Δ12-Oleate Desaturase-related Enzymes Associated with Formation of Conjugated trans-Δ11, cis-Δ13 Double Bonds

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Conjugated linolenic acids are present as major seed oils in several plant species. Punicic acid (or trichosanic acid) is a conjugated linolenic acid isomer containing cis-Δ9, trans-Δ11, cis-Δ13 double bonds in the C18 carbon chain. Here we report cDNAs, TkFac and PgFac, isolated from Trichosanthes kirilowii and Punica granatum, that encode a class of conjugases associated with the formation of trans-Δ11, cis-Δ13 double bonds. Expression of TkFac and PgFac in Arabidopsis seeds under transcriptional control of the seed-specific napin promoter resulted in accumulation of punicic acid up to ~10% (w/w) of the total seed oils. In contrast, no punicic acid was found in lipids from leaves even when the conjugases were driven under control of the cauliflower mosaic virus 35S promoter. In yeast cells grown without exogenous fatty acids in the culture medium, TkFac and PgFac expression resulted in punicic acid accumulation accompanied by 16:2 Δ10cis,12trans and 18:2 Δ10cis,12trans production. Thus, TkFac and PgFac are defined as bifunctional enzymes having both conjugase and Δ12-oleate desaturase activity. Furthermore, we demonstrate that 16:2 Δ10cis,12trans and 18:3 Δ10cis,12trans are potential substrates for the conjugases to form trans-Δ11 and cis-Δ13 double bonds.

A large number of fatty acid species have been found in plant seed oils. Typically, plant seeds contain saturated and unsaturated fatty acids, such as palmitic (16:0), palmitoleic (16:1 Δ9cis), stearic (18:0), oleic (18:1 Δ9cis), linoleic (18:2 Δ10cis,12trans), and α-linolenic (18:3 Δ12cis,15cis,18cis) acids. These are typical fatty acids with all other fatty acids regarded as unusual. Typically, polynsaturated fatty acids in the culture medium, TkFac and PgFac expression resulted in conjugated linoleic acid accumulation accompanied by 16:2 Δ10cis,12trans and 18:2 Δ10cis,12trans production. Thus, TkFac and PgFac are defined as bifunctional enzymes having both conjugase and Δ12-oleate desaturase activity. Furthermore, we demonstrate that 16:2 Δ10cis,12trans and 18:3 Δ10cis,12trans are potential substrates for the conjugases to form trans-Δ11 and cis-Δ13 double bonds.

Positional and geometrical isomers of CLNA, three 8,10,12-trienes and four 9,11,13-trienes, have been reported to occur naturally (1). Five CLNA isomers occur as major seed oils of several plants: α-eleostearic (cis-Δ9, trans-Δ11, trans-Δ13), calendic (trans-Δ8, trans-Δ10, cis-Δ12), punicic (cis-Δ9, trans-Δ11, cis-Δ13), jarcic (cis-Δ8, trans-Δ10, cis-Δ12), and catalpic (trans-Δ9, trans-Δ11, cis-Δ13) acids (1). These isomers have closely related structure; for example, α-eleostearic and jarcic acids are geometrical isomers of punicic and calendic acids, respectively. CLNA are major seed oils in plants such as tung (Aleurites fordii), karela (Momordica charantia), marigold (Calendula officinalis), and pomegranate (Punica granatum). Tung oil contains high levels of 9,11,13-triene (α-eleostearic acid) and is used mainly in quick-drying enamels and varnishes. There also is growing evidence showing that supplementation with CLNA has cytotoxic effects on tumor cells and that uptake of CLNA has an effect on lipid metabolism (2–4). Conjugated eicosapentaenoic and docosahexaenoic acids with conjugated trienoic structure also exhibit cytotoxic effects on tumor cells (5). It has been further suggested that the biological action of each conjugated fatty acid may not be equivalent (3).

Previous studies have indicated that linoleic acid is the acyl precursor of α-eleostearic acid and linoleoyl phosphatidylcholine is the precursor of α-eleostearoyl phosphatidylethanolamine (6). Recently cDNAs encoding enzymes that catalyze the formation of the conjugated double bonds in CLNA have been identified. These enzymes were termed conjugases and were shown to be divergent forms of Δ12-oleate desaturase (FAD2). Two types of conjugases associated with the formation of conjugated double bonds in trans-configuration have been identified: one catalyzes the conversion of a cis-Δ12 double bond into the conjugated trans-Δ11, trans-Δ13 double bonds found in α-eleostearic acid (7), and the other modifies a cis-Δ9 double bond into the trans-Δ8, trans-Δ10 double bonds of calendic acid (8, 9). Very recently a class of conjugase associated with the formation of cis-Δ11, trans-Δ13 double bonds of punicic acid was identified (10). Although the primary structures of these conjugases are similar, each enzyme specifically catalyzes the formation of α-eleostearic, punicic, or calendic acids. Other classes of conjugases associated with the formation of conjugated double bonds in jarcic and catalpic acids have not been reported.

It is known that Trichosanthes kirilowii and P. granatum accumulate punicic acid specifically in seed oil up to ~40 and ~80% (w/w) of the total seed oil, respectively (11, 12). The biochemical process resulting in high punicic acid accumulation in the seeds of these plants, however, has not been clear. In this study, for the first step in understanding CLNA accumulation in plant seed oils, we isolated cDNAs that encode enzymes associated with the formation of punicic acid from T. kirilowii and P. granatum and expressed them in Arabidopsis plants. We also analyzed the function of these enzymes in yeast cells. Interestingly the enzymes were demonstrated to possess
both Δ12-oleate desaturase and conjugase activities. The finding supports the idea that conjugases have diverged from Δ12-oleate desaturase. The growing number of identified primary structures of fatty acid-modifying enzymes provides valuable information to understand the functional divergence of these enzymes.

**EXPERIMENTAL PROCEDURES**

**FAD2-related cDNA Isolation**—Total RNA was isolated from maturing seeds of *T. kirkiiouii* and *P. graminum* by the methods of Carpenter et al. (13). The first strand cDNA was synthesized with an oligo-dT primer and SuperScript II reverse transcriptase (Invitrogen) from the total RNA. PCR amplification with PCR primers designed to degenerate primers were designed to target conserved amino acid sequences in FAD2-related enzymes. For *T. kirkiiouii*, a set of degenerate primers, 5'-TGGGNNCAAYCGGNTTCAYGATTACRT-3' (forward primer) and 5'-GGGRTGNTGRTGRTGNARNMGRT-3' (reverse primer) were used to target the amino acid sequences CGHHAADSYYQ and TYYAILQTHQP, respectively. For *P. graminum*, 5'-TGGGNNCAYMRRNCNTYWSNATAYCAR-3' (forward primer) and 5'-KYNNCCNCNARCARTYCCAYCTC-3' (reverse primer) were used to target the amino acid sequences CGH/HR/A/FSQD/Y and EWD/NWL/RG/A/N, respectively. PCR amplification was performed with Takara Ex Taq (Takara Shuzo) with 30 cycles of 30 s at 94°C, 1 min at 50°C, and 40 s at 72°C. For an extension step for the reverse primer corresponded to the sequence 5'-atgagctc-3'. The PCR-amplified products (~0.5 kb) were cloned into pGEM-T Easy plasmid vector (Promega) and sequenced using a PRISM DyeDeoxy Terminator Cycle Sequencing System (Applied Biosystems). The sequence analysis revealed that two types of cDNA fragments closely related to FAD2 were isolated in each experiment for *T. kirkiiouii* (TkFac and TkFad2) and *P. graminum* (PgFac and PgFad2). The second strand cDNA was synthesized with a Marathon cDNA Amplification Kit (Clontech). Adapter ligation to the double strand cDNAs and 5' and 3'-rapid amplification of cDNA ends was performed according to the manufacturer's protocol. cDNA fragments containing 5' and 3' regions were cloned into pGEM-T Easy and sequenced. Finally, full-length cDNAs of four FAD2-related cDNA fragments were isolated by PCR amplification with Pyrobest DNA polymerase (Takara Shuzo) and TaKaRa Ex Taq DNA polymerase (Takara Shuzo) using a set of primers corresponding to the sequences in 5'- and 3'-untranslated regions. The PCR amplification consisted of 25 cycles of 30 s at 94°C, 1 min at 55°C, and 2.5 min at 72°C followed by an extension step for 10 min at 72°C. The PCR product was incubated with TaKaRa Ex Taq DNA polymerase (Takara Shuzo) for 10 min at 72°C. The PCR products from four independent amplifications for a set of primers were cloned into pGEM-T Easy and sequenced as described above.

**Expression of TkFac and PgFac in Arabidopsis thaliana**—The coding regions of TkFac and PgFac were amplified by PCR using Pyrobest DNA polymerase. For amplification of TkFac, the forward primer was designed to be 5'-atgagctc-3' from the stop codon of TkFad2 with a flanking SacI site (5'-atgagctc-3'), and the reverse primer corresponded to the sequence 5'-27 bp downstream from the stop codon of TkFac with a flanking SacI site (5'-atgagctc-3'). For amplification of PgFac, the forward primer was designed with flanking EcoRV/Xhol sites (5'-atgagctc-3') and with a restriction site (Xhol) in the coding region and cloned into pBluescript II. The TkFad2 cDNA was then released with SacI digestion and cloned into pYES2. The PgFad2 cDNA fragment was released from pGEM-T Easy by HindIII/SacI digestion and cloned into pYES2. The resulting plasmid as well as pYES2 were introduced into *S. cerevisiae* D452-2 cells (19) using an S. c. EasyComp Transformation kit (Invitrogen). Transformed cells were selected for on yeast synthetic minimal medium plates lacking uracil (SC-Ura) (20).

Individual colonies of the transformed cells were grown in glucose culture medium (SC-Ura/2% glucose) for 1 day at 28°C with shaking. Cells were then collected by centrifugation, washed in sterilized water, and dissolved in galactose culture medium (SC-Ura/2% galactose). The cell suspension was diluted to A600 = 0.2 in galactose culture medium containing 0.1% (w/v) Tergitol-type Nonidet P-40 (Sigma) and grown at 20°C for 3 days with shaking in the dark. The culture was then harvested by centrifugation at 3,000 × g for 10 min, washed with 0.5 μM sodium methoxide in methanol at 50°C for 1 h. After the tubes were cooled to room temperature, the mixture was extracted with hexane. The pooled extracts were dried under vacuum and then dissolved in a small volume of hexane, and 1 μl was used for gas chromatography (GC) or GC-mass spectrometry (MS) analysis. For fatty acid analysis of Arabidopsis seeds, materials (2 mg) were ground with a mortar and pestle and then extracted with 1 ml of 0.5 M sodium methoxide in methanol. Leaf tissues (4 mg) were homogenized in 1 ml of 0.5 M sodium methoxide in methanol. For *T. kirkiiouii* and *P. graminum*, 2 mg of seeds without seed coats were used for fatty acid extraction. The homogenates were transferred to glass tubes and incubated at 50°C for 1 h. After the tubes were cooled to room temperature, 1.5 ml of 0.9% (w/v) sodium chloride was added to the tubes, and fatty acids were extracted with 1 ml of hexane. After centrifugation at 1,000 × g for 5 min, the hexane layer was transferred to a new tube and dried under vacuum. The dried copper pellets were then incubated with 0.5 μM sodium methoxide in methanol at 50°C for 1 h. After the tubes were cooled to room temperature, the mixture was extracted with hexane. The pooled extracts were dried under vacuum and then dissolved in a small volume of hexane, and 1 μl was used for gas chromatography (GC) or GC-mass spectrometry (MS) analysis. For fatty acid analysis of Arabidopsis seeds, materials (2 mg) were ground with a mortar and pestle and then extracted with 1 ml of 0.5 M sodium methoxide in methanol. Leaf tissues (4 mg) were homogenized in 1 ml of 0.5 M sodium methoxide in methanol. For *T. kirkiiouii* and *P. graminum*, 2 mg of seeds without seed coats were used for fatty acid extraction. The homogenates were transferred to glass tubes and incubated at 50°C for 1 h. After the tubes were cooled to room temperature, 1.5 ml of 0.9% (w/v) sodium chloride was added to the tubes, and fatty acids were extracted with 1 ml of hexane. After centrifugation at 1,000 × g for 5 min, the hexane layer was transferred to a new tube and dried under vacuum. The dried samples were dissolved in a small volume of hexane, and 1 μl was used for GC or GC-MS analysis. Fatty acid methyl esters were analyzed and quantified using a gas chromatograph (GC18A, Shimadzu) equipped with a TC-50 fused silica column (0.5 × 0.25 mm inner diameter, 0.25-mm film thickness; GL Science). The oven temperature was programmed to rise from 150°C to 240°C at a rate of 3°C/min and then hold for 6 min. GC-MS analysis was performed in standard EI mode using a JMS-600H MS route mass spectrometer.
RESULTS

Isolation of cDNAs Encoding FAD2-related Enzymes—Total fatty acids extracted from maturing seeds of *T. kirilowii* and *P. granatum* were analyzed by GC. Both seeds contained punicic acid at levels of more than ~40% (w/w) (*T. kirilowii*) and ~70% (w/w) (*P. granatum*) of total fatty acids (see Figs. 2D and 4D). We prepared RNA from these materials and isolated four cDNAs from *T. kirilowii* (TkFad2 and TkFac) and *P. granatum* (PgFad2 and PgFac) that encode polypeptides related to FAD2. These polypeptides contain three clusters of histidine residues that are thought to act as ligands to the catalytic iron atoms that have been proposed to form a di-iron oxo group (21). Comparison of amino acid sequences of FAD2-related fatty acid-modifying enzymes (GenBank™ accession numbers: hydroxylase, T09839 and AAC32755; epoxyxygenase, CA76156; acetylase, CA76158; and conjugase,AAF05915, AAF05916, AAG42259, AAG42260, and AAK26632) with 12-oleate desaturases (GenBank™ accession numbers: P46313, T14269, AAG42259, AAG42260, and AAK26632) revealed that several amino acids at certain positions were strictly conserved in 12-oleate desaturases (Table I). We prepared RNA from leaves of transgenic plants expressing FAD2-related polypeptides, each full-length cDNA was expressed under control of the constitutive CaMV 35S promoter (pKS-TkFad and pKS-PgFac) or the seed-specific napin promoter (pKN-TkFad and pKN-PgFac). Transformants (T1) were selected by drug resistance and then fatty acid methyl esters (FAMEs) from leaves of T1 plants were analyzed by gas chromatography. Although there was no difference in the fatty acid composition of vegetative tissues between transgenic and untransformed plants (data not shown), a prominent peak of FAME (Fig. 2, B and C, arrowheads), which was not present in seeds from untransformed plants, was detected in those from transgenic plants. The mass spectrum of this FAME was characterized by an abundant molecular ion at *m/z* = 320 and other diagnostic ions, which were identified as a methyl ester of an isomer of eicosatetraenoic acid (20:3). This peak displayed a gas chromatographic retention time different from that of the methyl 20:3Δ11cis,14cis,17trans present in both untransformed and transformed Arabidopsis seeds (Fig. 2). We analyzed FAMEs of yeast cells fed with 20:2Δ11cis,14cis to test whether this fatty acid was utilized as a substrate of TkFac and PgFac to produce conjugated 20:3 isomer. However, we could not find a peak corresponding to the methyl 20:3 isomer found in Arabidopsis seeds (data not shown). We supposed that a part of punicic acid was elongated to form the conjugated 20:3 (20:3Δ11cis,13trans,15 cis) as Arabidopsis seeds possess microsomal fatty acid elongase activity (22, 23).

In all transgenic plants, punicic acid was detected in seeds but not in vegetative tissues (data not shown). We compared the fatty acid composition of seed oils in transgenic and untransformed plants (Table I). Maximal concentration of punicic acid in seed oils was 10.2% (w/w) in pKN-TkFac transformants and 4.4% (w/w) in pKN-PgFac transformants (data not shown). The average concentration of punicic acid was higher in seeds from pKN-TkFac and pKN-PgFac transformants carrying the napin promoter, 3.5 and 2.3% (w/w), respectively. In contrast, concentrations in pKS-TkFac and pKS-PgFac transformants were ~0.4% (w/w). The concentration of the fatty acid tentatively identified as 20:3Δ11cis,13trans,15cis was in proportion to that of punicic acid (Table I) and accumulated to as much as 1% (w/w) of seed oils in the T2 seeds accumulating 10.2% (w/w) punicic acid (data not shown). Punicic acid accumulation in Arabidopsis seeds was accompanied by changes in relative amounts of other fatty acids (Table I). The change was remarkable in seeds of transformants with cDNAs driven by the napin promoter. Relative amounts of linoleic (18:2Δ9cis,12cis) and linolenic acids (18:3Δ9cis,12cis,15cis), which were 30% (w/w) and 19% (w/w) in untransformed seeds, were significantly lower in T2 seeds, at 23–24 and 12–15% (w/w), respectively. In contrast, the concentration of oleic acid was higher in seeds of transgenic plants (23–26% (w/w)) than in those of untransformed plants (15% (w/w)).

Functional Analyses of FAD2-related Enzymes in *S. cerevisiae*—To further characterize the function of the FAD2-related polypeptides, each full-length cDNA was expressed under control of the GAL1 promoter in yeast cells. First, each FAD2-related polypeptide was expressed in yeast cells grown in culture medium without exogenous fatty acids, and fatty acid compositions were compared with control yeast cells transformed with pYES2. In yeast cells expressing TkFad2 and PgFad2, two peaks of FAMEs were detected that were not present in those of pYES2-transformed control cells (Fig. 3 and data not shown). These were determined to be methyl esters of 16:2Δ9cis,12cis and 18:2Δ9cis,12cis by gas chromatographic retention time (Fig. 3) and mass spectral analyses (data not shown). We could not detect the peak corresponding to the methyl punicic acid in Fad2-expressing cells, although a large amount of linoleic acid was present (~19% (w/w) of the total fatty acids, Fig. 3B and data not shown). We concluded that TkFad2 and PgFad2 were Δ12-oleate desaturases. Interestingly, yeast cells expressing PgFac produced 16:2Δ9cis,12cis (~1% (w/w)) and 18:2 Δ9cis,12cis (~0.2% (w/w)), which were also found in TkFac-expressing cells although in lower concentrations of ~0.2% (w/w) and ~0.1% (w/w), respectively (Fig. 3, C and D). Although TkFac and PgFac were demonstrated to be the enzymes asso-
FIG. 1. Sequence analyses of FAD2-related proteins from *T. kirilowii* and *P. granatum*. A, comparison of amino acid sequences of FAD2-related proteins from *T. kirilowii* (*TkFad2* and *TkFac*) and *P. granatum* (*PgFad2* and *PgFac*) with FAD2 (Δ12-oleate desaturase) of *A. thaliana* (*AtFad2*). Three clusters of histidine residues are indicated by bars. Amino acids identical to those in the *TkFac* sequence are indicated by shading. Gaps in alignments are indicated by dashes. B, phylogenetic analysis of FAD2 and conjugase proteins using the ClustalW program. The distance along the horizontal axis corresponds to the extent of sequence divergence. Sequences were obtained from GenBank™ accession numbers: *ImpFadX*, *I. balsamina* conjugase, AAF05915.1; *CoFadX1*, *CoFadX2*, and *CoFac2*, *C. officinalis* conjugases, AAG42259, AAG42260, and AAK26632; *MomoFadX*, *M. charantia* conjugase, AAF05916.1; *CpFad2*, *Crepis palustina* 12-oleate desaturase, CAA76157.1; *CoFad2*, *C. officinalis* Δ12-oleate desaturase, AAK26633; and *AtFad2*, *A. thaliana* Δ12-oleate desaturase, P46313.
Fig. 2. Gas chromatographic analyses of FAMEs from transgenic Arabidopsis seeds. FAMEs from seeds of an untransformed Arabidopsis plant (A), from those of a T1 plant transformed with TkFac (B) and PgFac (C), and from T. kirilowii seeds (D) were analyzed by GC. The labeled peaks corresponding to methyl esters of fatty acids are as follows: 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, α-linolenic acid; 20:0, eicosanoic (arachidic) acid; 20:1, eicosenoic (gondoic) acid; 20:2, eicosadienoic acid; 20:3, eicosatrienoic acid; 22:0, docosanoic (behenic) acid; and 22:1, docosenoic (erucic) acid. Arrowhead, methyl punicic acid; arrow, a methyl ester of octadecatetraenoic acid (18:4), which was shown. By mass spectral analysis, the FAME was identified as 20:3Δ12,15Octyne, 18:4).

In addition to 16:2Δ9cis, 12cis and 18:2 Δ9cis, 12cis, two novel peaks of FAMEs (Fig. 3D, arrow and arrowhead) were found in PgFac-expressing cells grown in the absence of exogenous fatty acids. The peaks indicated by the arrow was determined to be methyl punicic acid by GC-MS analysis (data not shown) and by gas chromatographic retention time (Fig. 4D). Relative amounts of punicic acid produced in yeast cells grown with linoleic acid were greater than in those grown without linoleic acid (Figs. 3 and 4). With the addition of 0.3 ml linoleic acid to the medium, punicic acid was produced at concentrations of −0.1 and −0.8% (w/w) of the total fatty acids of TkFac- and PgFac-expressing cells, respectively (Fig. 4, B and C). In contrast, no or a very small amount of punicic acid was produced in TkFac- or PgFac-expressing cells grown without exogenous linoleic acid (Fig. 3, C and D). Another peak indicated by an arrowhead (Fig. 3) was also found in yeast cells grown with linoleic acid (Fig. 4C, arrowhead). This was characterized by a molecular ion at m/z = 264 and other diagnostic ions consistent with that of a methyl ester of hexadecatrienoic acid (16:3) but showed a different gas chromatographic retention time from 16:3Δ9cis, 12cis, 15cis (data not shown). The relative amount of 16:2Δ9cis, 12cis produced in PgFac-transformed cells was smaller in the cells grown with exogenous linoleic acid than in those grown without linoleic acid probably because of incorporation of linoleic acid from the medium into the lipid components of the cells instead of 16:2Δ9cis, 12cis (Fig. 4). Accompanying the 16:2Δ9cis, 12cis reduction in cells fed linoleic acid, the amount of the 16:3 isomer was also reduced. This result suggests that the isomer of 16:3 was 16:3Δ9cis, 11trans, 13cis specifically produced by PgFac expression. We detected a small amount of FAMEs of the 16:3 isomer and punicic acid through analysis of a large amount of FAMEs extracted from TkFac-expressing cells grown without exogenous fatty acids (data not shown). Therefore, we concluded that TkFac and PgFac are similar in function to each other.

Lastly, yeast cells expressing TkFac and PgFac were grown with exogenous α-linolenic acid, and FAMEs of these cells were compared with those of control cells by gas chromatography. We detected a novel although very small peak of FAME, which was not found in cells grown with linoleic acid (data not shown). By mass spectral analysis, the FAME was identified as a methyl ester of octadecatetraenoic acid (18:4), which was characterized by a molecular ion at m/z = 290 and other diagnostic ions (data not shown). Taken together the conjugases

| Fatty acid composition of Arabidopsis seeds from untransformed plants and from transgenic plants expressing TkFac and PgFac |  |  |  |  |
|---|---|---|---|---|
| Fatty acid | Untransformed (n = 6) | 3SS (n = 6) | Napin (n = 6) | 3SS (n = 9) | Napin (n = 9) |
| 16:0 | 7.6 ± 0.5 | 7.6 ± 0.2 | 7.2 ± 0.3 | 7.3 ± 0.3 | 6.9 ± 0.3 |
| 18:0 | 2.6 ± 0.4 | 2.9 ± 0.1 | 3.2 ± 0.1 | 2.7 ± 0.1 | 2.9 ± 0.1 |
| 18:1Δ9cis | 15.2 ± 1.5 | 16.2 ± 1.9 | 22.8 ± 3.7 | 19.1 ± 1.8 | 26.4 ± 4.2 |
| 18:2Δ9cis, 12cis | 30.3 ± 1.0 | 28.4 ± 1.3 | 23.3 ± 2.5 | 28.9 ± 1.2 | 24.2 ± 2.9 |
| 18:3Δ9cis, 12cis, 15cis | 19.4 ± 1.5 | 20.0 ± 1.0 | 14.6 ± 3.6 | 15.1 ± 1.4 | 11.5 ± 2.4 |
| 20:0 | 1.8 ± 0.3 | 1.9 ± 0.1 | 1.7 ± 0.2 | 1.7 ± 0.1 | 1.5 ± 0.1 |
| 20:1Δ9cis | 16.8 ± 0.9 | 17.8 ± 0.6 | 18.7 ± 0.8 | 17.0 ± 0.9 | 17.3 ± 1.0 |
| 20:2Δ9cis, 11cis, 13cis | 1.8 ± 0.2 | 1.7 ± 0.2 | 1.1 ± 0.3 | 1.3 ± 0.2 | 0.9 ± 0.2 |
| 20:3Δ9cis, 11cis, 14cis, 17cis | 0.4 ± 0.1 | 0.5 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.2 ± 0.1 |
| 22:1Δ9cis | 1.7 ± 0.3 | 1.7 ± 0.1 | 1.5 ± 0.1 | 1.4 ± 0.1 | 1.2 ± 0.2 |
| Punicic acid | ND | 0.4 ± 0.4 | 3.5 ± 3.3 | 0.4 ± 0.3 | 2.3 ± 1.1 |
| 20:3 | ND | 0.1 ± 0.1 | 0.4 ± 0.3 | 0.1 ± 0.1 | 0.2 ± 0.1 |
| Otherb | <3.0 | <2.0 | <2.0 | <4.0 | <5.0 |

a Tentatively identified as 20:3Δ12,15Octyne, 18:4.

b Includes 18:1Δ11cis, 20:1Δ13cis, and 22:0.
were shown to have the ability to form conjugated trienoic fatty acids of C16 and C18 carbon chains and the conjugated tetraenoic fatty acid of the C18 carbon chain. No conjugated dienoic fatty acids were detected in cells grown without exogenous fatty acid in the medium (Fig. 3) or with oleic acid (data not shown). These results suggest that TkFac and PgFac do not modify the cis-H90049 double bonds of oleic and palmitoleic acids.

**DISCUSSION**

In this study we isolated cDNAs encoding enzymes (TkFac and PgFac) involved in the formation of conjugated trans-Δ11, cis-Δ13 double bonds of punicic acid. Very recently cDNA encoding PuFADX, an enzyme identical to PgFac, was isolated from *P. granatum* (10). We have found a notable feature of TkFac and PgFac: they have bifunctional enzymatic activity. Yeast cells transformed with TkFac and PgFac accumulated 16:2H90049 cis,12cis and 18:2H90049 cis,12cis (Fig. 3). TkFac and PgFac were thus demonstrated to have both Δ12-oleate desaturase and conjugase activities. This observation supports the idea that a number of fatty acid-modifying enzymes have diverged from fatty acid desaturases (7, 24, 25). LFAH12 of *Lesquerella fendleri* has been shown to be a bifunctional enzyme having both Δ12-oleate hydroxylase and Δ12-oleate desaturase (FAD2) activities (26). Replacement of seven amino acids in LFAH12 with ones strictly conserved in FAD2 changed the catalytic preference of the enzyme from hydroxylase to desaturase (27). It is thus possible that a small number of amino acids determine the enzymatic specificity of conjugases. Punicic acid and α-eleostearic acid are closely related isomers in which a double bond at the Δ13 position is in cis- or trans-configuration. Comparison of amino acid sequences of the conjugases revealed that several amino acids conserved in MomoFADX and ImpFADX (which form conjugated trans-Δ11, trans-Δ13 double bonds) were replaced in TkFac and PgFac (which form conjugated trans-Δ11, cis-Δ13 double bonds). It is likely that several specific amino acids may be involved in the divergent functions of these enzymes.

Punicic acid accumulation was examined in transgenic *Ara-*
Bidopsis plants. All transgenic plants accumulated punicic acid in seeds (Table I), but not in leaves, even when the conjugases were driven under control of the CaMV 35S promoter (data not shown). A similar observation has been reported in Arabidopsis transformed with Δ12-oleate hydroxylase (26). In nature, seed-specific accumulation of unusual fatty acids results from seed-specific expression of their anabolic enzymes (7–9, 24, 26). It is not known whether conjugated fatty acids are eliminated from lipids in vegetative tissues because of their toxicity to cell function. It has been suggested that hydroxy fatty acids are broken down at a higher rate than typical fatty acids in vegetative tissues (26). Ectopic accumulation of very long chain fatty acids in vegetative tissues was accompanied by abnormalities in cell growth and chloroplast membrane integrity (28).

Expression of the conjugases under control of either the CaMV S35 or napin promoter led to accumulation of punicic acid in seeds (Table I). The concentration of punicic acid accumulation, several changes in fatty acid composition were observed (Table I). Oleic acid (18:1) increased in concentration (Table II) to a remarkable level in transgenic seeds with −10% (w/w) punicic acid, reaching a 1.8-fold higher concentration than in untransformed seeds. In contrast, linoleic (18:2) and α-linolenic acid (18:3) decreased from 30% (w/w) and 20% (w/w) in untransformed seeds (Table I) to 19% (w/w) and 8% (w/w) in transgenic seeds with 10% (w/w) punicic acid, respectively. A similar effect, suppression of Δ12-oleate desaturase, was reported in plants transformed with Δ12-acetyl-modifying enzymes. In Arabidopsis seeds expressing Δ12-oleate hydroxylase (26, 29) and Δ12-linoleate epoxidase (30) and in somatic soybean embryos expressing conjugases (7), accumulation of modified fatty acids was accompanied by a marked increase in oleic acid. A recent study suggested that exogenously expressed Δ12-acetyl-modifying enzymes lead to an increase in oleic acid by interfering with endogenous Δ12-oleate desaturase at the translational or post-translational levels (30). In transgenic Arabidopsis seeds, a novel fatty acid occurred that was identified as an isomer of 20:3 by GC-MS analysis. We analyzed the FAMEs of PgFac-expressing yeast cells grown with 20:2 Δ9cis, 12cis but did not find the peak corresponding to the 20:3 isomer (data not shown). Therefore, it is unlikely that the 20:3 isomer resulted from conjugase-mediated direct conversion of 20:2 Δ9cis, 12cis to conjugated 20:3. Alternatively, as Arabidopsis seeds contain polyunsaturated very long chain fatty acids (Fig. 2), in contrast to T. kirilowii and P. granatum which accumulate neither very long chain fatty acids nor α-linolenic acid (Figs. 2D and 4D), it is possible that the 20:3 isomer resulted from fatty acid elongation of punicic acid by the activity of microsomal fatty acid elongase (22, 23).

The substrate specificity of the conjugases was revealed by their expression in yeast cells. 16:2Δ9cis, 12cis occurred in yeast expressing the conjugases and could be converted into the 16:3 isomer, which was proposed to be a conjugated fatty acid containing cis-Δ9, trans-Δ11, cis-Δ13 double bonds (Figs. 3D and 4C). Thus, the conjugases can utilize fatty acids with C16 carbon chains as substrates as well as those with C18 carbon chains. In addition, we analyzed the FAMEs of PgFac-expressing yeast cells grown with α-linolenic acid (18:3 Δ9cis, 12cis, 15cis) and found a novel peak representing a methyl ester of 18:4 isomer, which was proposed to be 18:4 Δ9cis, 11trans, 13cis, 15cis. By GC-MS analysis of FAMEs from transgenic Arabidopsis seeds, we also found a small amount of methyl ester of 18:4 isomer, which had a chromatographic retention time identical to that of the 18:4 isomer found in yeast cells (data not shown). In Arabidopsis seeds, α-linolenic acid compose −10% (w/w) of the total seed oils but, in contrast, is absent in seed oils of T. kirilowii and P. granatum. In addition, transgenic studies using yeast cells have demonstrated that α-linolenic acid is a possible substrate for conjugases associated with the formation of both trans-Δ11, trans-Δ13 double bonds and trans-Δ8, trans-Δ10 double bonds (7–9). In our study, yeast cells accumulated large amounts of 16:1 Δ9cis and 18:1 Δ9cis; however, no novel dienoyl fatty acids were found in T. Fac- and PgFac-expressing cells (Figs. 3 and 4). This result indicates that these enzymes exclusively modify a Δ12 double bond in contrast to CoFac2/CoFADxs, which modifies a Δ9 double bond to produce conjugated trans-Δ8, trans-Δ10 double bonds (8, 9).

Fatty acids with conjugated double bonds have been studied in therapeutic medical applications, and a large number of beneficial effects of dietary supplementation with conjugated linoleic acid (CLA) have been reported. CLA has been shown to reduce the incidence of mammalian tumors in mice, inhibit the proliferation of cancer cells in culture (31–33), and reduce body fat in rodents and humans (34–37). Recent studies have shown that CLNAs are more cytotoxic to tumor cells than is CLA (2). In addition, perirenal and epididymal adipose tissues are reduced in rats fed free fatty acids rich in CLNAs (4). CLNAs have been suggested to modulate body fat and triacylglycerol metabolism differently than CLA (4), but the exact mechanism of the anticarcinogenic and anti-obese action of CLA and CLNA remains unknown. In addition to increasing our understanding of fatty acid biochemistry, identification of conjugases may lead to the discovery of methods to produce CLNAs in common oil crops.

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