Conjugated Fatty Acid Synthesis

**RESIDUES 111 AND 115 INFLUENCE PRODUCT PARTITIONING OF MOMORDICA CHARANTIA CONJUGASE**

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**Background:** *Momordica* conjugase produces α-eleostearic acid, whereas *Punica* or *Trichosanthes* conjugases produce punicic acid.

**Results:** *Momordica* conjugase residues 111 and 115 affect total eleostearic acid accumulation levels in transgenic *Arabidopsis*.

**Conclusion:** Interactions of *Momordica* side chains 111 and 115 influence α-eleostearic acid versus punicic acid formation.

**Significance:** *Momordica* conjugate accumulated punicic acid in addition to α-eleostearic acid.

Conjugated linolenic acids (CLNs), 18:3 Δ9,11,13, lack the methylene groups found between the double bonds of linolenic acid (18:3 Δ9,12,15). CLNs are produced by conjugase enzymes that are homologs of the oleate desaturases FAD2. The goal of this study was to map the domain(s) within the *Momordica charantia* conjugase (FADX) responsible for CLN formation. To achieve this, a series of *Momordica* FADX-*Arabidopsis* FAD2 chimera were expressed in the *Arabidopsis* fad3/fad4 mutant, and the transformed seeds were analyzed for the accumulation of CLN. These experiments identified helix 2 and the first histidine box as a determinant of conjugase product partitioning into punicic acid (18:3 Δ9cis,11trans,13cis) or α-eleostearic acid (18:3 Δ9cis,11trans,13trans). This was confirmed by analysis of a FADX mutant containing six substitutions in which the sequence of helix 2 and first histidine box was converted to that of FAD2. Each of the six FADX2 substitutions was individually converted back to the FADX equivalent identifying residues 111 and 115, adjacent to the first histidine box, as key determinants of conjugase product partitioning. Additionally, expression of FADX G111V and FADX G111V/D115E resulted in an approximate doubling of eleostearic acid accumulation to 20.4% and 21.2%, respectively, compared with 9.9% upon expression of the native *Momordica* FADX. Like the *Momordica* conjugase, FADX G111V and FADX D115E produced predominantly α-eleostearic acid and little punicic acid, but the FADX G111V/D115E double mutant produced approximately equal amounts of α-eleostearic acid and its isomer, punicic acid, implicating an interactive effect of residues 111 and 115 in punicic acid formation.

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Oils rich in conjugated linolenic acids (CLNs) are important medicinally as a source of nutraceuticals and industrially as drying agents in paints, inks, and varnishes (1). CLNs are found as triglycerides in the seed oils of various plant species belonging to the Cucurbitaceae, Punicaceae, Bignoniacae, Rosaceae, Chrysobalanaceae, Lythraceae, Balasamiae and Euphorbiaceae families as either C18 trienes or C18 tetraenes (2, 3). α-Eleostearic acid (α-ESA, 18:3 Δ9cis,11trans,13trans) is the most widespread CLN. Tung (Aleurites fordii) and bitter gourd (*Momordica charantia*) seeds are rich source of α-ESA and accumulate >80% and >60% of α-ESA, respectively. Other geometrical isomers of α-ESA that are found in nature are punicic acid (18:3 Δ9cis,11trans,13cis), calpatic acid (18:3 Δ9trans,11trans,13cis), and β-ESA (18:3 Δ9trans,11trans,13trans). Punicic acid is found in pomegranate (*Punica granatum*) and snakegourd (*Trichosanthes kirilowii*) seeds, and calpatic acid is present in catalpa (*Catalpa bignonioides* and *Catalpa ovata*) seeds, and β-ESA is present in pomegranate, bitter gourd, and catalpa seeds (4, 5). Additional positional isomers of α-ESA known are calendic acid (18:3 Δ9trans,10trans,12cis) and jaccacic acid (18:3 Δ9cis,10trans,12cis) and are present in pot marigold (*Calendula officinalis*) and jacaranda (*Jacaranda mimosifolia*) seeds, respectively (6, 7).

In recent years cDNAs encoding enzymes that catalyze the formation of conjugated double bonds in CLNs have been identified (8–13). These enzymes were named conjugases (FADXs) and have been shown to be divergent forms of Δ12-oleate desaturases (FAD2). Like FAD2s, FADXs contain histidine motifs that are characteristic of membrane-bound diiron proteins (14). FADXs utilize linoleic acid (18:2 Δ9cis,12cis) and/or linolenic acid (18:3 Δ9cis,12cis,15cis) as a precursor to produce CLNs. Most of the FADXs are bifunctional, possessing both desaturase and conjugase activities (Fig. 1). For example, besides CLNs, Tung FADX can produce the desaturase product, 18:2 Δ9cis,12trans, an isomer of the FAD2-like desaturase product (10). Intriguingly, *Punica* and *Trichosanthes* FADXs can produce a FAD2-like desaturase product, 18:2 Δ9cis,12cis, suggesting that these conjugase enzymes can produce their own substrates (13).

The crystal structures of the soluble desaturases from castor (*Ricinus communis*) and the English Ivy (*Hedera helix*) have given valuable insights into novel catalytic activities and speci-
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Arabidopsis FAD2 amino acids 149–383), Chimera 2 (FADX aa 1–210; FAD2 aa 202–383), Chimera 3 (FADX aa 1–252; FAD2 aa 244–383), Chimera 4 (FADX aa 1–326; FAD2 aa 317–383), Chimera 5 (FAD2 aa 1–52; FAD2 aa 61–399), Chimera 6 (FAD2 aa 1–83; FAD2 aa 93–399), Chimera 7 (FAD2 aa 1–116; FAD2 aa 126–399), and Chimera 8 (FAD2 aa 1–148; FAD2 aa 158–399) were constructed in this manner, and the sequence of the chimeras was confirmed by complete nucleotide sequencing.

Site-directed Mutagenesis—Modified FADX genes containing single or multiple mutations were constructed using appropriate templates by overlap-extension PCR as described above. The primers used for site-directed mutagenesis are shown in supplemental Table S1.

Arabidopsis Growth and Transformation—The plants were grown at 22 °C under a 16-h day/8-h night photoperiod. Plants were transformed by employing the floral dip method (29) using the Agrobacterium tumefaciens strain GV3101. Individual T1 seeds carrying the transgenes were identified based on DsRed2 expression (30).

Fatty Acid Analysis—Fatty acid methyl esters (FAMES) were prepared from seeds (single or three seeds) by incubation with 30 μl of 0.2 M trimethylsulfonium hydroxide in methanol (31). After drying the samples under a nitrogen stream, the samples were resuspended in hexane. FAMES were analyzed with the use of either a Hewlett-Packard 6890 gas chromatograph–flame ionization detector (Agilent Technologies, Santa Clara, CA) fitted with 60 m × 250 μm × 0.25 μm SP-2340 capillary column (Supelco, Bellefonte, PA), or an HP7890A gas chromatograph–mass spectrometer (Hewlett-Packard, Palo Alto, CA) equipped with a HP5975C selective detector (GC/MS) and a 30 m × 250 μm × 0.25 μm HP-Innowax capillary column (Agilent Technologies). The injector was maintained at 225 °C, and the oven temperature was varied from 100 to 240 °C at 15 °C/min, then held at 240 °C for 6 min. GC peaks were identified by comparison with authentic standards and by characterization via GC/MS. CLN methyl esters were routinely identified by the presence of an abundant molecular ion at m/z = 292 and specific isomers identified by GC retention times compared with an authentic standard mixture of CLN FAMES. The standard for CLN was obtained from seeds that accumulate different geometrical isomers of C18-conjugated trienoic fatty acids, namely P. granatum, M. charantia and C. bignonoides. P. granatum seeds accumulate punicic acid (18:3 Δ9cis,11trans,13cis); M. charantia seeds accumulate α-eleostearic acid (18:3 Δ9cis,11trans,13cis); and C. bignonoides seeds accumulate catalpic acid (18:3 Δ9trans,11trans,13cis). The percentage values indicating the composition in the fatty acid samples reported are mean values based on at least three individual measurements.

RESULTS

Construction of FADX-FAD2 Chimeras—To identify structural elements that are functionally important in FADX and FAD2, we constructed chimeras comprising domains of Momordica FADX fused to those of Arabidopsis FAD2. These two enzymes share ~62% sequence identity. To construct chimeras in a rational fashion we first generated a topology model for Momordica conjugase based on online computational algo-
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![Diagram of conjugase product partitioning](image)

**FIGURE 2. Construction of chimeras.** A, proposed topology model for *Momordica* FADX. H1, H2, and H3 depict the location of conserved histidine boxes and are shown coordinating to a putative diiron center. Seven cleavage sites where the portions of *Momordica* FADX were replaced with the corresponding *Arabidopsis* FAD2 sequences have been marked on the model. B, schematic of chimeric enzyme sequences of *Momordica* FADX and *Arabidopsis* FAD2. Regions in white boxes originate from *Momordica* FADX, and regions in black boxes from *Arabidopsis* FAD2. For detailed description of the chimeras, see “Experimental Procedures.” C, amino acid sequence alignment of putative transmembrane helix 2 and first histidine box of *Arabidopsis* FAD2 and *Momordica* FADX. Six residues that differ between the two have been underlined.

There are no experimental data available for the topology of either FAD2 or FADX, and the structural models available for other membrane-bound desaturases are derived from their primary structure and in vivo activities in yeast (32). Based on the hydropathy plots, the integral membrane fatty acid desaturases and desaturase-like enzymes are predicted to share the same membrane topology (four transmembrane helices and two hairpin domains) and overall three-dimensional structures. Therefore, replacing a particular domain of one of these enzymes with the corresponding domain from the other enzyme is not expected to disrupt the overall structure, and quantification of the fatty acid accumulation would help us to identify domains containing amino acids that control particular properties. To implement this approach seven sites were selected as domain borders that isolate individual helices or hairpin domains within *Momordica* FADX that could be replaced with the corresponding FAD2 sequence as shown in Fig. 2.

**Functional Characterization of FADX-FAD2 Chimeras in Arabidopsis fad3fae1**—Because the expression of FADX from *Momordica* fad3fae1 seeds accumulate 13% total ESA (34). Consistent with this, FAD2-transformed *fad3fae1* T1 seeds in this study showed 9.9% of total ESA accumulation with an isomer distribution of 7.7% α-ESA, 0.2% punicic acid, and 2.1% β-ESA. Chimeras 1–4 and 8 produced lower total ESA (7.5%) compared with WT-FAD2. Chimeras 5 and 6 accumulated ~11% total ESA, whereas Chimera 7 accumulated ~17% total ESA (Fig. 3A and supplemental Table S2). Intriguingly, Chimera 7 displayed a different product distribution of CLN isomers relative to that of WT-FAD2 in accumulating 8.9% of punicic acid in addition to 7.8% α-ESA (Figs. 4 and 5A). In the absence of protein quantitation data we are unable to determine whether changes in ESA accumulation result from changes in enzyme activity or changes in enzyme levels.

**Functional Characterization of FADX-FAD2 Chimeras in fad2fae1**—To test the presence of FAD2-like desaturase functionality, the individual chimeras and the WT enzymes were transformed into the *Arabidopsis* fad2fae1 background as these lines contain ~86% of the FAD2 substrate 18:1Δ9cis and ~1% of 18:2. Additionally, because *fad2fae1* seeds contain only ~0.6% of 18:2 Δ9cis,12cis, the accumulation of any ESA above this level must result from FAD2-like desaturase activity of the introduced enzyme. Analysis of the transformed *fad2fae1* seeds showed that the *Arabidopsis* WT-FAD2 produced ~43.7% of plasmidic reticulum ω-3 desaturase (FAD3) and the fatty acid elongation 1 (FAE1)-condensing enzyme, and contains elevated level of the FADX substrate, linoleic acid (~52% of the total fatty acids, compared with ~19% in WT) (33).
was much lower than in
Although the accumulation of total ESA in
Besides previously, WT-FADX showed diverse functionality (35).
Functionalities—Chimeras 6 and 7 are fusions between the N terminus of FAD2 and the C terminus of FADX, the difference being that Chimera 7 contains the first two helices and first portion of the tripartite conserved histidine motif 1 (13) of FAD2, whereas Chimera 6 only contains the first helix of FAD2, the balance of the protein consists of FADX. That Chimera 7 produced a mixture of α-ESA and punicic acid, whereas Chimera 6 produced primarily α-ESA (like Momordica) suggests that both the ability to produce punicic acid and the increase in total ESA accumulation must be specified by the six amino acid differences between FAD2 and FADX in helix 2 and first histidine box (Fig. 2). To test this hypothesis, the FADX_{mut} was engineered consisting of V97L, F100A, A104C, M109I, G111V, and D115E (using Momordica FADX numbering), in which the six WT-FADX residues were replaced by the corresponding residues from WT-FAD2.

Analysis of the transformed seeds confirmed that the FADX_{mut} was functionally similar to Chimera 7 in producing 20.1% total ESA in fad3fae1 lines (Fig. 5A and supplemental Table S4); and 7.8% total ESA in fad2fae1 lines (Fig. 5B and supplemental Table S5). Further, the FADX_{mut} like Chimera 7 produces both punicic and α-ESA (9.5% punicic acid and 9.5% α-ESA in fad3fae1 lines, and 4.1% punicic acid and 3.3% α-ESA in fad2fae1 lines). Expression of the FADX_{mut} in fad2fae1 Arabidopsis background shows that it also produces 7.1% of 18:2 Δ^9cis,12cis, i.e. it has a desaturase activity that is responsible for the production of substrate for its own conjugase activity.

Substitutions of G111V and D115E in Momordica FADX Are Sufficient for Significant Punicic Acid Accumulation—To determine whether any single amino acid of the six substitutions in FADX_{mut} has a major impact on product partitioning between α-ESA and punicic acid, we constructed a series of six drop-out FADX_{mut} genes in which each of the six amino acid substituents was individually converted back to its FADX equivalent. The constructs were transformed into both fad3fae1 and fad2fae1 Arabidopsis backgrounds. Analysis of the transformed seeds showed that four of the six dropout mutants, i.e. FADX_{mut}-V97L (contains all of the six FAD2 substitutions except V97L), FADX_{mut}-F100A, FADX_{mut}-A104C, and FADX_{mut}-M109I had conjugase activities similar to that of the FADX_{mut} (Figs. 5 and 6), indicating that substitutions at positions 97, 100, 104, and 109 do not contribute to the increase in ESA accumulation or the product distribution of FADX_{mut} enzyme.

In contrast, FADX_{mut}-G111V resulted in much lower ESA accumulation relative to the FADX_{mut} and eliminated the accumulation of punicic acid (Fig. 5 and supplemental Tables S4 and S5), suggesting a key role of residue 111 in imparting Trichosanthes-like (punicic acid-producing) conjugase activity to FADX_{mut}. Supporting this observation, the single mutant FADX G111V produced 20.4% ESA in fad3fae1- and 11.6% ESA in fad2fae1-transformed seeds. However, G111V accumulated higher α-ESA compared with FADX_{mut} (16.1% versus 9.5% in fad3fae1, and 8.4% versus 3.3% in fad2fae1), but considerably lower amounts of punicic acid compared with the FADX_{mut} (2.8% versus 9.5% in fad3fae1, and 2.2% versus 4.1% in fad2fae1). Furthermore, it is interesting to note that the single mutant G111V produced 5.6% of 18:2 Δ^9cis,12cis (Fig. 6 and supplemental Table S5), suggesting that Val at position 111 near the active site is important for imparting FAD2-like desaturase functionality.

Although FADX_{mut}-D115E showed a modest reduction of ESA accumulation relative to the FADX_{mut} (15.0% versus 20.1%, supplemental Table S4), punicic acid accumulation was decreased by ~75% compared with FADX_{mut} (2.1% versus...
9.5% in fad3fae1, and 1.1% versus 4.1% in fad2fae1, supplemental Tables S4 and S5). This result suggests that position 115 also plays a role in imparting Trichosanthes-like conjugase activity to WT-FADX. However, the single mutant D115E displayed activity like WT-FADX, with respect to total ESA and punicic acid accumulation. This implies that D115E alone cannot enhance ESA accumulation.

We therefore investigated the possibility that D115E and G111V substitutions in combination are responsible for additional ESA accumulation by analyzing the expression of a FADX G111V/D115E double mutant. Analysis of the transformed seeds show that the conjugase double mutant produced 21.2% of total ESA compared with 9.9% for WT-FADX in fad3fae1 and 10.8% versus 1.6% in fad2fae1, respectively (supplemental Tables S4 and S5). Besides increased α-ESA, the double mutant resulted in significant punicic acid accumulation (10.5% punicic acid and 9.7% α-ESA in fad3fae1; and 5.8% punicic acid and 4.2% α-ESA in fad2fae1, Fig. 5). This result suggests that just two amino acid changes, G111V/D115E, are sufficient to impart significant Trichosanthes-like conjugase activity.

**FIGURE 5. Levels of ESA accumulated in wild-type or mutant Momordica FADX transformed fad3fae1 (A) and fad2fae1 (B) seeds.** To test the contribution of individual amino acid substitutions to the activity of FADXmut gene, each of the six amino acid was individually substituted for its FAD2 equivalent to create six modified FADX genes. For example, enzyme FADXmut-V97L contains five substitutions F100A, A104C, M109I, G111V, and D115E, and so on. Values represent means ± S.D. (error bars) of at least three independent analyses.

**FIGURE 6. Levels of 18:2 (c, c) product and total eliestearic acid accumulating in Arabidopsis fad2fae1 seeds upon the expression of wild-type Arabidopsis FAD2, wild-type Momordica FADX, or mutant Momordica FADX constructs as described in Fig. 5 legend.** Values represent means ± S.D. (error bars) of at least three independent analyses.
functionality to the Momordica conjugase. Further, the double mutant had increased FAD2-like functionality and produced 6.4% of 18:2 Δ9cis,12cis compared with 1.7% for WT-FADX (Fig. 6 and supplemental Table S5).

DISCUSSION

Through our approach of analyzing the products of Momordica FADX-Arabidopsis FAD2 chimeric enzymes by expression in various Arabidopsis mutant backgrounds we identified Chimera 7, which accumulated higher ESA relative to WT-FADX. In this analysis our interpretation is restricted to accumulation of total ESA in transformed seeds because no determination of differential fatty acid turnover was made. Further analysis revealed that the increase of ESA upon expression of Chimera 7 was associated with substantially elevated punicic acid production and equivalent levels of α-ESA production relative to WT-FADX. Subsequent targeted mutagenesis identified residues 111 and 115 as the two key residues that influence both ESA accumulation and reaction partitioning. Expression of FADX G111V in fad3fae1-transformed seeds resulted in a 14-fold increase in punicic acid and 2-fold increase in α-ESA accumulation relative to WT-FADX. Although FADX D115E showed similar ESA accumulation as WT-FADX, the double mutant FADX G111V/D115E led to a 50-fold increase in punicic acid accumulation in the same background. This result implies that the difference with respect to partitioning of conjugase product into its geometrical isomers is directly determined by the identity of the amino acid residues at positions 111 and 115. Further, the bulkier residues in the G111V/D115E double mutant appear to interact because we observed an increase in punicic acid formation but no change in total ESA accumulation (relative to G111V). We further tested the substitution of Gly at position 111 with a range of side chains of increasing (relative to G111V). We further tested the substitution of Gly at position 111 with a range of side chains of increasing side chain bulk at position 111.

Conjugases from Trichosanthes and Punica accumulate punicic acid in WT-Arabidopsis seeds when heterologously expressed under the control of napin promoter (13). The average amounts of punicic acid in Trichosanthes and Punica conjugase transformants have been reported to be 3.5% and 2.3%, respectively, with maximal amounts reaching 10.2% and 4.4%, respectively. Interestingly, in transformed fad3fae1 Arabidopsis seeds, besides 9.7% of 18:2 Δ9cis,12cis compared with 1.7% for WT-FADX (Fig. 6 and supplemental Table S5), suggesting that Val has an optimal side chain bulk at position 111.

The determination of differential fatty acid turnover was made. Further analysis revealed that the increase of ESA upon expression of Chimera 7 was associated with substantially elevated punicic acid production and equivalent levels of α-ESA production relative to WT-FADX. Subsequent targeted mutagenesis identified residues 111 and 115 as the two key residues that influence both ESA accumulation and reaction partitioning. Expression of FADX G111V in fad3fae1-transformed seeds resulted in a 14-fold increase in punicic acid and 2-fold increase in α-ESA accumulation relative to WT-FADX. Although FADX D115E showed similar ESA accumulation as WT-FADX, the double mutant FADX G111V/D115E led to a 50-fold increase in punicic acid accumulation in the same background. This result implies that the difference with respect to partitioning of conjugase product into its geometrical isomers is directly determined by the identity of the amino acid residues at positions 111 and 115. Further, the bulkier residues in the G111V/D115E double mutant appear to interact because we observed an increase in punicic acid formation but no change in total ESA accumulation (relative to G111V). We further tested the substitution of Gly at position 111 with a range of side chains of different bulk, i.e. the larger Leu and Phe, and the smaller Ala compared with Val. We made each of these mutations with Asp or Glu at position 115. Although these changes had little effect on product partitioning, each of these substitutions resulted in a decrease in ESA accumulation relative to G111V and G111V/D115E (supplemental Tables S4 and S5), suggesting that Val has an optimal side chain bulk at position 111.

Determination of Conjugase Product Partitioning

Various chemical mechanisms have been proposed to describe the biological origin of conjugated trienoic acids. It has been shown that during conversion of linoleic acid to calendic acid in C. officinalis developing seeds, hydrogen is abstracted sequentially from the C8 and C11 methylene groups that flank the cis-Δ9 double bond of linoleic acid, a mechanism known as “1,4-desaturation” (36, 37). The production of α-ESA is proposed to involve the removal of hydrogens from the C11 and C14 methylene groups that flank the cis-Δ12 double bond of linoleic acids (34). In contrast, little mechanistic information is presently known about the formation of geometrical isomers of ESA such as punicic acid and α-ESA.

In previous work on the evolutionarily distinct acyl-ACP desaturases, for which the geometry of the substrate binding cavity is defined from crystal structures, the production of allylic hydroxy fatty acids was rationalized by a relative shift of the fatty acid substrate with respect to the active site Fe-O oxidant. The boomerang shape of the substrate binding cavity imposes an eclipsed conformation on the acyl chain that causes the pro-R hydrogens to be presented to the active site oxidant bound to the diiron active site (15, 16). Although the amino acid sequences of acyl-ACP desaturases and integral membrane FAD2 desaturases differ, the common chemical imperative imposed by the stereochemistry of desaturation implies that the shapes of the substrate binding cavities relative to the active site iron oxidant species will be similar.

Based on the data presented here, and by analogy to mechanisms proposed for the acyl-ACP desaturases (38) involving shifting the register of the bound fatty acid substrate relative to the active site, the formation of either punicic acid or α-ESA can perhaps be rationalized in terms of distinct proposed substrate binding modes relative to the position of the oxidant and the envisaged bend in the substrate binding cavity. It is likely that punicic acid results from the placement of C12 and C13 of the 18:2 Δ9cis,12cis substrate at the bend in the binding cavity wherein the bulkier residues constrain the product in a cis configuration at C13, whereas α-ESA is produced when the substrate 18:2 Δ9cis,12cis binds deeper into the binding cavity wherein the smaller residues allow the product to adopt a more stable anti-conformation that results in pro-S hydrogen removal at C14 and thence trans-double bond formation. In the case of the single mutant, G111V, steric bulk presented by a larger Val residue causes the substrate to bind two carbons less deeply in the active site leading to conversion of 18:2 Δ9cis,12cis to punicic acid, and in the case of the double mutant, G111V/D115E, conversion of 18:2 Δ9cis,12cis to punicic acid is much enhanced due to larger steric bulk presented by the presence of larger residues Val and Glu near the active site.

Position 111 was also found to be crucial for imparting FAD2-like desaturase functionality to Momordica conjugase (Fig. 6). In fad3fae1 lines, the single mutant G111V produced 3-fold more 18:2 Δ9cis,12cis and 8-fold more ESA compared with WT-FADX. Perhaps this higher FAD2-like desaturase function
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attributable to the GI11V mutation allows the enzyme to perform the two consecutive reactions to produce ESA from 18:1 without release of the product of the desaturation reaction. Such a mechanism could help explain higher ESA accumulation in seeds transformed with G111V. As described above, 9cis,12trans can form.

In other FAD2-related work, subtle changes near the active site have been shown to play a key role in influencing desaturation in seeds transformed with G111V. As described above, 9cis,12trans can form.

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