Expression of a Δ⁹ 14:0-acyl carrier protein fatty acid desaturase gene is necessary for the production of ω⁵ anacardic acids found in pest-resistant geranium (Pelargonium xhortorum)

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ABSTRACT Anacardic acids, a class of secondary compounds derived from fatty acids, are found in a variety of dicotyledonous families. Pest resistance (e.g., spider mites and aphids) in Pelargonium xhortorum (geranium) is associated with high levels (~81%) of unsaturated 22:1ω5 and 24:1ω5 anacardic acids in the glandular trichome exudate. A single dominant locus controls the production of these ω5 anacardic acids, which arise from novel 16:1Δ11 and 18:1Δ13 fatty acids. We describe the isolation and characterization of a cDNA encoding a unique Δ⁹ 14:0-acyl carrier protein fatty acid desaturase. Several lines of evidence indicated that expression of this desaturase leads to the production of the ω5 anacardic acids involved in pest resistance. First, its expression was found in pest-resistant, but not susceptible, plants and its expression followed the production of ω5 anacardic acids in segregating populations. Second, its expression and the occurrence of the novel 16:1Δ11 and 18:1Δ13 fatty acids and the ω5 anacardic acids were specific to tall glandular trichomes. Third, assays of the recombinant protein demonstrated that this desaturase produced the 14:1Δ9 fatty acid precursor to the novel 16:1Δ11 and 18:1Δ13 fatty acids. Based on our genetic and biochemical studies, we conclude that expression of this Δ⁹ 14:0-ACP desaturase gene is required for the production of ω5 anacardic acids that have been shown to be necessary for pest resistance in geranium.

In the garden geranium (Pelargonium xhortorum), inbred genotypes resistant to pests (e.g., spider mites and aphids) and inbred genotypes susceptible to pests have been identified (1, 2) (Fig. 1A). Pest-resistant and pest-susceptible plants produce anacardic acids (6-alkyl-salicylic acid) in exudates of tall glandular trichomes. However, the composition of anacardic acids differs between resistant and susceptible genotypes (3, 4). The trichome exudate from the resistant genotype has a predominance (~81% of exudate profile) of unsaturated 22:1ω5 and 24:1ω5 anacardic acids. In contrast, trichome exudates from the susceptible genotype lack the ω5 products and have saturated 22:0 and 24:0 anacardic acids (Fig. 1B) (3, 4).

The desaturase status of the anacardic acid exudate affects the physical properties of the exudate and the effectiveness of pest resistance. The anacardic acid exudate of the resistant genotype is fluid and acts as a “sticky trap” that impedes pest movement and adheres to their exoskeleton (2, 8). This results in enhanced pest exposure to anacardic acids that have toxic properties and have been shown to inhibit enzymatic steps in pest reproduction (1, 9). In contrast, the anacardic acid exudate of the susceptible genotype is solid, does not act as an effective sticky trap and does not adhere to the exoskeleton. Therefore, exposure to the toxic exudate is minimized.

Fatty acids have been shown to be direct precursors of anacardic acids. Saturated and unsaturated 14C-labeled fatty acids applied to floral tissue and leaves produce corresponding 14C-labeled saturated and unsaturated anacardic acids (6, 7). The production of anacardic acids is consistent with the addition of six carbons to the labeled fatty acid (e.g., supplying a 16:0 fatty acid results in the production of a 22:0 anacardic acid) (6, 7). Thus the novel 16:1Δ11 and 18:1Δ13 fatty acids are direct precursors to the 22:1ω5 and 24:1ω5 anacardic acids, respectively, which are associated with pest resistance (6, 7). Consistent with this, the 16:1Δ11 and 18:1Δ13 fatty acids and corresponding ω5 anacardic acids are specifically localized in the trichomes of the resistant genotype (3, 4, 10).

Early analysis of inbred resistant and susceptible genotypes suggested that pest resistance is correlated with a quantitative difference in the levels of ω5 anacardic acids (1, 2, 8, 11–13). Subsequent refinement of the anacardic acid analysis showed that ω5 anacardic acids are either present at high levels in the resistant plants or undetectable in the susceptible plants (3, 4). Analysis of an F₂ population (n = 160) resulting from a cross of inbred resistant and inbred susceptible genotypes confirmed a 3:1 segregation ratio (χ² = 0.03, P ≥ 0.86) for a single dominant locus controlling the production of ω5 anacardic acids (R.O.M. and R.C., unpublished data). To confirm the linkage between ω5 anacardic acids and pest resistance, 10 plants containing and 9 plants lacking ω5 anacardic acids were subjected to mite bioassays. All plants containing ω5 anacardic acids were pest resistant, and all plants deficient for ω5 anacardic acid were pest susceptible (R.O.M. and R.C., unpublished data).

We report the isolation and characterization of a novel plant fatty acid desaturase cDNA that encodes a Δ⁹ 14:0-ACP desaturase. We demonstrate a close correlation between expression of this desaturase to the accumulation of 16:1Δ11 and 18:1Δ13 fatty acids and 22:1ω5 and 24:1ω5 anacardic acids, as well as to the pest-resistant genotype. Collectively, these data indicate that expression of the Δ⁹ 14:0-ACP desaturase is a critical factor for pest resistance.

MATERIAL AND METHODS

Plant Materials. All plant genotypes described originated from a resistant inbred (71-17-7) and susceptible inbred (71-10-1) that were maintained by vegetative propagation (1, 2). The resistant and susceptible inbreds were crossed reciprocally to produce F₁ hybrids. The F₁ plants were self-pollinated to produce the F₂ generation. Backcross generations were made by crossing the F₁ to each parental genotype. The backcross

Abbreviations: ACP, acyl carrier protein; SSPE, standard saline phosphate/EDTA.

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EDTA (SSPE), used for molecular membranes will
x2 generation population chemically
Expression isolated A9 18:0-ACP desaturase cDNA
and is the branch by required exudate.

Fig. 1. (A) Geranium flower pedicel of the pest-resistant genotype covered with the glandular trichomes (T). The trichomes have been observed to be an effective barrier to pests such as the aphid (P) (pictured is the potato aphid, Macrosiphum euphorbiae). (Insert) Close-up view of the glandular trichomes. Analysis of the trichome exudate (E) from pest-resistant plants shows it is rich in 22:1ω5 and 24:1ω5 (3, 4). (B) Structures of anarcardic acids. The 22:1ω5 and 24:1ω5 anarcardic acids are the major component (≈81%) of the pest-resistant trichome exudate. In contrast, the 22:0 and 24:0 anarcardic acids are the major component (≈52%) of the pest-susceptible genotype trichome exudate. (C) The proposed biosynthetic pathway model [starting from 14:0-acyl carrier protein (ACP)] of the 22:1ω5 and 24:1ω5 anarcardic acids of the pest-resistant genotype. Expression of the novel desaturase is required to place a double bond at the Δ5 position of a 14:0-ACP substrate, and is the branch point between the resistant and susceptible genotypes. Expression (+) of the ΔΔ9 14:0-ACP desaturase may require other factors such as a positive regulator (P). The 14:1ω7 product is subsequently elongated to 16:1Δ11- and/or 18:1ω13-ACP in a manner similar to that reported (5). The 16:1Δ11- and 18:1ω13-ACP fatty acids will then be converted to the 22:1ω5 and 24:1ω5 anarcardic acids as previously proposed (6, 7).

population resulting from the cross to the susceptible parent was used for molecular analysis. This backcross population was chemically characterized for the presence or absence of ω6 anarcardic acids and grouped accordingly. χ2 analysis of this backcross generation for a 1:1 ratio (dominant heterozygote to the recessive homozygote) provides an acceptable fit (ν = 38, χ2 = 0.72, P ≥ 0.46). Both parents were selfed to produce the inbred resistant line (88-51-10) and inbred susceptible line (88-50-10) used for molecular analysis. All tissue was harvested and frozen under liquid nitrogen. Samples were then stored at −80°C until used.

Isolation and Characterization of Desaturase cDNA Clones. A geranium λ-Zap II cDNA library, prepared from RNA isolated from tissue rich in glandular trichomes (14) was screened under nonstringent conditions with the cospan vector D9 18:0-ACP desaturase cDNA clone (15) radiolabeled with [32P]dCTP by a Prime-It II synthesis kit (Stratagene). The membranes were prehybridized and hybridized at 42°C in a solution of 25% formamide, 5× standard saline phosphate/EDTA (SSPE), 5× Denhardt’s solution, 100 μg of denatured salmon sperm DNA per ml, and 1% SDS (16). Positive plaques were identified following autoradiography, and each positive plaque was rescreened through two further rounds to isolate a single pure bacteriophage. After rescreening, the plasmid (pBlueScript, SK+) was excised as recommended (Stratagene), and plasmid inserts were manually sequenced by dideoxy chain termination with the use of Sequenase version 2.0 (United States Biochemical) (16).

RNA Gel Blot Analysis. RNA was isolated from geranium tissues (leaves with intact trichomes, pedicel tissue stripped of trichomes, and trichomes taken from the pedicel tissue) as described (17). Total RNA (30 μg) was electrophoresed through a 1.4% agarose gel with formaldehyde and blotted onto a nylon membrane (GeneScreenPlus, DuPont) (16). The blot was probed with the 3′ untranslated region (HindIII/EcoRI, 3 fragment) of the geranium type B clone which was [32P]dCTP labeled with the Prime-It II synthesis kit (Stratagene). The blot was prehybridized at 65°C in a solution of 10% dextran sulfate, 1% SDS, and 1 M NaCl. Hybridization was performed using an identical solution, but contained 100 μg of salmon sperm DNA per ml. The membrane was washed twice for 5 min at room temperature in 2× SSPE/1% SDS followed by a single wash for 30 min at 65°C in 1× SSPE/0.1% SDS and one wash for 15 min at 68°C in 0.5× SSPE/0.1% SDS. Expression of the gene was analyzed with autoradiography (Kodak XAR-5 film), and the radioactive signal was quantified with a PhosphorImager 445 SI (Molecular Dynamics). The blot was then stripped of the labeled probe by washing in 0.1× SSPE/0.1% SDS at 100°C until no signal could be detected. The Arabidopsis thaliana 18S ribosomal DNA 0.4-kb EcoRI, HindIII fragment (from plasmid Sbg10IT79; Arabidopsis Stock Center, Ohio State University) was then hybridized as described above to verify equal loading of each lane.

Expression in Escherichia coli. To express the clone in E. coli, the cDNA corresponding to the mature peptide was cloned into the pET3d vector (Novagen). PCR primers were designed to allow amplification of the mature peptide by synthesis of oligonucleotides encoding the amino acids denoted in boldface type in Fig. 2. A Ncol site was incorporated into the upstream primer and a BgII site was incorporated into the downstream primer to allow directional cloning into the pET3d vector. The primers are: (i) 5′-ggggccatggcttctacttc-

and (ii) 5′-ggggagatctcactagacttt-

Expression of the novel desaturase was confirmed by gel electrophoresis, autoradiography, and the radioactive signal was quantified with a PhosphorImager 445 SI (Molecular Dynamics). The blot was then stripped of the labeled probe by washing in 0.1× SSPE/0.1% SDS at 100°C until no signal could be detected. The Arabidopsis thaliana 18S ribosomal DNA 0.4-kb EcoRI, HindIII fragment (from plasmid Sbg10IT79; Arabidopsis Stock Center, Ohio State University) was then hybridized as described above to verify equal loading of each lane.

Fatty Acid Desaturase Assay in E. coli. The geranium type B clone (pPXH-B) was expressed in E. coli as described (18). Protein expression was induced by addition of isopropyl β-D-thiogalactopyranoside to 0.1 mM. Cells were then grown for 15 h at 20°C with moderate shaking. Cells were pelleted and resuspended in 0.5 ml boron trifluoride in methanol and incubated at 100°C for 1 h. After hexane extraction, the fatty acid methyl esters were analyzed by gas chromatography (GC)

* MvGlnlncs pfpvvaass TG5IKSKNNHR KVGTYGTVAP -40
* QKIEIFKSEM EGWGHNLPL AKPVEKSWQP TDFLPDSSE -80
GFMSEYNNAPK ERTFLPEDY FVVLAGDIT EALPTQTYL -120
UNRPVEVADE TGSHSSEFW VSRWATAEEN RHGDLLNKLY -160
VLQDQDCMQ VEBTQGQLA LGQLOGETQ PHPLTPVSP -200
QERATFFISHA NTAKLQAGHQ DLKQACIOT IADDEKRET -240
AYTIVKLDL ELDQBFMTSC LARHMKRFIT MPALRMGRD -280
DHPFLQHPSS VASNQGTVT MVIIINITHEF VERMNKEIT -320
AGLSDGREGA QDVVCGLKER LRKVEEARQ RVQVPADIEP -360
SWIDPDKR

FIG. 2. Deduced amino acid sequence derived from the type B desaturase clone. Lowercase amino acids with an overstrike denote a putative transit peptide. The starting methionine is designated by an asterisk. Amino acid sequences in boldface type denote regions used to design PCR primers for cloning the desaturase into expression vectors (see Materials and Methods).
with a Hewlett-Packard 5890 GC equipped with a 30 m RTX2330 column (Restek, Bellefonte, PA). Samples were further analyzed to identify each chromatogram peak. Dimethyl disulfide adducts of fatty acid methyl esters were prepared as described (19). Methyl esters of unsaturated fatty acids and their dimethyl disulfide derivatives were identified by mass spectral (MS) analysis with a Hewlett-Packard 5890 Series II GC equipped with a SE-54 column (Alltech Associates) and a Hewlett-Packard 5971 mass selective detector. The oven was programmed at an initial temperature of 40°C to a final temperature of 300°C with a ramp of 6°C/min.

In Vitro Fatty Acid Desaturase Assays. In vitro reactions were incubated for 30 min with a crude protein extract from E. coli expressing the type B clone (pPXH-B) as described (20), except we included 500 μM cerulenin to inhibit E. coli fatty acid elongation enzymes (21). Four acyl-ACP substrates, [1-14C]12:0, 14:0, 16:0, and 18:0-ACP, synthesized as described (22) were tested. Each assay contained 124 pmol of labeled substrate and 81 μg of crude protein. Reactions were terminated, derivatized and extracted as described (20) except methyl esters were made with the use of boron trifluoride. Samples were then analyzed on TLC plates treated with 15% AgNO3 and toluene as the mobile phase (5). Radioactivity was quantified by an AMBIS 400 TLC plate reader AMBIS core software version. 4.0 at a scan time of 15 h. Detected signal (cpm) of unsaturated product and reaction saturated substrate was used to determine the percent unsaturated product. The mol quantity of unsaturated product was determined by multiplying the percent unsaturated product by 124 pmol.

Double bond placement was verified by a modified in vitro assay. In vitro reactions were as described (20) except the reactions were scaled-up 20-fold, incubation time was 40 min, and 150-ACP (1.5 μM) was used as the substrate. Reactions were terminated, derivatized, and extracted as described (20), and dimethyl disulfide derivatives were prepared (19) and analyzed by GC/MS.

RESULTS

Isolation of a Geranium Desaturase-Like cDNA. The Δ⁹ 18:0-ACP desaturase gene exists as part of a gene family in Thunbergia alata (black-eyed susan vine), where three independent Δ¹⁸ 18:0-ACP desaturase-like genes have been isolated (23). In addition, two novel acyl-ACP desaturases (Δ⁴ 16:0-ACP and Δ⁸ 16:0-ACP) with high homology to the Δ¹⁸ 18:0-ACP desaturase have been identified (20, 24). These desaturases recognize a shorter acyl chain and place double bonds at distinct positions. Hence, we reasoned that a variant acyl-ACP desaturase could be involved in the production of 16:1α¹¹ and 18:1α¹³ found in the resistant genotype of geranium.

When a castor bean Δ¹⁸ 18:0-ACP desaturase probe was used in geranium Southern blot analysis under low stringency conditions, a small gene family was detected. To identify acyl-ACP desaturase clones that may be involved in pest resistance, a trichome-enriched cDNA library was screened with the castor bean Δ⁹ 18:0-ACP desaturase probe. Two classes of clones were isolated and designated type A and type B based on restriction endonuclease analysis.

Type A clone was sequenced and the longest open reading frame was found to have a 89% amino acid similarity to the castor bean Δ⁹ 18:0-ACP desaturase. Hence, the type A clone likely represents a Δ⁹ 18:0-ACP desaturase homologue. In contrast, the type B clone was sequenced and found to contain an open reading frame with 79% amino acid similarity to the castor bean Δ⁸ 18:0-ACP desaturase, suggesting it may represent a novel desaturase. More detailed studies focused on the type B clone.

Fig. 2 shows the deduced amino acid sequence for the 1.3 kb type B clone. The ATG (nucleotides 8–10) is likely the initiation codon because a G is found at position +4, consistent with the consensus for plant translation initiation sites (25).

The open reading frame does not continue upstream of the ATG. Though there is divergence between the transit peptides of different acyl-ACP desaturases, there is homology between the transit peptide cleavage sites (20). Such a consensus cleavage site is found between residues 18 and 19 (26). This indicates that either the type B clone has a very short transit peptide or that the clone is truncated at the 5' end. In either case, the identification of a conserved cleavage site suggested that the entire mature peptide coding sequence is represented in this type B clone.

RNA Gel Blot Analysis. Expression of the type B gene was analyzed in order to determine if it follows the expected pattern for pest resistance. Fig. 3 shows that the type B gene was expressed only in RNA from trichomes of the resistant genotype (compare lanes 1 and 2 to 5 and 6). All other tissues analyzed (pest-resistant leaves and pedicels; pest-susceptible trichomes, leaves and pedicels) displayed no significant type B expression levels. PhosphorImager quantification indicated that there is at least 20-fold greater expression in the resistant trichomes compared with the susceptible trichomes. Expression of the type B gene in the resistant genotype trichomes was consistent with the production of the novel fatty acids (16:1α¹¹ and 18:1α¹³) as well as the ω⁶ anacardic acids (22:1ω⁶ and 24:1ω⁵). In addition, type B gene expression was at least 31-fold greater in trichomes of the resistant genotype than in all other “nontrichome” tissues. Expression in trichomes from homozygous plants was found to be 1.9 fold higher than expression in trichomes from heterozygous plants (Fig. 3A, lanes 1 and 2). This pattern is consistent with the effects predicted for two copies of the dominant allele in the homozygous plants compared with one copy of the dominant allele in the heterozygous plants.
**Fatty Acid Desaturase Assay in E. coli.** The type B gene was placed under the control of an inducible promoter in E. coli to determine if its product functions as a desaturase. Upon induction, a protein of ~39 kDa was produced. This peptide has a similar size to that predicted to be encoded by the cDNA sequence and cross-reacts with an antibody prepared against the Δ⁹ 18:0-ACP desaturase of avocado (data not shown).

E. coli fatty acids were analyzed by GC to determine if the 39 kDa type B polypeptide had desaturase activity. The fatty acid profiles of the E. coli cell line BL21(DE3) grown with expression of the type B clone were compared with the fatty acid profile of the native cell line. In controls, the only unsaturated moieties detected were methyl esters of palmitoleic acid (16:1Δ⁹) and cis-vaccenic acid (18:1Δ¹¹) (Fig. 4A). When the 39 kDa type B gene product was expressed (Fig. 4B), two new fatty acids were detected, identified as methyl esters of 16:1Δ¹¹ and 18:1Δ¹³ by GC/MS of their dimethyl disulfide adducts. These could either result from a Δ¹¹ 16:0 desaturation reaction or desaturation of a shorter acyl chain and subsequent elongation to 16:1Δ¹¹ and 18:1Δ¹³. To distinguish between these two possibilities, chain length specificity of the type B desaturase was assessed.

**In Vitro Fatty Acid Desaturase Assays.** The chain length specificity of the type B clone was determined with in vitro assays and [1-14C]12:0-, 14:0-, 16:0-, and 18:0-ACP substrates (22). Assays contained crude E. coli protein extract and cerulenin (500 μM) to inhibit the E. coli fatty acid elongation enzymes (20, 21). Methyl esters were made, separated by TLC, and quantified to determine desaturase activity with each substrate (see Materials and Methods). Fig. 5A shows that the type B desaturase was 3-fold more active with [1-14C]14:0-ACP than with [1-14C]16:0-ACP and was much less active toward [1-14C]12:0-ACP and [1-14C]18:0-ACP substrates.

The methyl ester product of the [1-14C]14:0-ACP substrate had the same mobility on argentation TLC as that of a methyl ester standard for 14:1Δ⁹, suggesting that the type B gene product functioned as a Δ⁹ 14:0-ACP desaturase. The mobility of the [1-14C]16:0-ACP desaturation product on argentation TLC was also consistent with that of a Δ⁹ isomer. To confirm the presence of double bond placement, assays were repeated with a 15:0-ACP substrate. Because E. coli does not synthesize odd chain fatty acids, ambiguities caused by the presence of bacterial acyl-ACPs were eliminated. GC/MS analysis of the dimethyl disulfide adducts of 15:1 methyl esters from this assay showed that the double bond is placed at the Δ⁹ position of the 15:0-ACP substrate (Fig. 5B). Collectively, results from the three assays with the recombinant protein, indicate that the type B gene product functions as a Δ⁹ 14:0-ACP desaturase.

**DISCUSSION**

We have identified a novel acyl-ACP desaturase whose expression in inbred pest-resistant geranium genotypes was closely correlated with pest resistance and the presence of the novel 16:1Δ¹¹ and 18:1Δ¹³ fatty acids. To determine if the type B gene functions as a desaturase that could produce the novel 16:1Δ¹¹ and 18:1Δ¹³ fatty acids, we expressed this gene in E. coli. Three distinct assays—(i) in vivo E. coli assays, (ii) in vitro chain length specificity assay, and (iii) the double bond position analysis of the in vitro product—led us to conclude that the type B desaturase functions to place a double bond at the Δ⁹ position of a 14:0-ACP substrate. This represents the only report of a 14:0-ACP desaturase to date.

In E. coli control lines, the only unsaturated products detected were methyl esters of palmitoleic acid (16:1Δ⁹) and cis-vaccenic acid (18:1Δ¹¹). Both of these unsaturated products result from the elongation of 10:1Δ²-ACP (21). The efficiency of this elongation process in E. coli is evident because the intermediates (12:1Δ⁵ or 14:1Δ⁷) are not detected (Fig. 4A). In E. coli expressing the type B gene, the major additional fatty acids detected are 16:1Δ¹¹ and 18:1Δ¹³, likely elongation products of a 14:1Δ² fatty acid.

In resistant geranium trichomes, we have also identified 16:1Δ¹¹ and 18:1Δ¹³ fatty acids but have not identified a 14:1Δ⁹ fatty acid (10). We propose that the pest-resistant genotype is characterized by the production of 14:1Δ⁹ which is efficiently elongated, as in E. coli, to the two unique fatty acids 16:1Δ¹¹ and 18:1Δ¹³ (Fig. 1C). A similar plant fatty acid elongation mechanism has been identified in the production of 18:1Δ⁸ fatty acids found in Coriandrum sativum (5). Based on biochemical analysis (6, 7), the 16:1Δ¹¹ and 18:1Δ¹³ fatty acids likely are the precursors to the 22:1Δ⁹ and 24:1Δ⁷ anacardic acids, respectively.

Small Pest resistance in geranium is closely correlated with the presence of a single dominant locus that directs the production of 14:1Δ⁹ anacardic acids. Several possibilities exist for the gene(s) encoded by this locus. One possibility is that the locus encodes the Δ⁹ 14:0-ACP desaturase gene we have isolated. Geranium is one of the few plants that remains recalcitrant to transformation. We therefore are unable to attempt complementation of the susceptible genotype with the novel desaturase gene to test the hypothesis that our gene is the dominant factor that controls resistance. An alternative possibility is that the dominant factor encodes a positive regulator that directly controls expression of the Δ⁹ 14:0-ACP desaturase gene (Fig. 1C). However, because expression of the Δ⁹ 14:0-ACP desaturase showed quantitative differences between ho-
mozygous and heterozygous pest-resistant plants, any putative positive regulator must interact quantitatively with our gene. Regardless of the scenario for the protein encoded by this dominant factor, the expression of the \( \Delta^9 \) 14:0-ACP desaturase gene is required for the production of the \( \omega^6 \) anacardic acids, and therefore is necessary for pest resistance.

Unsaturated anacardic acids provide a novel defense against pests. The identification of a trichome-specific \( \Delta^9 \) 14:0-ACP desaturase defines the biosynthetic pathway of the specific \( \omega^6 \) anacardic acids that are necessary for pest resistance. Although plants produce a wide range of secondary metabolites, their effects on pests are largely unknown. Plant secondary metabolites and the genes encoding their biosynthetic enzymes represent a vast resource for future genetic engineering of plant pest resistance. The isolation and characterization of the novel \( \Delta^9 \) 14:0-ACP desaturase represents a step toward this goal.

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