Progeny Analysis of Tobacco That Express a Mammalian Δ9 Desaturase

W.S. Grayburn[a] and D.F. Hildebrand[b,*]

[a]USDA, ARS, Northern Crop Science Lab, Fargo, North Dakota 58105
and [b]Department of Agronomy, University of Kentucky, Lexington, Kentucky 40546-0091

ABSTRACT: Various tissues of *Nicotiana tabacum* plants transformed with a rat stearyl-CoA desaturase gene were analyzed for lipid composition. Gas chromatographic analysis showed a decrease in saturated 16 and 18 carbon fatty acids in leaves and roots of several progeny. Polymerase chain reaction analysis of total DNA from leaves indicated the presence of the desaturase gene in kanamycin-resistant progeny with altered fatty acid composition. Western immunoblot analysis confirmed the presence of introduced desaturase protein. Data on the inheritance of kanamycin resistance is presented.
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Recently there has been interest in reducing the content of saturated fatty acids in foods, with an emphasis on edible oils. In response, efforts have been initiated to develop vegetable oils with higher monounsaturated fatty acid contents.

The two major saturated fatty acids found in most plant tissues are palmitic (16:0) and stearic (18:0) acids. To reduce saturation, double bonds are formed by consecutive desaturation reactions. In addition to desaturases, these reactions also require electron transport components. In plants, the first desaturation step occurs in chloroplasts (1). Genes that encode the desaturase which converts 18:0 to 18:1 have been cloned from rat (2), mouse (3), yeast (4), and plants (5,6).

In higher plants, 16:1 and 18:1 are formed from palmitic and stearic acids esterified to acyl carrier protein (7). Intermembrane forms of fatty acids in the cytoplasm can exist as CoA derivatives. Antisense RNA for a *Brassica* desaturase has been used to increase fatty acid saturation (8). Conversely, we used an approach to reduce fatty acid saturation. A desaturase that normally uses the CoA forms of fatty acids and functions in the cytoplasm (9) was introduced into tobacco (10). The stearyl-CoA desaturase was placed in a plant transformation vector driven by a duplicated 35S promoter which should give near-constitutive expression of the desaturase. We previously reported fatty acid changes in transgenic calli and leaves (10). In this report we present data on the progeny of transgenic tobacco plants with altered desaturase.

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*To whom correspondence should be addressed.

EXPERIMENTAL PROCEDURES

Determination of kanamycin resistance. Seeds were distributed on the surface of Petri dishes that contained 1% Phytagar (Gibco BRL, Gaithersburg, MD) supplemented with 200 mg/L kanamycin monosulfate. Untransformed *Nicotiana tabacum* cv. Xanthi seeds were placed on a separate plate as a negative control. Germinated seedlings were scored as yellow or brown (kanamycin-sensitive) or green (kanamycin-resistant) 2 wk after plating.

Preparation of antibody to rat desaturase and immunoblotting. An 18-amino acid synthetic peptide was prepared corresponding to amino acid residues 46 to 62 of the rat liver stearyl-CoA desaturase (2), but with a cysteine added to the amino terminus. This synthetic peptide was linked to keyhole limpet hemacyanin as described by Sambrook *et al.* (11). This synthetic peptide was also linked to bovine serum albumin to serve as an immunochemical control. The peptide–KLH conjugate was used to immunize rabbits using standard procedures. Stearyl-CoA desaturase-specific antibodies were isolated using a Pierce Chem. Co. (Rockford, IL) SulfoLink affinity column with the synthetic peptide linked to the gel using the manufacturer's procedures.

Western immunoblotting was performed by extracting proteins with a solution containing 3% sodium dodecyl sulfate (SDS), 1.5% 2-mercaptoethanol, 1% glycerol, and 1 mM phenylmethylsulfonyl fluoride. Proteins were separated on 8.3% SDS gels and electroblotted to nitrocellulose membranes. The nitrocellulose membranes were incubated with the desaturase antibodies in blocking solution, then in a goat anti-rabbit second antibody linked to alkaline phosphatase (Promega Co., Madison, WI). The membranes were then incubated in alkaline phosphatase substrate for visualization of the cross-reacting protein(s).

Preparation of nucleic acids and polymerase chain reaction (PCR). Plant DNA isolation and PCR amplification with primers specific desaturase gene were carried out as previously described (10).

Preparation of lipid extracts. Mature seeds were placed in a drying oven at 60°C for 12 to 18 h then transferred to paper envelopes and stored in a desiccator under vacuum until ready for extraction. Dried seeds (5 mg) were then ground in a mortar with 5 mg of carborundum in the presence of 2 mL (2:1)

chloroform/methanol. Liquid was transferred to a glass culture tube, and seeds were ground two additional times in chloroform/methanol. Most of the seed residue and 1 to 2 mL of extract were dried in a speedvac (Savant, Farmingdale, NY). Fatty acid methyl esters of lipids from seeds and leaves were then prepared as previously described (10).

RESULTS

Tobacco plants (*N. tabacum* cv. Xanthi) transformed with a gene encoding rat stearyl-CoA desaturase have been described previously (10). A number of these plants were allowed to self-pollinate. Seeds were screened for their ability to germinate and become green in the presence of kanamycin (Table 1). The calculated χ^2 values for each hypothesis are presented. A hypothesis was rejected if $P < 0.05$. The last column indicates retained hypotheses. In experiments with untransformed tobacco seed, a total of 513 seedlings in five different experiments were all sensitive to kanamycin (data not shown). Progeny of plants transformed with plasmids conferring kanamycin resistance and either lacking (plant E) or including the rat stearyl-CoA desaturase gene (plants T, U, W, X, Y) showed varying segregation for kanamycin resis-

tance. Nearly all progeny of plant T were resistant to kanamycin.

Fatty acid composition of seeds from primary transformants was determined to assess changes in saturation (Fig. 1). Since monounsaturated fatty acids may serve as substrates for further desaturation reactions, data are presented as ratios of saturated fatty acids to the sum of unsaturated fatty acids with 1, 2, or 3 double bonds. Panel A summarizes 16C fatty acids, while 18C fatty acid data are presented in panel B. It should be noted that scales may vary in different graphs. In mature seeds from primary transformants, the only clear difference observed was between plant T and controls for 16C fatty acids. For seed data, multiple seeds were pooled.

The remaining data in Figure 1 are from kanamycin-resistant plants. The letter of the progeny plant indicates the parent, while individual progeny are distinguished from each other by a number following a letter. Since preliminary studies indicated that progeny of plants T and U exhibited the greatest reduction in saturated fatty acids, these plants were studied in more detail. Compared to control (E) progeny, a reduction in 16:0 is apparent in leaves of most of the other plants tested. A smaller number of these plants show a reduction in 18:0. The opposite trend is seen in roots, where a

TABLE 1
Inheritance of Kanamycin Resistance for Progeny of Selfed Transgenic *Nicotiana tabacum* cv. Xanthi Plants[a]

Plant	Observed segregation		χ^2	Number of segregating alleles
	Km[R]	Km[S]		
E cap A	31	8	0.4 (0.5 < P < 0.75)	1
E cap B	54	16	0.2 (0.5 < P < 0.75)	1
E pool	54	11	2.3 (0.1 < P < 0.25)	1
T cap A	39	0	0.2 (0.5 < P < 0.75)	2–4
T cap B	70	1	0.01 (0.9 < P < 0.95)	2–4
T cap C	92	0	0.4 (0.5 < P < 0.75)	3–4
T cap D	85	0	0.3 (0.5 < P < 0.75)	3–4
T cap E	7	0	0.03 (0.75 < P < 0.9)	2–4
T cap F	31	0	0.1 (0.5 < P < 0.75)	2–4
T pool 1	22	0	0.09 (0.75 < P < 0.9)	2–4
T pool 2	55	0	0.2 (0.5 < P < 0.75)	2–4
U cap A	39	8	1.6 (0.1 < P < 0.25)	1
U cap B	63	21	0 (P > 0.9)	1
U cap D	98	35	0.1 (0.5 < P < 0.75)	1
U cap E	89	21	2.0 (0.1 < P < 0.25)	1
U cap F	55	27	2.7 (0.05 < P < 0.1)	1
U cap G	73	36	3.7 (0.05 < P < 0.1)	1
U cap H	37	3	0.1 (0.5 < P < 0.75)	2
W pool	46	6	2.5 (0.1 < P < 0.25)	2
X cap A	32	16	1.8 (0.1 < P < 0.25)	1
X cap B	38	8	1.4 (0.1 < P < 0.25)	1
X cap C	42	17	0.5 (0.25 < P < 0.5)	1
Y cap B	37	6	2.8 (0.05 < P < 0.1)	1

[a]"Pool" represents seed pooled from several capsules. "Cap" represents seed from individual capsules from a given plant; Km[R] and Km[S] = kanamycin-resistant and susceptible. For 1, 2, 3, or 4 segregating alleles, the best fit for a segregation ratio 3:1, 15:1, 63:1, or 255:1.
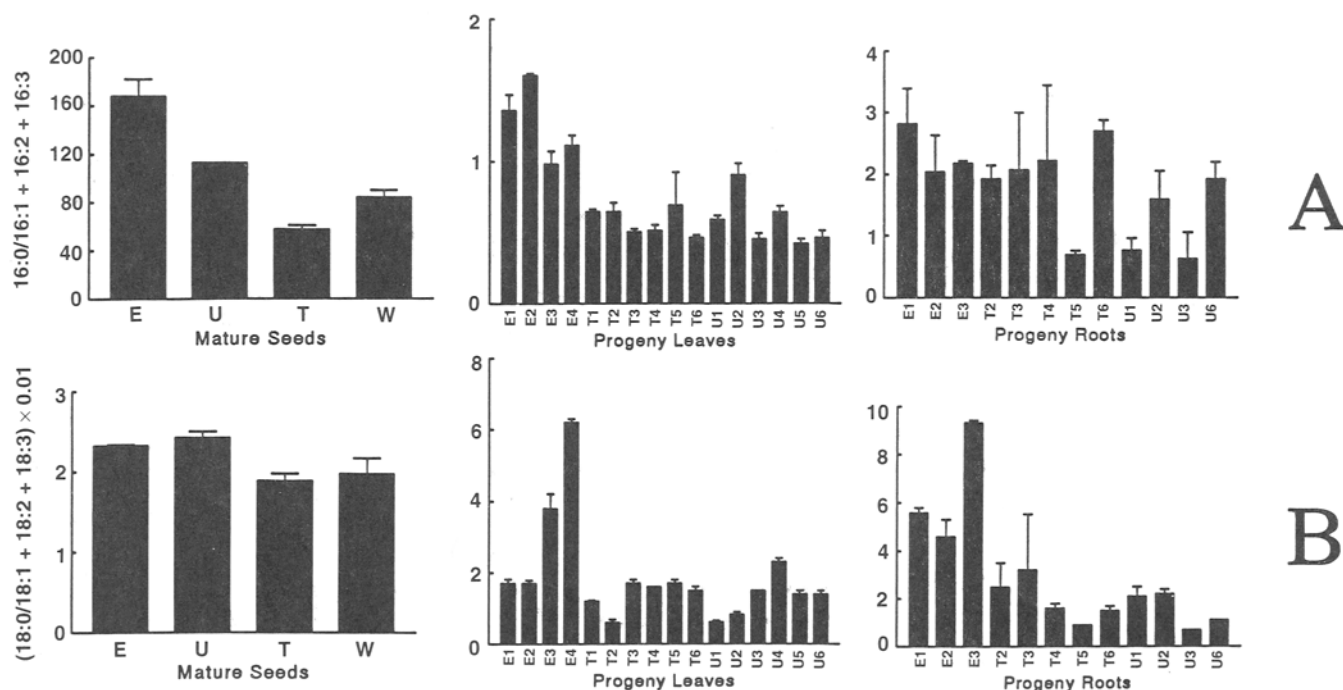
FIG. 1. Ratios of palmitic acid/16 carbon unsaturated fatty acids [16:0/(16:1 + 16:2 + 16:3)]; 16:1 in this case is palmitoleic or Δ9 hexadecenoic acid (A), or stearic acid/18 carbon unsaturated fatty acids [18:0/(18:1 + 18:2 + 18:3)] (B) for mature seeds, leaves, and roots from plants transformed with the plasmid containing the Δ9 desaturase coding sequence, p71²RDS (plants U and T) or the control plasmid pKYLX71:35S² (plants E). For leaves and roots, data are presented for individual kanamycin-resistant progeny of primary transformants. Graphs represent the means of three replications. Standard errors are indicated.

greater number of progeny (of desaturase transformants) show roots with reduced 18:0 than with reduced 16:0. The progeny varied in the expression of the introduced desaturase and in alteration of fatty acid composition. Some showed no desaturase or change in fatty acid composition relative to controls. Others showed somewhat larger changes in fatty acid desaturation than the parental plant material (primary transformants).

Concentrations of individual fatty acids in leaves and mature seeds are presented in Figure 2. Data from pooled seeds of primary transformant U showed a reduction in 16:0 and 18:0 levels relative to the control. Slight increases in 16:3 and 18:3 concentrations were seen in U seeds. Seeds from desaturase transformant T showed higher levels of all major fatty acids. In progeny leaves, concentrations of 16:1 were higher in plants with the introduced desaturase gene than in the control. A reduction in the 18:0 level was also seen in plants T1 and U1.

To demonstrate the presence of the rat stearyl-CoA desaturase gene in kanamycin-resistant progeny with altered fatty acid saturation, PCR amplification of total leaf DNA was performed (Fig. 3). A band with the expected mobility of 1.1 kb was seen in progeny of plants transformed with the rat desaturase gene but not in progeny of plants transformed with the vector not containing desaturase sequences. Evidence for the presence of protein encoded by the rat desaturase gene is shown in Figure 4. As indicated by reaction with a desaturase

antibody, the apparent size of the protein produced in a leaf from plant U6 was 35 kD.

DISCUSSION

This study demonstrates heritable alteration of fatty acid composition in transgenic tobacco. The cDNA for stearyl-CoA desaturase from rat consists of a 1.1 kb coding sequence followed by a 3.5 kb untranslated sequence (2). We have previously reported the use of this coding sequence in a plant expression plasmid to transform tobacco (10). Although the most common substrate for rat desaturase is stearyl-CoA (18:0 CoA), palmityl-CoA (16:0 CoA) is also an effective substrate (12). Consequently, it is not unexpected that 16:0 is altered, as well as 18:0, in tobacco transformed with the gene encoding rat desaturase (Figs. 1 and 2). In several cases, the reduction of 16:0 is more pronounced than the reduction of 18:0. This may be due to the presence of a larger available pool of 16:0 for a desaturase substrate.

Inheritance of kanamycin resistance is presented in Table 1. Resistance to kanamycin in plant T is consistent with two possible explanations. If this is an example of cytoplasmic inheritance (100% kanamycin-resistant seedlings), foreign DNA is expected to be present in the chloroplast or mitochondrial genomes. Since PCR amplification of desaturase-encoding DNA from these fractions did not show a significantly stronger signal than PCR amplification of DNA from the nu-
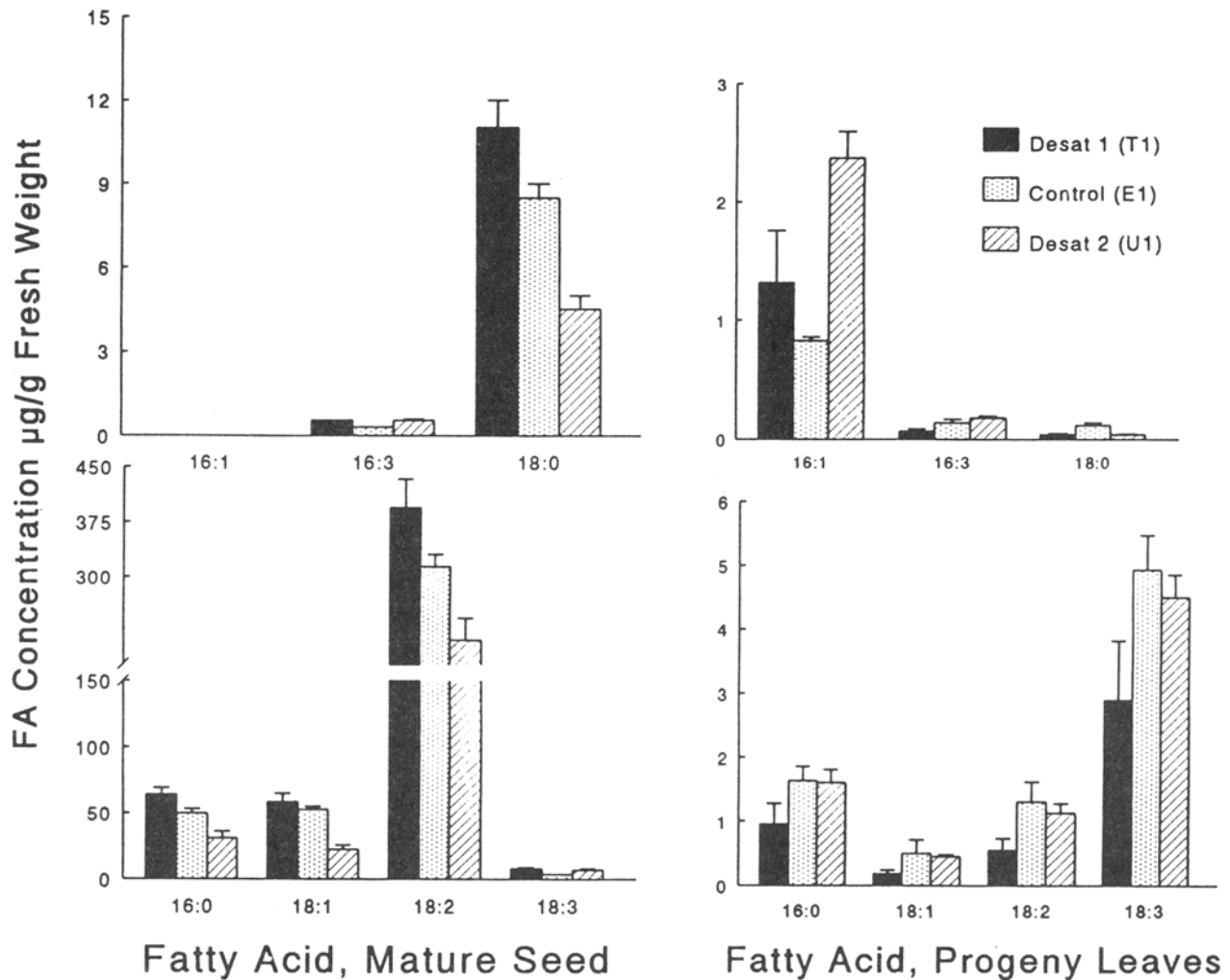
FIG. 2. Concentrations of abundant fatty acids (FA) in mature seeds and progeny leaves. Seeds from primary transformants were pooled. Leaves were from individual progeny plants. Seed data represent the means of two replications. Leaf data represent the means of three replications. Desat, desaturase.
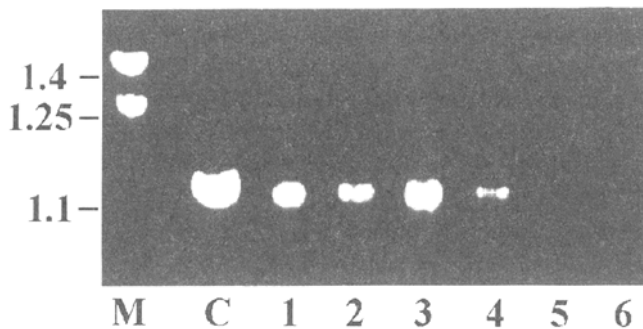

FIG. 3. Scanned ethidium bromide-stained agarose gel of polymerase chain reaction amplifications. DNA from cell fractions enriched in nuclei was amplified with primers homologous to the gene encoding the rat liver Δ9 desaturase. Data are presented for different kanamycin-resistant progeny of plants transformed with p71²RDS (lanes 1–4) or the control plasmid pKYLX71:35S² (lanes 5 and 6) (Ref. 10). A control amplification (C) used 10 ng of pRDSBS DNA digested with Hind III as substrate. This plasmid contains the rat stearoyl desaturase gene. Size markers are indicated in kb.

clear-enriched fraction (data not shown), this explanation appears unlikely. The preferred explanation of uniform (or near-uniform) inheritance of kanamycin resistance in progeny of plant T is that foreign DNA was integrated at two or more independently segregating loci.

The hypothesis of two independent integrations of foreign DNA is also retained for a single capsule from plant U (capsule H). The same interpretation can be applied to pooled seed from plant W. In the remaining cases, kanamycin resistance segregates as a single Mendelian trait.

An earlier study of inheritance in transgenic tobacco indicated that the majority of transformed plants transmitted kanamycin resistance as a single Mendelian factor (13). The observation of higher frequencies of kanamycin-resistant progeny in Table 1 may be correlated with increased activity of the introduced desaturase. Those primary transformants that were originally selected for increased desaturase activity may have had more than one copy of the stearyl-CoA desaturase gene, and consequently more than one copy of the (linked) gene conferring kanamycin resistance.

— BSA

— BSA–D9P

— TB1

— TB1 Desat

— Contr LF

— Desat LF

— Desat LF
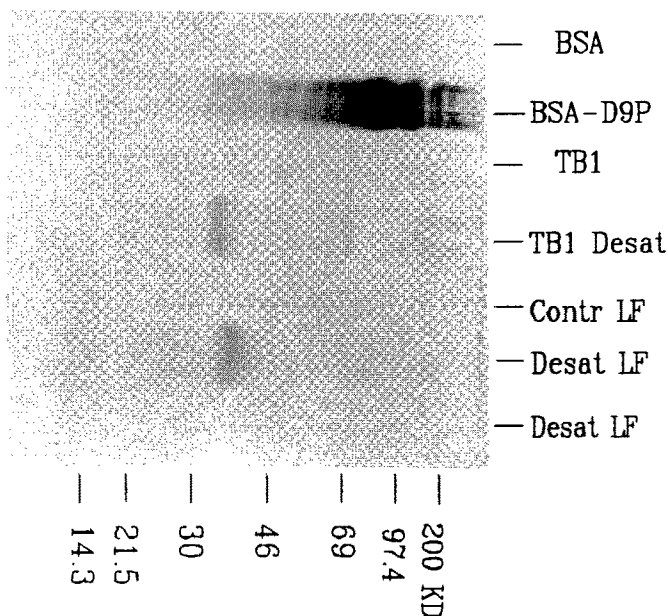
14.3 21.5 30 46 69 97.4 200 KD

FIG. 4. Western immunoblot of an 8.3% acrylamide sodium dodecyl sulfate gel reacted with a polyclonal antibody for rat stearyl CoA desaturase. The first lane from the left contains bovine serum albumin (BSA); the second BSA linked to a peptide of the desaturase; the third an extract of *Escherichia coli* TB1; the fourth *E. coli* TB1 containing the Δ9) desaturase (Desat) construct; the fifth a control (contr) progeny leaf (LF) extract from plant E; lane 6 a desaturase transgenic progeny leaf extract from plant U6; lane 7 an extract of a desaturase transgenic plant not expressing the introduced desaturase gene.

The magnitude of reduction for 16:0 differed in roots and leaves, depending on the individual plant (Fig. 1). For example, in plant T5, 16:0 is reduced more in roots than in leaves. In plant T6, a reduction in 16:0 is seen in leaves but not in roots. These differences may result from nonuniform gene expression in different tissues.

Several lines of evidence indicate that a stearyl-CoA desaturase that was functional in primary tobacco transformants is also functional in progeny. Altered fatty acid profiles (Figs. 1 and 2) are expected if the introduced desaturase is active. Inheritance of kanamycin resistance (Table 1) suggests that

the (linked) desaturase gene is also present in these plants. Direct evidence for presence of the desaturase gene is indicated by PCR products of the expected size (Fig. 3). Finally, a Western immunoblot (Fig. 4) demonstrates the presence of novel desaturase protein in transgenic tobacco.

ACKNOWLEDGMENTS

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