Production of Linolenic Acid in Yeast Cells Expressing an Omega-3 Desaturase from Tung (*Aleurites fordii*)

John M. Dyer*, Dorselyn C. Chapital, Jui-Chang W. Kuan, Hurley S. Shepherd, Fuqiang Tang, and Armand B. Pepperman

USDA, ARS, SRRC, New Orleans, Louisiana 70124

ABSTRACT: Tung oil is an industrial drying oil containing *ca.* 90% PUFA. We previously reported on enzymes required for the synthesis of linoleic (6% of FA) and eleostearic (80%) acids and here describe the cloning and functional analysis of an omega-3 FA desaturase (FAD3) required for the synthesis of linolenic acid (1%). The tung FAD3 cDNA was identified by screening a tung seed cDNA library using the polymerase chain reaction and degenerate primers encoding conserved regions of the FAD3 enzyme family. Expression of this cDNA in yeast cells, cultured in the presence of linoleic acid, resulted in the synthesis and accumulation of linolenic acid, which accounted for up to 18% w/w of total cellular FA. Tung FAD3 activity was significantly affected by cultivation temperature, with the greatest amount of linolenic acid accumulating in yeast cells grown at 15°C. The amount of linolenic acid synthesized in yeast cells by tung FAD3 is *ca.* 10-fold higher than that observed by expression of a rapeseed (*Brassica napus*) FAD3 in yeast, suggesting that tung FAD3 might be useful for biotechnological production of omega-3 FA in transgenic organisms.

Paper no. J10676 in *JAOCS 81,* 647–651 (July 2004)

KEY WORDS: *Aleurites fordii,* linolenic acid, omega-3 fatty acid desaturase, *Saccharomyces cerevisiae,* tung oil.

Tung oil is a high-value industrial oil derived from seeds of the tung tree, *Aleurites fordii.* The oil dries to a clear, hard, protective finish and is considered one of the highest-quality drying-oils known to man (1). The oil is used in pure form as a wood lacquer and also may be used in formulations of inks, dyes, coatings, and resins. The unique drying qualities of tung oil are determined primarily by the FA composition of the oil, which is dominated by an unusual FA called α-eleostearic acid (*cis*-9,*trans*-11,*trans*-13 18:3). This conjugated, trienoic FA accounts for about 80% of tung oil FA and is readily oxidized when exposed to air, resulting in formation of the unique polymer that is characteristic of a tung oil finish.

Availability of genes encoding enzymes for tung oil biosynthesis would represent a valuable tool for production of tung-like drying oils in more conventional oilseed crops. Progress to date has indicated, however, that transfer of a single gene encoding an enzyme for synthesis of an unusual FA into crop plants generally results in much lower levels of the FA than observed in the oil of the source plant (2). Thus, it is clear that multiple enzymes from a source plant will be required for efficient synthesis and accumulation of exotic FA in a transgenic host plant.

Our laboratory is interested in identifying all of the enzymes involved in the synthesis of tung oil. Since this oil is enriched in PUFA, our initial focus has been on the identification of enzymes for PUFA biosynthesis. Production of PUFA in developing seeds is generally well understood (3). FA biosynthesis occurs in the plastids, and a soluble fatty acid desaturase (FAD) that introduces the first double bond into the FA structure is present in the stroma. Oleoyl-CoA is exported from the plastids, and then the oleoyl side chain is transferred to the phospholipid fraction of the endoplasmic reticulum (ER) for further desaturation by membrane-bound FAD. These enzymes act upon the fatty-acyl side chains of PC. The FAD2 enzyme converts oleic acid (*cis*-9 18:1) to linoleic acid (*cis*-9,*cis*-12 18:2), which is further desaturated to α-linolenic acid (*cis*-9,*cis*-12,*cis*-15 18:3) by FAD3. In some plants, including tung (4), a diverged FAD2 enzyme is present that converts linoleic acid to exotic FA containing conjugated or acetylenic bonds, or additional functional groups such as epoxy moieties (5).

Previously, we identified the tung FAD2 and diverged-FAD2 enzymes that catalyzed the synthesis of linoleic and eleostearic acids, respectively (4), and here we describe the cloning and functional analysis of a tung FAD3 enzyme. Collectively, these three enzymes synthesize all of the PUFA found in tung oil. Surprisingly, the tung FAD3 enzyme exhibited robust activity in yeast cells, suggesting that this enzyme might be a valuable tool for biotechnological production of omega-3 FA in transgenic organisms.

EXPERIMENTAL PROCEDURES

Identification of omega-3 desaturases using the polymerase chain reaction (PCR). To identify omega-3 desaturases expressed in developing tung seeds, a tung seed cDNA library was screened using PCR and degenerate primers encoding conserved regions of the FAD gene family (5′-GCTTGTTGGACTGCAATGGC-3′ and 5′-GGGATYTGHGGGAARAGATGATG-3′). PCR products of expected size (~750 bp) were cloned into pCR2.1 (Invitrogen, Carlsbad, CA), and their DNA sequences were determined. Three desaturase-like

*To whom correspondence should be addressed at USDA-ARS Southern Regional Research Center, 1100 Robert E. Lee Blvd., New Orleans, LA 70124. E-mail: jdyer@srrc.ars.usda.gov

Present address of fifth author: National Center for Infectious Disease, Centers for Disease Control and Prevention, 1600 Clifton Rd., Atlanta, GA 30333.

fragments were identified, two of which encoded putative plastid-localized enzymes that are described elsewhere in sequence reports (6,7). The remaining portions of the FAD3-like fragment were obtained using the 5′/3′ RACE Kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's protocol. Finally, the full-length open reading frame was PCR-amplified from the cDNA library using primers 5′-ATCCACCATGAAGCAGCAACAGTACAAA-GAC-3′ and 5′-TCATCAGTTCAACTTGCTTTTATCAAC-3′ and cDNA library as template. The resulting DNA fragments were gel purified and ligated into the yeast expression vector pYES2.1 (Invitrogen); several clones were sequenced in their entirety. As a positive control for FAD3 enzyme activity in yeast, the *Brassica napus* FAD3 open reading frame was amplified from plasmid pRTL2-mycFAD3 (8) using primers 5′-GGGATCCACCATGGTTGTTGCTATGGAC-3′ and 5′-GGAGCTCTTAATTGATTTTAGATTTGTCAGAAGC-3′, and products also were subcloned into a pYES2.1 expression vector.

Yeast expression and lipid analysis. Plasmids were transfected into yeast cells using a lithium acetate protocol (9), and transformants were selected on synthetic dextrose media (2% wt/vol dextrose, 0.67% yeast nitrogen base without amino acids) containing synthetic complete supplements lacking uracil (Bufferad, Lake Bluff, IL). Yeast cells were cultured in galactose media, then cells were harvested, lipids extracted, and cellular FA compositions determined as described previously (4). Briefly, cells were grown overnight in dextrose media and then diluted into galactose media containing (in some cases) 0.1% (vol/vol) linoleic acid. Cultures were incubated with continuous shaking for 40 h at 20°C, unless indicated otherwise. Cells were harvested by centrifugation, lipids extracted using chloroform/methanol (10), and FAME prepared using sodium methoxide. FAME were analyzed by GC/FID, and peaks were identified by comparison with authentic standards run using the same conditions. Methyl heptadecanoate was used as an internal standard.

RNA isolation and Northern blot analysis. Seed and leaf tissues were immersed in liquid nitrogen immediately after harvest at the American Tung Oil Corporation (Lumberton, MS) and then stored at –80°C until isolation of RNA. RNA was isolated using the hot borate method of Wan and Wilkins (11). RNA (15 μg) was size-fractionated on a 1% glyoxal-agarose gel using the NorthernMax kit and RNA Millennium Markers (Ambion, Austin, TX). Seed RNA from a time at which linolenic acid was known to be present in the seed (late July) was used for the Northern blot analysis. Equal loading of RNA was assessed by UV quantification and by ethidium bromide staining of agarose gels. RNA was blotted onto charged nylon membrane (Ambion). Probe for the blot was a gel-purified *Xho*I/*Bam*HI 1,252 bp fragment of the tung FAD3 open reading frame. Labeling of the probe (Psoralen-Biotin), hybridization, and detection (BrightStar BioDetect) were carried out according to manufacturer's protocols (Ambion) using high stringency conditions. Chemiluminescent blots were analyzed using a Fuji LAS-1000Plus (Fuji Medical Systems, Stamford, CT).

RESULTS AND DISCUSSION

Cloning and sequence analysis of tung FAD3. As part of an ongoing project to identify all of the enzymes involved in the biosynthesis of tung oil, a tung seed cDNA library was screened to identify enzymes responsible for synthesis of linolenic acid, a minor component of tung oil (approximately 1% w/w total FA). PCR reactions were conducted using degenerate primers encoding conserved regions of the omega-3 desaturase enzyme family (see the Experimental Procedures section for details). Three different desaturase cDNA were identified, two of which encoded putative plastid-localized omega-3 desaturases likely involved in the synthesis of plastid PUFA (6,7). The third cDNA, described in detail here, encoded a putative ER-localized polypeptide of 387 amino acids.

Alignment of the deduced tung FAD3 polypeptide sequence with the sequence of *B. napus* FAD3 (Fig. 1A) illustrates the presence of several conserved features of the FAD enzyme family, including several potential transmembrane-spanning domains and three highly conserved histidine boxes, which are thought to coordinate two iron atoms at the active site of the enzyme (5,12). Like other plant FAD3 reported to date, the tung FAD3 sequence lacks a recognizable N-terminal ER targeting signal but does contain a di-lysine motif (KxKxx) at the C-terminus previously shown to be important for localization of membrane proteins in the ER (13). Phylogenetic analysis indicated that tung FAD3 was grouped with other FAD3 enzymes, which formed a distinct clade separate from the plastid-localized FAD7/8 enzymes (Fig. 1B).

Functional analysis of tung FAD3 expressed in yeast cells. To determine the enzymatic function of tung FAD3, the cDNA encoding FAD3 was expressed in *Saccharomyces cerevisiae* cells on a high-copy plasmid (pYES2.1) using a galactose-inducible gene promoter. *Saccharomyces cerevisiae* cells do not normally synthesize PUFA (Fig. 2, top panel) but can readily acquire a variety of FA substrates initially provided in the growth media (14). Incubation of control yeast cells (harboring an empty expression plasmid) in galactose media supplemented with linoleic acid resulted in substantial uptake and incorporation of this FA, which accounted for *ca*. 54% w/w of total cellular FA (Fig. 2). As a positive control for linolenic acid biosynthesis, the *Brassica* FAD3 cDNA was expressed in yeast cells, resulting in accumulation of *ca.* 1.5% linolenic acid in yeast cells, an amount consistent with previous observations (15,16). Expression of tung FAD3 in yeast, however, resulted in a notable reduction of linoleic acid content and an increase in linolenic acid content to about 15% (Fig. 2). The amount of linolenic acid produced in yeast cells by tung FAD3 is *ca.* 10-fold higher than the amount produced by *Brassica* FAD3. It is also greater than threefold higher than the highest amount of linolenic

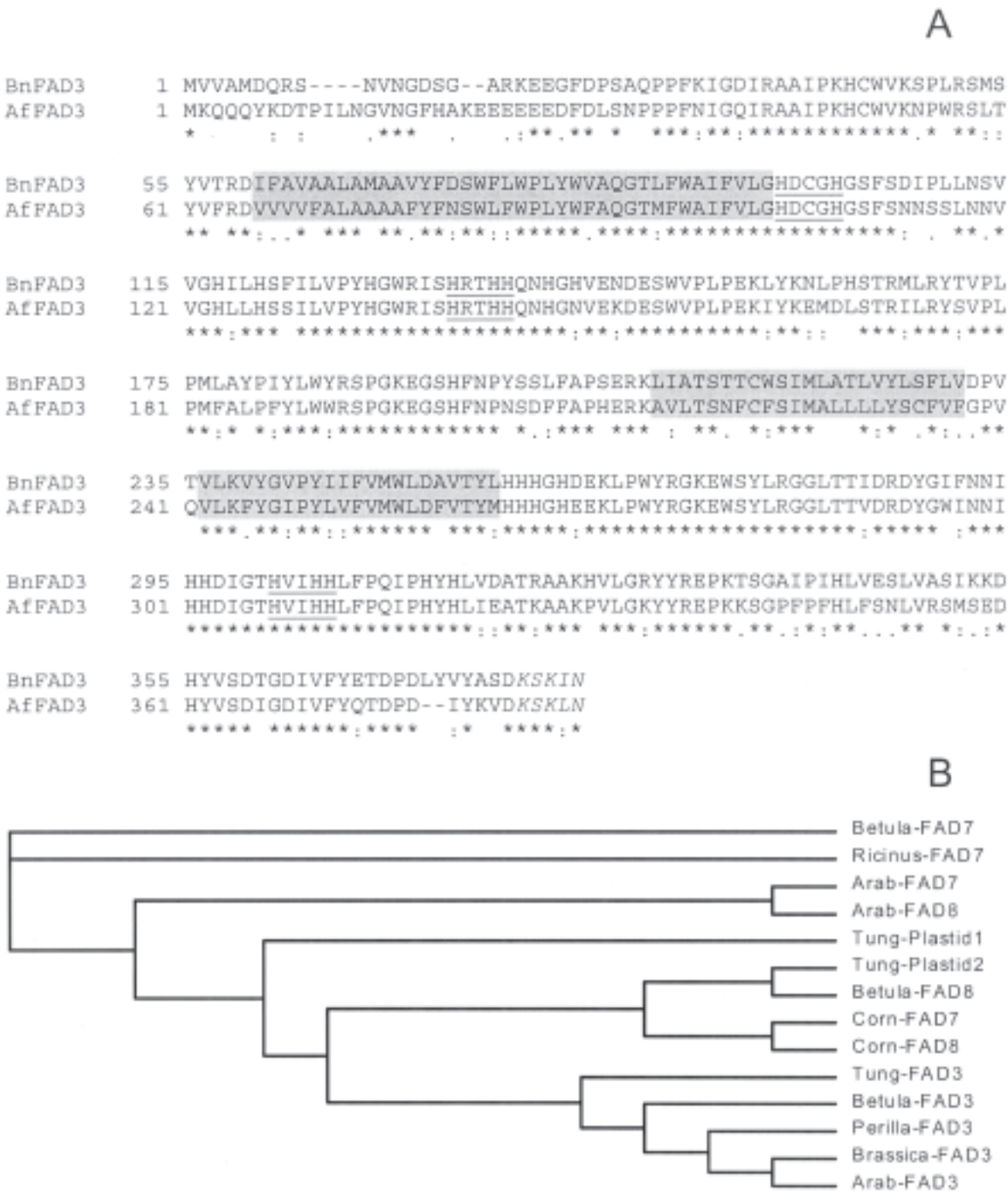

FIG. 1. Sequence analysis and phylogenetic relationships of tung omega-3 fatty acid desaturases (FAD). (A) Amino acid alignment of deduced *Brassica napus* FAD3 (BnFAD3; GenBank accession number L01418) and tung FAD3 (AfFAD3; accession number AJ0110004). Conserved hydrophobic regions predicted to form membrane-spanning domains are shaded. Three histidine-rich boxes are underlined. Putative endoplasmic reticular membrane retention signals at the C-termini are italicized. (B) Dendrogram showing phylogenetic relationships of various FAD3 and FAD7/8 omega-3 FAD. Amino acid sequences of the indicated proteins were aligned and compared using the CLUSTALW algorithm (version 1.82), and the resulting dendrogram was generated with TreeView (Win32), version 1.6.6. Sequences represented in the dendrogram, from top to bottom, are *Betula pendula* FAD7 (accession number AY135565), *Ricinus communis* FAD7 (L25897), *Arabidopsis thaliana* FAD7 (D14007), *A. thaliana* FAD8 (L27158), *Aleurites fordii* (tung) putative plastid isoform 1 (AF200717), *A. fordii* putative plastid isoform 2 (AF061027), *B. pendula* FAD8 (AY135564), *Zea mays* FAD7 (D63954), *Z. mays* FAD8 (D63953), *A. fordii* FAD3, *B. pendula* FAD3 (AY135566), *Perilla frutescens* FAD3 (AF047039), *B. napus* FAD3, and *A. thaliana* FAD3 (D26508).

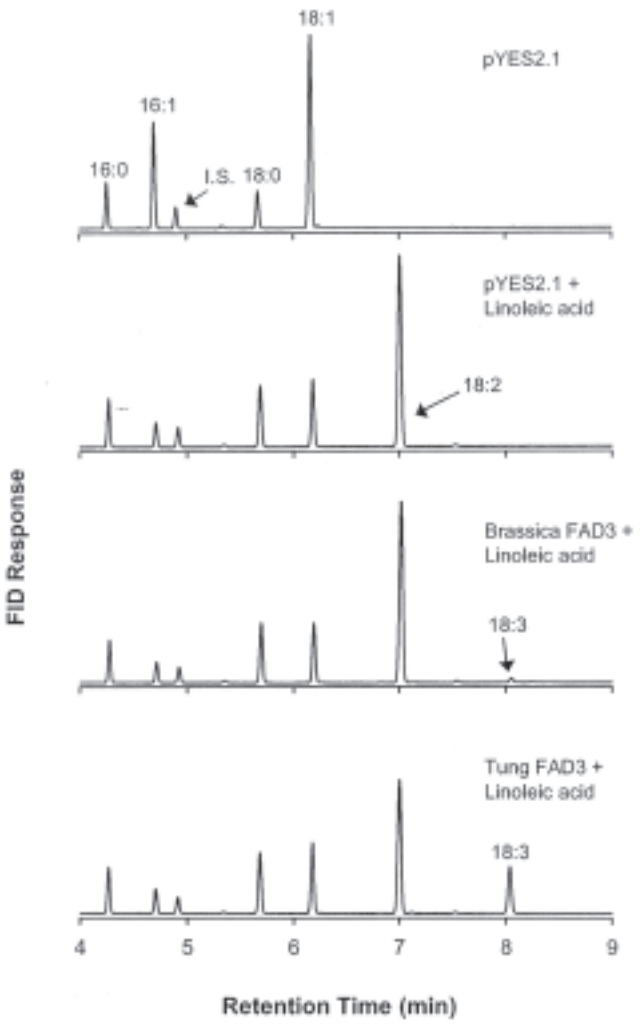

FIG. 2. Functional analysis of plant omega-3 desaturases expressed in yeast. Yeast cells harboring a control plasmid (pYES2.1), high-copy plasmid containing *Brassica* FAD3, or high-copy plasmid bearing tung FAD3 were cultured at 20°C in galactose media containing linoleic acid, then cells were harvested, lipids extracted, and FA composition determined using GC/FID. Labeled peaks correspond to the methyl esters of palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), linoleic (18:2), and α-linolenic (18:3) acids. Methyl heptadecanoate (17:0) was included as an internal standard (I.S.). Specific plasmids and growth conditions are shown on each panel. Each chromatogram is representative of three independent experiments. See Figure 1 for abbreviation.

acid reported previously in transgenic yeasts (4.1%), which was detected during functional analysis of a *Caenorhabditis elegans* desaturase expressed in yeast cells (17).

It is interesting to note the extraordinary difference in tung and *Brassica* FAD3 activity in yeast cells, despite 70% amino acid identity (88% similarity). Since identical gene expression vectors and promoters were used in our comparison of tung and *Brassica* FAD3 described here, the differences in activity are likely due to posttranscriptional events such as mRNA stability, translational efficiency, enzyme half-life, or enzyme catalytic efficiency. A detailed analysis of these factors is underway, and this information should aid in the use of these enzymes in lipid metabolic engineering strategies geared toward production of PUFA in microbes (18).

Effects of temperature on tung FAD3 activity. We previously reported that the chilling of yeast cells expressing the *Brassica* FAD3 enzyme resulted in a significant increase in linolenic acid content in cellular lipids (16). To determine whether tung FAD3 was also sensitive to cultivation temperature, yeast cells expressing this cDNA were grown at temperatures ranging from 10 to 30°C, then FA composition was determined using GC/FID. As shown in Figure 3, the amount of linolenic acid accumulating in yeast cells was significantly increased by cultivation at cooler growth temperatures, with *ca.* 15% linolenic acid observed at 10–20°C and only 5% observed at 25 or 30°C. The increases in linolenic acid content at cooler temperatures are consistent with the trends previously observed with *Brassica* FAD3 (16), except that the amounts of linolenic acid produced at each temperature by tung FAD3 are at least 10-fold higher than amounts with the *Brassica* enzyme.

Northern blot analysis of tung FAD3 in tung tissues. To determine whether the tung FAD3 gene was expressed exclusively in developing tung seeds, Northern blots were performed on RNA isolated from both tung leaf and seed tissues. As shown in Figure 4, the tung FAD3 gene was expressed in both tissues, and mRNA content was higher in leaf tissue in

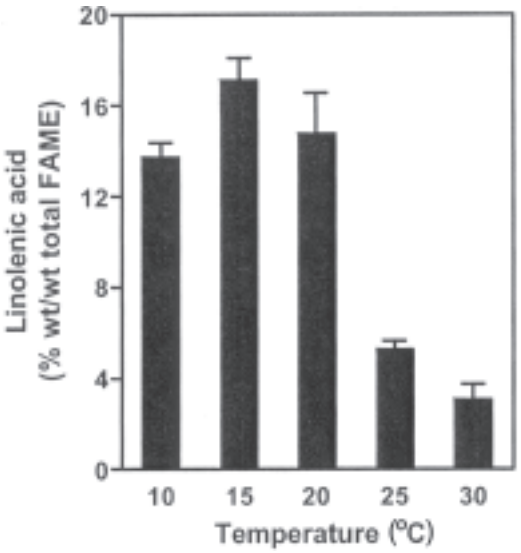

FIG. 3. Effects of temperature on tung FAD3 activity. Yeast cells expressing tung FAD3 were cultured in the presence of linoleic acid and at the indicated temperatures, then cells were harvested and FA composition determined using GC/FID. Bars represent the amount of linolenic acid detected in yeast cells at each temperature (average and SD of three independent experiments). See Figure 1 for abbreviation.

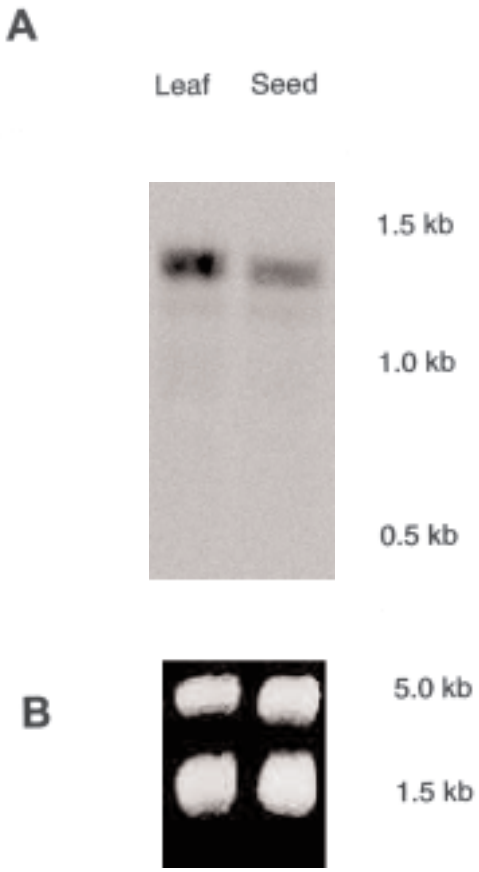

FIG. 4. Northern blot analysis of tung FAD3 gene expression in tung leaf or seed tissue. (A) Total RNA (15 μg) was isolated from either tung seed or leaf tissue, then size-fractionated on an agarose gel, transferred to membrane, and probed with labeled tung FAD3 under stringent wash conditions. (B) Ethidium bromide-stained gel of total RNA demonstrating equal loading of samples. See Figure 1 for abbreviation.

comparison with seed tissue. This pattern of gene expression was similar to that of the tung FAD2 gene (4), which encodes the enzyme for synthesis of linoleic acid. The only seed-specific oil-related gene that we have identified to date is tung FADX, which encodes the enzyme for production of eleostearic acid. These patterns of gene expression correlate with the known FA compositions of each tissue: Linoleic and linolenic acids are found in both leaf and seed tissues, whereas eleostearic acid is found exclusively within developing seeds.

ACKNOWLEDGMENT

This work was supported by the United States Department of Agriculture, Agricultural Research Service (CRIS project no. 6435-41000-083-00D).

REFERENCES

1. Sonntag, N.O.V., Composition and Characteristics of Individual Fats and Oils, in *Bailey's Industrial Oil and Fat Products,* 4th edn., edited by D. Swern, John Wiley & Sons, New York, 1979, Vol. 1, pp. 289–477.
2. Thelen, J.J., and J.B. Ohlrogge, Metabolic Engineering of Fatty Acid Biosynthesis in Plants, *Metab. Eng. 4:*12–21 (2002).
3. Ohlrogge, J., and J. Browse, Lipid Biosynthesis, *Plant Cell 7:*957–970 (1995).
4. Dyer, J.M., D.C. Chapital, J.C. Kuan, R.T. Mullen, C. Turner, T.A. McKeon, and A.B. Pepperman, Molecular Analysis of a Bifunctional Fatty Acid Conjugase/Desaturase from Tung. Implications for the Evolution of Plant Fatty Acid Diversity, *Plant Physiol. 130:*2027–2038 (2002).
5. Shanklin, J., and E.B. Cahoon, Desaturation and Related Modifications of Fatty Acids, *Annu. Rev. Plant Physiol. Plant Mol. Biol. 49:*611–641 (1998).
6. Tang, F., J.M. Dyer, A.R. Lax, D.S. Shih, D.C. Chapital, and A.B. Pepperman, Nucleotide Sequence of a cDNA Clone for Omega-3 Fatty Acid Desaturase (Accession No. AF061027) from *Aleurites fordii* Seeds (PGR99-009), *Plant Physiol. 119:*364 (1999).
7. Shepherd, H.S., J.M. Dyer, F. Tang, D.S. Shih, and A.B. Pepperman, Nucleotide Sequence of a cDNA Clone of a Plastid-Type Omega-3 Fatty Acid Desaturase (Accession No. AF200717) from Tung (*Aleurites fordii*) Seeds (PGR00-009), *Ibid. 122:*292 (2000).
8. Dyer, J.M., and R.T. Mullen, Immunocytological Localization of Two Plant Fatty Acid Desaturases in the Endoplasmic Reticulum, *FEBS Lett. 494:*44–47 (2001).
9. Gietz, R.D., and R.A. Woods, High-Efficiency Transformation in Yeast, in *Molecular Genetics of Yeast: Practical Approaches,* edited by J.A. Johnston, Oxford University Press, New York, 1994, pp. 121–134.
10. Bligh, E.G., and W.J. Dyer, A Rapid Method of Total Lipid Extraction and Purification, *Can. J. Biochem. Physiol. 37:*911–917 (1959).
11. Wan, C.Y., and T.A. Wilkins, A Modified Hot Borate Method Significantly Enhances the Yield of High-Quality RNA from Cotton (*Gossypium hirsutum* L.), *Anal. Biochem. 223:*7–12 (1994).
12. Shanklin, J., E. Whittle, and B.G. Fox, Eight Histidine Residues Are Catalytically Essential in a Membrane-Associated Iron Enzyme, Stearoyl-CoA Desaturase, and Are Conserved in Alkane Hydroxylase and Xylene Monooxygenase, *Biochemistry 33:*12787–12794 (1994).
13. Jackson, M.R., T. Nilsson, and P.A. Peterson, Identification of a Consensus Motif for Retention of Transmembrane Proteins in the Endoplasmic Reticulum, *EMBO J. 9:*3153–3162 (1990).
14. Bossie, M.A., and C.E. Martin, Nutritional Regulation of Yeast Δ-9 Fatty Acid Desaturase Activity, *J. Bacteriol. 171:*6409–6413 (1989).
15. Reed, D.W., U.A. Schäfer, and P.S. Covello, Characterization of the *Brassica napus* Extraplastidial Linoleate Desaturase by Expression in *Saccharomyces cerevisiae*, *Plant Physiol. 122:*715–720 (2000).
16. Dyer, J.M., D.C. Chapital, J.W. Cary, and A.B. Pepperman, Chilling-Sensitive, Post-transcriptional Regulation of a Plant Fatty Acid Desaturase Expressed in Yeast, *Biochem. Biophys. Res. Commun. 282:*1019–1025 (2001).
17. Meesapyodsuk, D., D.W. Reed, C.K. Savile, P.H. Buist, S.J. Ambrose, and P.S. Covello, Characterization of the Regiochemistry and Cryptoregiochemistry of a *Caenorhabditis elegans* Fatty Acid Desaturase (Fat-1) Expressed in *Saccharomyces cerevisiae, Biochemistry 39:*11948–11954 (2000).
18. Dyer, J.M., D.C. Chapital, J.W. Kuan, R.T. Mullen, and A.B. Pepperman, Metabolic Engineering of *Saccharomyces cerevisiae* for Production of Novel Lipid Compounds, *Appl. Microbiol. Biotechnol. 59:*224–230 (2002).

[Received June 19, 2003; accepted June 14, 2004]