5±0.6	15±0.7	18.7±0.4	14.1±1.3	14.3±1.2
TD6-Q276D										
score	-84.5±10.3	-77±5.3	-72.2±3.9	-70.8±7.3	-68.4±6.5	-66.2±4.2	-64.6±2.6	-63.8±4.5	-62±12.7	-59.1±5.5
rmsd(d4)	7.7±0.2	14.2±0.2	10.2±0.5	14±0.8	15.1±0.4	14.1±0.1	13.1±0.9	9.6±0.4	15.4±0.9	12.2±0.4
rmsd(d6)	2.8±0.4	18.4±0.3	13.7±0.9	14.3±3.1	18.3±0.8	13.5±0.1	11±1	13.2±0.4	17.5±1.3	16.6±0.4
rmsd(d9)	9.8±0.5	19±0.2	16.3±0.3	13.5±1.7	18.6±0.8	12.3±0.9	13.4±1.7	12.7±0.5	14.3±0.9	13±1.1
TD6-Q276D, R263H										
score	-83.7±1.9	-76.2±6.4	-67±1.1	-66.8±14.5	-59.6±4.8	-52.3±12.1	-48.4±1.9	-43.9±10.6		
rmsd(d4)	12.9±0.1	9.4±0.7	9±0.2	14.8±1	17.6±0.3	15.9±1.3	14.1±0.1	14.1±1		
rmsd(d6)	13.5±0.3	12.9±0.3	12.3±0.5	18.7±0.9	11.6±0.3	14.9±1.1	15.6±0.3	12.1±2		
rmsd(d9)	11.3±0.4	13.1±0.2	11.8±0.2	19.2±1	17.4±0.5	19.9±1.5	10.7±1	12.3±2.2		
TD6-Q276D, R263H, K255R, M256K										
score	-87±9.5	-76.7±9	-74.2±9.5	-70.3±8.3	-65.6±2.3	-63.8±4.9	-62±2.6	-60.6±6.2	-58.1±4.2	-52.2±5.8
rmsd(d4)	13.4±0.9	6.2±0.9	14.6±0.4	13.4±0.8	14.2±0	9.5±0.5	15.1±2.1	15.6±0.7	14.3±0.2	14.5±0.8
rmsd(d6)	14.2±0.8	3.7±1.3	18.4±0.7	10.7±0.9	13.4±0.3	13±0.7	20.9±2.4	17.8±0.9	13.7±0.1	11±0.5
rmsd(d9)	16.7±1.5	9.7±0.6	19±0.4	14±1.2	13.5±0.5	13.3±0.7	14.9±1.7	14.3±0.9	13.7±0.3	14.2±1.7

Table S1: Docking results for TD6 and various mutants comparing the 10 highest ranked structures. For each mutation the Haddock2.4 score and the RMSD for the backbone atoms of the recognition helix (residue 38-53) of the ACP are given with respect to the ivy (Δ^4), castor (Δ^9) and *T. alata* (Δ^6). For each mutant, the RMSD values smaller than 5 Å, or the smallest values are highlighted in green.

TD6-Q276K										
score	-85±9.2	-81.9±5.6	-72.9±9.9	-70±6.7	-66.8±4.4	-65.8±2	-65.4±3.1	-64.7±8.4	-59.1±16.1	-58±4
rmsd(d4)	8.3±0.8	9.7±1.1	14.2±0.6	10±0.6	13.9±0.3	7.4±0.8	9.3±0.9	17±0.8	16.1±1.4	13.8±0.8
rmsd(d6)	3.1±1.5	12.4±1.2	16.9±1.3	12.4±0.8	13.3±0.3	4.4±2.8	12.9±0.7	15.2±0.3	21.4±1.9	11.9±0.7
rmsd(d9)	9.8±1.3	15.8±0.9	18.3±0.2	15.4±0.7	13.4±0.4	9±0.4	12.7±0.5	20.3±0.5	16.9±1.8	14.8±1.4
TD6-Q276K, R263H										
score	-90.8±1.3	-82.8±6	-79.2±8	-64.6±16.1	-35±9.9					
rmsd(d4)	12.9±0.2	5.2±0.6	9.6±0.2	13±1.2	15.2±0.6					
rmsd(d6)	13.8±0.3	6.6±0.6	12.8±0.6	8.7±0.8	15.1±1.4					
rmsd(d9)	11.6±0.2	11.5±0.4	12.9±0.4	7.4±1.8	13.9±1.3					
TD6-Q276K, R263H, K255R, M256K										
score	-90.3±22.5	-77.6±2.9	-70.6±2.4	-66.3±4.5	-65±13	-64.5±13.3	-64.4±8.5	-54.1±8.9	-52.5±3.7	-52.1±8.5
rmsd(d4)	10.2±0.5	16.6±1	8.2±1.2	14±0.2	13.9±0.4	16.3±0.5	14±0.3	12.5±0.2	8.7±0.6	16.2±0.8
rmsd(d6)	9.1±0.2	15.7±1	3.5±1.2	13.7±0.4	17.2±0.4	15.1±1	8.4±0.5	10.9±0.7	12.2±0.8	22.6±1.1
rmsd(d9)	13.5±0.6	20.6±1.3	9.7±1.6	13.4±0.6	18±0.4	20.3±1	11.7±0.4	13.1±0.3	12.6±0.5	16.2±1.3

Table S2: Docking results for TD6 mutants comparing the 10 highest ranked structures. For each mutation the Haddock2.4 score and the RMSD for the backbone atoms of the recognition helix (residue 38-53) of the ACP are given with respect to the ivy (Δ^4), castor (Δ^9) and *T. alata* (Δ^6). For each mutant, the RMSD values smaller than 5 Å are highlighted in green. Orange highlight shows orientations that are in between Δ^4 and Δ^6 .

CD9										
score	-105.9±9.2	-79.8±1.1	-75.8±2.6	-74.4±10.5	-67±1.7	-64.4±14.1	-63.2±5	-58.8±5.7	-56.5±3.1	-45.6±4.7
rmsd(d4)	13.6±0.3	8.3±1.8	16.1±0.5	14.2±0.5	14.7±0.6	13±0.8	12.5±1.3	13.4±0.3	9.7±0.9	15.3±0.7
rmsd(d6)	15.1±0.6	9.8±2.4	15.2±0.7	11.1±0.3	16.3±0.9	19.6±0.9	16.8±2.6	13.3±1.1	8.2±1.3	21.6±0.9
rmsd(d9)	12.1±0.2	3.8±0.2	20.2±0.8	11.7±1.5	12.6±0.6	14.1±1	13.3±3.1	11.3±0.8	4.2±0.7	17.6±1
CD9-D280Q, H268R										
score	-97.4±2.3	-90.4±16.3	-82.2±10.5	-80.3±5.8	-69.2±12.4	-67.4±13.3	-66.2±4.2	-54.5±4.4	-50.4±7.1	-43.8±8.9
rmsd(d4)	13.3±0.2	14.3±0.3	14.4±0.4	9.2±1.2	8.3±0.5	12.1±1.1	13.2±0.2	12.5±1	11.6±1.7	12.2±1.1
rmsd(d6)	14.6±0.4	16.1±0.5	11.1±0.3	10.2±0.3	7.2±1.5	17.2±0.6	13.8±0.7	17.3±1.9	15.1±1	15.9±1.2
rmsd(d9)	12.3±0.3	12.2±0.4	12.3±0.6	2.7±0.5	4.9±0.8	14.7±1.4	11±0.7	13.5±2	8.3±1.2	13.3±0.8
CD9-D280Q, H268R, K262A										
score	-86±7.1	-70.1±9	-68±1.1	-63.8±3	-54.4±6.1	-52.9±1.9	-50.9±8.6	-49±8	-46.8±6.8	-41.6±5
rmsd(d4)	14.3±0.6	13.6±0.3	12.4±1.6	12.6±2.7	9.5±0.7	14.6±1.2	16.8±0.3	14.9±0.6	17.6±0.9	14.4±1
rmsd(d6)	15.9±0.9	13.2±1.3	13.8±1.5	13.5±3.1	5.8±0.6	15±1.5	18.9±0.4	14.7±1	23.2±1	20.3±1.1
rmsd(d9)	12.3±0.4	12.2±1.6	6.2±1	5.2±2.5	7.6±2.2	7.8±0.9	14±0.3	11.9±0.8	15.8±1.2	14.3±1
CD9-D280Q, H268R, K262A, K346A										
score	-78.6±4.9	-69±4.5	-62.6±3	-62.4±7.6	-60.2±4.9	-59.9±1.8	-52.6±9.7	-44.7±5.2	-39.2±2.5	-32.8±6.2
rmsd(d4)	14.2±0.8	15.5±0.7	13.6±0.2	10.2±0.6	17.1±0.4	15.9±0.3	13.7±0.5	14.5±2.2	13.7±0.7	16.8±0.8
rmsd(d6)	15.8±1	16.5±1.1	12.8±1.3	11.1±2.8	17.2±0.5	17.2±0.6	14.4±1.4	14.8±2.9	15.6±2	19±0.8
rmsd(d9)	12±0.4	8±1	12.2±1	4.3±1.5	11.7±0.8	8.9±0.3	11.7±0.5	8.2±1.7	12.2±1	10.7±0.8
CD9-D280Q, H268R, K262A, K346A, R340A										
score	-80.3±3.6	-58.7±1.5	-53.8±4.7	-53.2±6.3	-45.5±2.9	-41.2±4.8	-33.2±5.2	-14.3±3.9		
rmsd(d4)	13.6±0.3	10.8±0.6	12.9±0.3	8.8±1.5	13.3±0.2	14±0.6	10.8±0.8	11.6±0.2		
rmsd(d6)	14.8±0.7	8.3±0.4	15.2±0.3	6.9±1.2	13.9±0.7	14.9±1.3	16.6±0.8	9±0.6		
rmsd(d9)	12.4±0.3	4.3±0.9	12.4±0.2	3.7±1.5	13.9±0.4	12.4±1.3	11±0.9	6.5±0.8		
CD9-R180D										
score	-102.1±5.2	-84.3±12.5	-69.8±1.6	-62.6±4.7	-61±2.5	-58.8±6.1	-48.7±5	-32.7±6.8		
rmsd(d4)	13.6±0.2	14.5±1.1	9.8±1.9	9.8±0.7	11.8±1.4	15.1±0.8	12.9±3.3	17.9±1		
rmsd(d6)	14.9±0.4	16.3±1.4	11.1±3.2	6.6±0.6	17.1±2.4	21.3±0.8	15.1±2.7	20±0.2		
rmsd(d9)	11.8±0.2	12.7±0.9	4.8±1	5.3±0.4	13±1.8	17.9±0.5	7.4±1.8	14.1±0.5		

Table S3: Docking results for CD9 and various mutants comparing the 10 highest ranked structures. For each mutation the Haddock2.4 score and the RMSD for the backbone atoms of the recognition helix (residue 38-53) of the ACP are given with respect to the ivy (Δ^4), castor (Δ^9) and *T. alata* (Δ^6). For each mutant, the RMSD values smaller than 5 Å, or the smallest values are highlighted in green.

CD9-R180D, D280Q										
score	-99.2±5.2	-77±14.2	-75.3±2.3	-63.2±13.5	-59.7±2.6	-56.6±4.6	-55.9±9.3	-51.7±4.6	-50±6.6	
rmsd(d4)	13.7±0.3	13.2±0.2	8.6±1.5	13.8±0.5	12.3±1	14.5±0.5	13.9±0.1	12.9±2.4	9.7±1	
rmsd(d6)	15±0.6	14.1±0.6	6.2±0.6	11±0.1	16.1±1.6	20.5±0.7	11.8±0.1	18.7±3.1	7.9±2.3	
rmsd(d9)	11.9±0.2	11.4±0.2	5.2±1	12.1±0.8	12±1.5	16.8±0.8	13.4±0.4	14.3±3.1	4.7±0.7	
CD9-R180D, D280Q, H268R										
score	-97.4±2	-73.5±2.7	-69.6±6.8	-59.1±5.9	-58.1±14.2	-57.1±3.1	-52.3±3.8	-48±10.2	-45±3	
rmsd(d4)	13.5±0.1	9.5±1.6	13.3±0.3	12.3±2	15.2±0.4	14.9±2	14±1.7	14±0.2	9.8±0.8	
rmsd(d6)	14.9±0.1	10±2.7	13.3±1.1	18.2±1.3	16.9±0.8	20.3±2.5	18.3±1.3	15.1±0.7	9.3±0.8	
rmsd(d9)	12.2±0.5	4.1±1.1	12.4±0.5	12±0.9	12.2±0.6	17±2.1	12±2.3	12±0.2	4.5±0.9	
CD9-R180D, D280Q, H268R, K262A										
score	-101.1±1.3	-76±4.2	-67.1±1.1	-62.8±6.6	-59.5±2.6	-57.6±8	-57.1±10	-55.9±4.2	-55.7±9.9	-42.2±7
rmsd(d4)	13.7±0.2	10.3±1.7	9.8±1.5	11.7±0.8	9.6±0.4	13.5±0.2	13.1±0.3	14.6±0.6	12.4±1.4	10.9±1.1
rmsd(d6)	15.1±0.5	11.9±2.2	11.6±2.5	16.5±0.9	7.7±0.7	13.2±0.6	13.6±1	20.8±0.9	16.4±1.5	17.1±1.3
rmsd(d9)	11.9±0.4	4±1.5	3.9±1.9	12.4±1.3	4.7±1.7	11.6±0.7	13.2±1.5	17±1	14.8±1.8	12.9±0.9
CD9-R180D, D280Q, H268R, K262A, K346A										
score	-77.8±8.6	-66.8±5.9	-63.7±1.6	-59.4±5.4	-58.6±1.2	-57.6±3.2	-54.1±9.4	-52±1.9	-49.2±7.4	-46.2±4
rmsd(d4)	13.5±0.4	15.8±0.4	7.8±1.3	13.9±2.4	18.4±0.6	17.8±0.8	14.7±0.4	17.1±0.9	15.2±0.5	15.3±1.1
rmsd(d6)	14.6±0.7	16.8±2	8.3±2.3	17.1±3.7	24±0.7	16.7±1.1	20.9±0.3	23±0.9	12.9±1.9	16.3±0.9
rmsd(d9)	11.5±0.6	14.2±0.7	5.3±0.8	8.8±4.2	16.5±0.7	11.2±1	17.1±0.7	16.5±1	13.6±2.9	9.5±1.3
CD9-R180D, H268R										
score	-99.3±2	-75.8±4.1	-66.7±8.4	-61±5.9	-57±9.4	-56.8±7.6	-53.7±3.1	-50±6.9	-47.7±6	-47.6±4.4
rmsd(d4)	13.8±0.2	9.1±1.4	11.5±2	11.2±0.9	13.2±0.2	14.9±0.8	8±1.1	14.2±0.4	16±0.7	10.2±1.1
rmsd(d6)	15.5±0.5	8.1±2.2	14.2±2.5	15.9±1.2	13.4±0.2	13.9±1	6.6±0.7	15.6±0.8	22.1±1.1	10.3±2.6
rmsd(d9)	12.1±0.2	4.2±1.4	5.9±2.4	12.1±1.3	11.3±0.7	18.8±1.6	6.1±0.7	12.1±0.2	18.1±0.7	5.1±1
CD9-R180D, D280Q, H268R, K262A, K346A, R344A										
score	-80.6±5.6	-58.2±2.4	-57.1±1.7	-53.8±4.2	-53.7±1.8	-53.3±4.1	-48.2±3.3	-47.6±3.1	-46.6±5.6	-40.6±11.5
rmsd(d4)	13.5±0.2	10.7±2.3	10.5±1.6	14.7±0.5	15.2±0.7	15.6±0.5	16.9±1.2	11±0.8	13.1±0.2	14±1.2
rmsd(d6)	15.1±0.7	11.2±4	11.8±2.1	21±0.7	21.4±1	16.7±0.8	21.9±1.2	17.1±0.7	13±0.9	16.1±1
rmsd(d9)	12.2±0.2	4.8±2.3	3.8±1.6	17.1±0.9	17.2±0.6	8.2±0.7	14.1±1.1	11.5±0.8	12.7±1	7.9±0.8

Table S4: Docking results for CD9 mutants comparing the 10 highest ranked structures. For each mutation the Haddock2.4 score and the RMSD for the backbone atoms of the recognition helix (residue 38-53) of the ACP are given with respect to the ivy (Δ^4), castor (Δ^9) and *T. alata* (Δ^6). For each mutant, the RMSD values smaller than 5 Å, or the smallest values are highlighted in green.

TD6										
score	-81.5±6.3	-74.6±7.1	-73.4±9.2	-71.2±14.3	-68.5±6.6	-66.3±3.3	-61.6±7.9	-60.4±3.3	-53.2±8.3	-51.9±2.7
rmsd(d4)	9.8±0.4	7.6±0.4	14.7±0.2	15.3±1	9.4±0.7	13.9±0.1	12.7±0.6	17.2±0.5	10.7±1.2	6.8±0.3
rmsd(d6)	12.3±0.8	3.1±0.3	18.6±0.5	9.6±0.9	13.1±0.6	13.8±0.9	11.6±0.5	15.1±0.4	13.3±1.6	6.9±1.8
rmsd(d9)	16±0.5	9.5±0.7	19±0.3	13.3±1.2	13.5±0.8	12.6±0.4	14.4±0.2	20.8±0.5	16.3±1.1	6.6±1.5
TV1										
score	-73.7±6.4	-72.6±1.3	-69.7±2	-69±12.1	-61.1±5	-56.6±4.6	-50.1±4.2	-44.1±2.3	-43.7±1.7	-41.1±2.9
rmsd(d4)	13.2±0.4	12.5±0.4	14±0.2	10.1±0.4	14.6±0.2	13.8±0.5	13±0.5	14.1±0.1	12.7±1.5	14.5±0.8
rmsd(d6)	11.5±0.4	17.8±0.6	13.7±0.5	9.6±1.3	14.2±0.8	12.4±0.9	17.5±0.8	13.8±0.2	16.9±2.1	19.4±1.1
rmsd(d9)	12.4±0.3	11.7±0.3	12.3±2.1	4.5±0.7	13.7±0.8	8.8±0.6	17±0.6	12.9±0.9	10.1±2	13.2±2.3
TV2										
score	-72.4±5	-71.5±3.6	-69±3.3	-62.5±12.3	-60±3.6	-57.7±6.9	-54.5±5.3	-51.5±10	-48.1±2.8	-45.7±8.6
rmsd(d4)	13.3±0.3	14.1±0.1	12.9±0.7	12.3±0.3	13.6±1.3	14.3±0.3	8.6±0.7	14.6±0.4	14±0.2	13.4±0.4
rmsd(d6)	11.4±0.3	13.3±0.6	11.7±0.9	15.6±1.2	19.1±1.7	13.1±0.9	14.6±0.7	14.2±2.3	14.6±0.9	17.3±0.9
rmsd(d9)	12±0.9	13.2±0.8	8.6±0.5	16±0.9	13.4±1.7	13.9±1.1	10.8±0.7	14.1±1.9	12.5±0.2	10.4±1.4
CD9										
score	-105.9±9.2	-79.8±1.1	-75.8±2.6	-74.4±10.5	-67±1.7	-64.4±14.1	-63.2±5	-58.8±5.7	-56.5±3.1	-45.6±4.7
rmsd(d4)	13.6±0.3	8.3±1.8	16.1±0.5	14.2±0.5	14.7±0.6	13±0.8	12.5±1.3	13.4±0.3	9.7±0.9	15.3±0.7
rmsd(d6)	15.1±0.6	9.8±2.4	15.2±0.7	11.1±0.3	16.3±0.9	19.6±0.9	16.8±2.6	13.3±1.1	8.2±1.3	21.6±0.9
rmsd(d9)	12.1±0.2	3.8±0.2	20.2±0.8	11.7±1.5	12.6±0.6	14.1±1	13.3±3.1	11.3±0.8	4.2±0.7	17.6±1
CV1										
score	-93.6±3.8	-74.2±1.6	-74±2.8	-58.9±6	-56.6±1.1	-56.5±3.6	-50.3±6.5	-43.2±0.9	-37.2±4.5	
rmsd(d4)	10.5±1	4.9±0.3	14.4±2.5	15.9±0.5	9±1.1	11.4±0.7	4.4±0.7	15.7±0.9	13.2±1.1	
rmsd(d6)	5.3±0.8	5.9±0.3	8.5±2.6	16.1±0.5	7.9±0.3	15.7±0.7	6.1±0.7	10.9±0.6	12.6±1.6	
rmsd(d9)	11.2±1	11.3±0.5	13.5±2.2	20.7±0.5	12±0.7	15.9±0.7	11±0.2	14.6±1.5	8.2±0.9	
CV2										
score	-87±3.3	-86.8±3.8	-75±6.5	-66.7±8.5	-66.6±3.2	-62.3±8.2	-57.4±3.6	-53.6±4.6	-48.3±6.5	-39.1±3
rmsd(d4)	5.2±0.8	9.4±0.9	8.4±0.3	8.4±1.4	10.5±0.6	14.5±0.9	9.5±1.5	16.2±0.5	11.8±0.8	15.6±1
rmsd(d6)	6.2±0.4	4.3±0.9	5.4±0.4	5.6±0.6	4.6±1	14.1±0.5	9±0.6	15.8±0.3	15.7±0.8	11.1±0.6
rmsd(d9)	11.4±0.9	10.2±0.4	12.5±0.8	13±0.9	9.7±0.9	18.2±1.1	13.1±0.9	20.4±0.4	16.3±0.8	14.9±1.1

Table S5: Docking results for TD6, TV1, TV2, CD9, CV1, and CV2 comparing the top 10 structures. For each desaturase the Haddock2.4 score and the RMSD for the backbone atoms of the recognition helix (residue 38-53) of the ACP are given with respect to the ivy (Δ^4), castor (Δ^9) and *T. alata* (Δ^6). For each mutant, the RMSD values smaller than 5 Å are highlighted in green, and orange highlights show orientations that have a RMSD values between 5 and 6 Å.

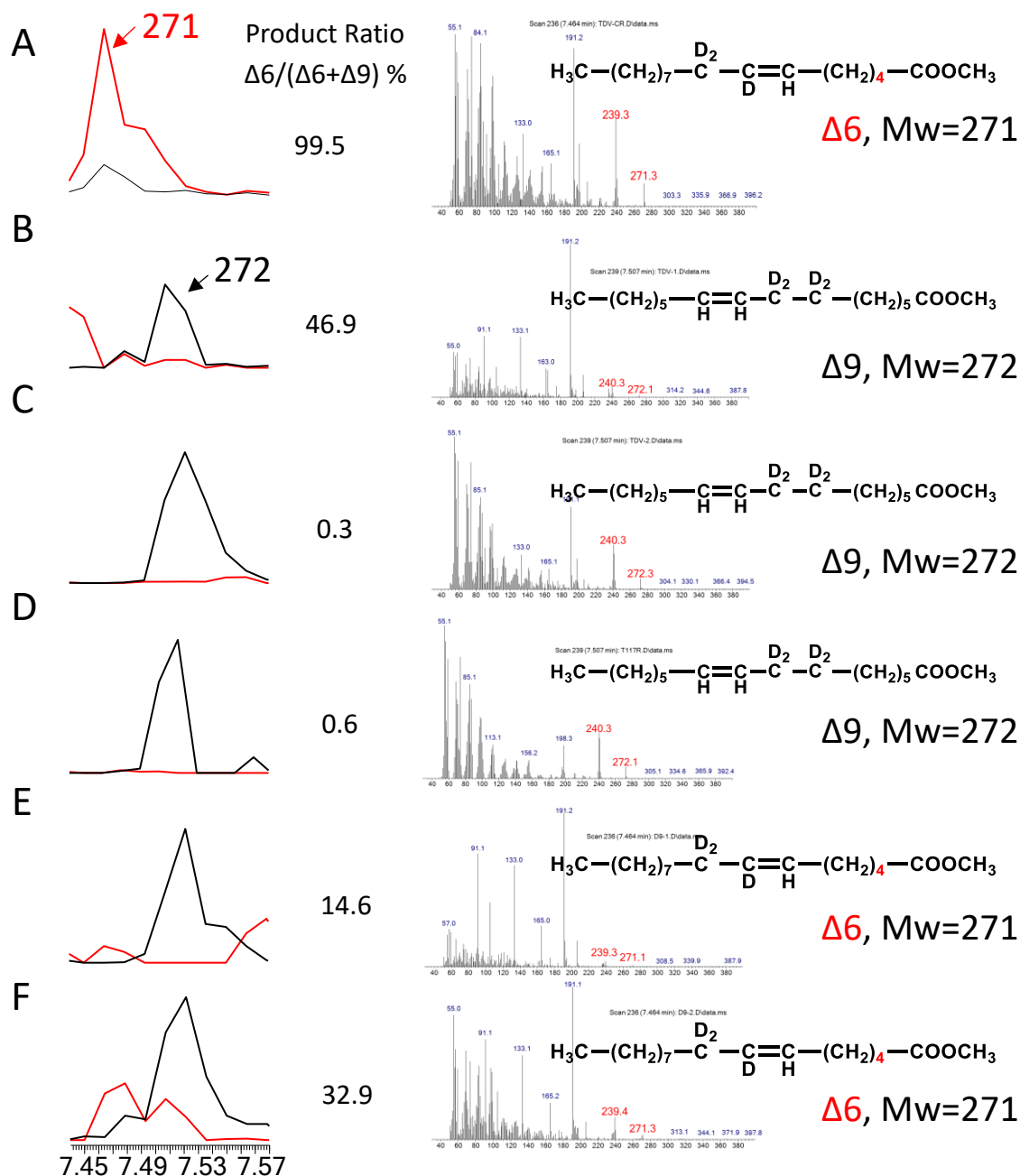


Figure S8. Single ion monitoring (SIM) results for the fatty acid methyl ester products of desaturase variants. Red traces show SIM of 271 AMU, which indicates Δ^6

desaturation, while Δ^9 desaturation-indicating traces of 272 AMU are shown in black traces. The percentage of Δ^6 desaturation specificity was shown as the ratio of Δ^6 desaturation over total desaturation products. The percentage of Δ^6 desaturation specificity was shown as the ratio of Δ^6 desaturation over total desaturation products. Traces: A, TD6; B, TV1; C, TV2; D, CD9; E, CV1; F, CV2 as defined in the main text.