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The Impact of Alteration of Polyunsaturated Fatty Acid Levels on C_6-Aldehyde Formation of *Arabidopsis thaliana* Leaves[1]

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C_6-aldehydes are synthesized via lipoxygenase/hydroperoxide lyase action on polyunsaturated fatty acid (PUFA) substrates in plant leaves. The source pools and subcellular location of the processes are unknown. A close relationship is found between the composition of PUFA and the composition of C_6-aldehydes. In the current study, this relationship was tested using the Arabidopsis PUFA mutant lines *act*1, *fad*2, *fad*3 *fad*5, *fad*6, and *fad*7. The results indicate that C_6-aldehyde formation is influenced by the alteration of C_{18} PUFA levels. Mutants *act*1 and *fad*5, which are deficient in C_{16} unsaturated fatty acids, had wild-type levels of C_6-aldehyde production. Mutants deficient in the chloroplast hexadecenoic acid/oleic acid desaturase (*fad*6) or hexadecadienoic acid/linoleic acid desaturase (*fad*7) had altered C_6-aldehyde formation in a pattern similar to the changes in the PUFA. Mutations that impair phosphatidylcholine desaturase activity, such as *fad*2 and *fad*3, however, resulted in increased *E*-2-hexenal formation. The enzymes involved in C_6-aldehyde production were partially characterized, including measurement of pH optima. The differences in C_6-aldehyde formation among the fatty acid mutants of Arabidopsis appeared not to result from alteration of lipoxygenase/hydroperoxide lyase pathway enzymes. Investigation of the fatty acid composition in leaf phospholipids, glycolipids, and neutral lipids and analysis of the fatty acid composition of chloroplast and extrachloroplast lipids indicate that chloroplasts and glycolipids of chloroplasts may be the source or major source of C_6-aldehyde formation in Arabidopsis leaves.

The C_6-aldehydes, hexanal and hexenals, along with the corresponding alcohols are the major volatile compounds responsible for the "green odor" of leaves (Hatanaka et al., 1987; Gardner, 1989). Because of their low-odor threshold, C_6-aldehydes may directly influence food quality. For example, *Z*-3-hexenal contributes to the fresh flavor of certain fruits and vegetables (Forss et al., 1962; Drawert et al., 1966; Kazeniac and Hall, 1970). Hexanal may contribute an undesirable aroma, which has limited the widespread use of soybean products (Wolf, 1975). Several investigations have shown that hexanal and hexenals inhibit growth of microorganisms in vitro, particularly fungal species, and might be involved in plant defense (Major et al., 1960; Nandi and Fries, 1976; Croft et al., 1993). Others have shown that the C_6-aldehydes inhibited the germination of several seed species (Nandi and Fries, 1976; Bradow and Connick, 1990; Gardner et al., 1990), and pollen germination (Hamilton-Kemp et al., 1991) with the α,β-unsaturated C_6-aldehyde, *E*-2-hexenal, showing considerably more inhibitory action than the saturated aldehyde hexanal. In addition, these compounds are found in some insect excretions as functioning attractants and repellents (Schauenstein et al., 1977).

C_6-aldehydes are synthesized by the enzymatic peroxidation of PUFAs in plant leaves (Hatanaka et al., 1987; Hatanaka, 1993). When plant leaves are damaged by wounding or freezing, the PUFAs can be released by LAH from glycerides and then dioxygenated by LOX into ω6-hydroperoxy fatty acids. These hydroperoxides can be cleaved by HPL to form C_6-aldehydes. ω6-PUFAs are utilized to form hexanal (Zhuang et al., 1991). ω3-PUFAs are used to form Z-3-hexenal. Z-3-Hexenal is usually either enzymatically or spontaneously isomerized rapidly to *E*-2-hexenal.

A close relationship has been reported between the composition of PUFAs and the composition of C_6-aldehydes in plant tissues. In green plant leaves, 18:3 composes more than 40% of TFAs, but 18:2 is less than 20%. Plant leaves also generally produce much higher levels of hexenals than hexanal (Hatanaka et al., 1983; Zhuang et al., 1992). In normal mature soybean seeds, more than 50% of the TFAs is 18:2 and the major C_6-aldehyde formed is hexanal (Hildebrand et al., 1990). The ratio of 18:2 to 18:3 in the black tea stem is much higher than that of the leaves or buds, and the stem has a higher potential for production of hexanal (Selvendran et al., 1978). In the summer tea leaves, the ratio of 18:3 to 18:2 is about 3, and the ratio increased to about 5 in the winter leaves. An increase in ratio of hexenals to hexanal is also found following the season change (Sekiya et al., 1984).

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Abbreviations: *act*1, a mutant of Arabidopsis deficient in chloroplast glycerol-3-phosphate acyltransferase activity; *fad*5, a mutant of Arabidopsis deficient in desaturation of palmitic acid in chloroplasts (Browse and Somerville, 1991); *fad*6, a mutant of Arabidopsis deficient in the chloroplast 16:1/18:1 desaturase; *fad*7, a mutant of Arabidopsis deficient in the chloroplast 16:2/18:2 desaturase; *fad*2, a mutant of Arabidopsis deficient in the PC 18:1 desaturase; *fad*3, a mutant of Arabidopsis deficient in the PC 18:2 desaturase; GL, glycolipid; HPL, hydroperoxide lyase; LAH, lipolytic acyl hydrolase; LOX, lipoxygenase; NL, neutral lipid; PC, phosphatidylcholine; PL, phospholipid; PUFA, polyunsaturated fatty acid; TFA, total fatty acid; X:Y, a fatty acyl group containing X carbon atoms and Y *cis* double bonds; WT, wild type.

Browse and Somerville (1991) recently isolated a group of PUFA mutant lines of *Arabidopsis thaliana* (L.). These include *fad*B (Kunst et al., 1989) and *act*1 (Kunst et al., 1988), with a much lower relative amount of 16:3 than WT; *fad*C, with reduced relative levels of both 18-carbon and 16-carbon PUFAs and increased levels of 18:1 and 16:1 (Browse et al., 1989); *fad*D, containing much higher relative levels of 16:2 and 18:2 and much lower levels of 16:3 and 18:3, especially in chloroplasts (Browse et al., 1986); *fad*2, with a much lower relative amount of 18:2 (Lemieux et al., 1990; Miquel and Browse, 1992); and *fad*3, with a small decrease in 18:3 levels in leaves (Lemieux et al., 1990). The mutations that define the *fad*B, *fad*C, and *fad*D loci have been renamed *fad*5, *fad*6, and *fad*7, respectively (Browse et al., 1995). In the present investigation, these mutants were used to study the impact of alteration of PUFA synthesis on the formation of C_6-aldehydes.

MATERIALS AND METHODS

Plant

Arabidopsis thaliana mutant lines were grown in a growth chamber with 16 h light/d at 22°C for 24 d and then at 28°C for 1 week before the leaves were collected for the experiments.

C_6-Aldehyde Analysis

Whole mature Arabidopsis leaves from different mutants were collected and put into screwcap vials (1.8 mL). The tightly sealed vials were then frozen at −80°C for at least 1 d. Before measurement of C_6-compounds the vials were placed in a water bath at 30°C for 20 min and then at 80°C for 5 min. Gases from the head space of the vials were withdrawn with a gas-tight syringe and injected directly into a Varian (Harbor City, CA) 3700 GC with a 30- × 0.53-m DB-Wax (PEG) fused silica column. The hexanal and hexenals were identified by MS and comparison of the GC retention times of the plant compounds with those of authentic compounds (Zhuang et al., 1992).

Enzyme Assay

LOX was extracted with nanopure water (1:1, w/v). HPL and LAH were extracted with Hepes buffer (pH 7.5, 50 mM) containing 0.5% reduced Triton X-100 on ice. The homogenates were centrifuged at 13,600*g* for 15 min to obtain supernatants. Before enzyme activities were measured, the pH optima were determined. LOX, HPL, and LAH activities were measured using spectrophotometric assays at their pH optima (Zhuang et al., 1992).

Lipid Analysis

It has been demonstrated that environmental factors or growth conditions can impact plant lipid composition and contents. Growth conditions influenced fatty acid composition in Arabidopsis leaves (J. Browse, personal communication). In the previous studies, Arabidopsis mutants were, in general, grown at only 23°C for lipid composition analyses. However, in our investigation, plants were transferred from 22 to 28°C for 1 week before analyses of C_6-aldehydes and lipids. Since the fatty acid compositions of these mutants have been reported by other groups, we focused on other aspects of lipid status in Arabidopsis leaves of the mutants. For the TFAs, we measured fatty acid contents as well as compositions. Little such information is available for those mutants, and also quantitative levels of fatty acids could influence production of C_6-aldehydes in plants. For lipid fractions, we were more interested in three major lipid fractions, GLs, PLs, and NLs, as well as in chloroplast lipid fractions and extrachloroplast lipid fractions. In the previous studies, most reports focused on fatty acid composition of individual lipid fractions, such as monogalactosyldiacylglycerol, digalatosyldiacylglycerol, PC, PG, and similar compounds. To our knowledge, only one paper about mutant line *fad*6 reported the fatty acid composition of both chloroplasts and extrachloroplasts in Arabidopsis leaves (Browse et al., 1989).

In the present investigation, Arabidopsis leaf lipids were extracted by following the procedure of Browse et al. (1986). Before extraction, 19:0 and 17:0-PC were added directly on the leaf surface to serve as internal standards. Since we were more interested in major lipid fractions instead of individual lipid fractions, the isolation of different lipid fractions was accomplished using silica gel Sep-Pak cartridges from Waters for GL and PL following the procedure of Parkin and Kuo (1989). NLs were purified by a one-dimensional TLC method on silica plates (Merck, Darmstadt, Germany) with a solvent system of hexane: ether:acetic acid (50:50:1, v/v/v). The identity of each fraction from a Sep-Pak cartridge was determined by analysis of the presence of 16:3, 17:0, and 19:0 in different fractions

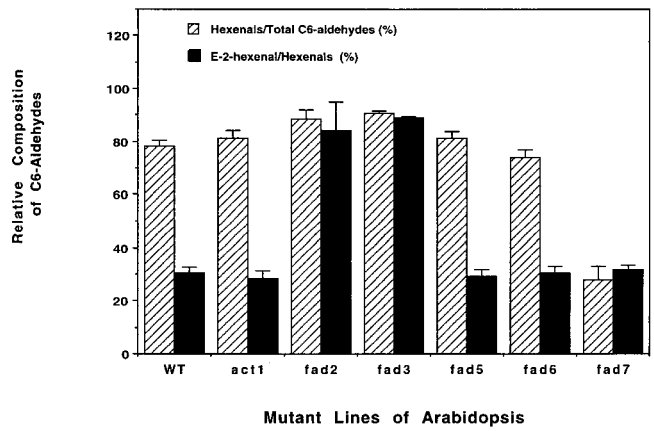

Figure 1. The relative composition of C_6-aldehydes produced by Arabidopsis fatty acid mutant lines. Arabidopsis plants were grown in a growth chamber at 22°C for 24 d and transferred to 28°C for 1 week. Whole, mature leaves were collected and placed into tightly sealed vials. The samples were frozen at −80°C for at least 1 d and incubated in a water bath at 30°C for 20 min and then at 80°C for 5 min before the head space samples were analyzed by GC. Hexanal and hexenals were identified by comparing GC-MS and GC data of plant components with those authentic chemicals.

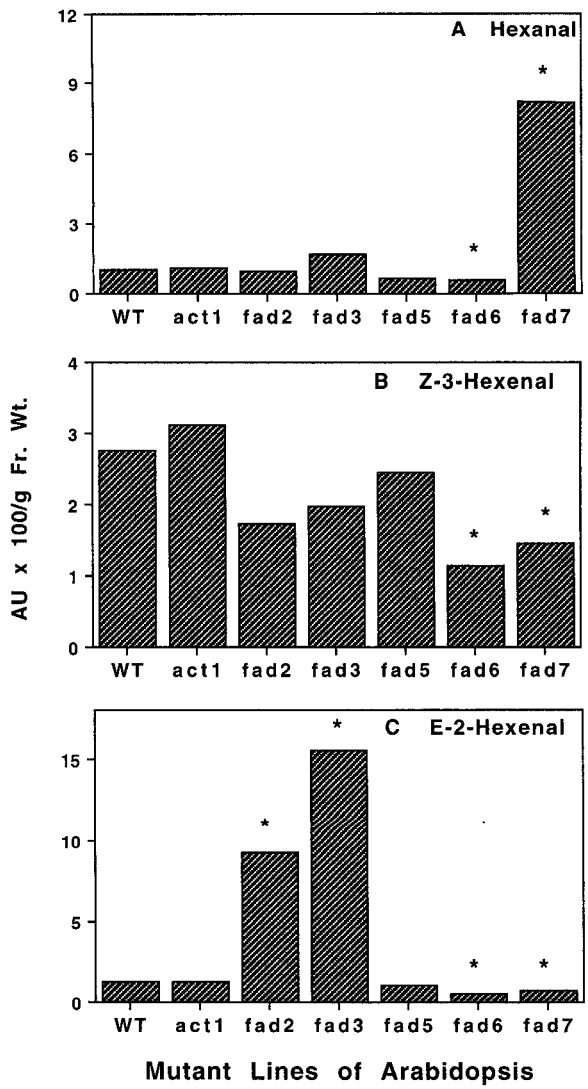

Figure 2. The formation of C_6-aldehydes by Arabidopsis fatty acid mutant lines (Browse et al., 1995). A, Hexanal; B, *Z*-3-hexenal; C, *E*-2-hexenal. The experimental procedure was the same as described in the legend to Figure 1. The hexanal yield from WT leaves was about 0.77 ng/g fresh weight (Fr. Wt.). The total hexenal yield from WT leaves was about 2.44 ng/g fresh weight. AU, Area units. Asterisks (*) indicate that the means were significantly different from the WT ($P < 0.05$). Statistical analysis was made by the SAS program (SAS Institute, Cary, NC).

or using TLC with specific staining. Chloroplast fractions and extrachloroplast fractions were prepared as reported by Palmer (1986). Fatty acid composition was measured by GC, after direct transmethylation, on an FFAP column (Hewlett-Packard) at 180 to 220°C at 4°C min^{-1} (Dahmer et al., 1989). The quantitative fatty acid levels were calculated based on internal standards. The contamination of extrachloroplast lipid by chloroplast fatty acids was corrected by 16:3 levels detected in extrachloroplast fatty acids (Browse et al., 1989).

RESULTS

C_6-Aldehyde Formation

Measurement of C_6-aldehydes showed that hexenals were the main C_6-aldehydes produced by mature Arabidopsis leaves (Fig. 1). *fad6* and *fad7* produced about one-half the levels of hexenals, but *fad2* and *fad3* synthesized more than 4-fold higher levels of hexenals than WT plants. *fad5* and *act1* formed as much hexenals as the WT (Fig. 2, B and C). The main component of hexenals produced by *fad2* and *fad3* was *E*-2-hexenal (Fig. 1), which was about 10 times more than that produced by WT plant leaves (Fig. 2C). For hexanal formation, *fad7* produced much higher (about 8 times) and *fad6* produced less than one-half of the hexanal compared to the WT. No significant difference was observed among the other mutants (Fig. 2A). Much more total C_6-aldehydes was formed by *fad7*, *fad2*, and *fad3* than those by the WT, whereas *fad6*, in contrast, produced much less total C_6-aldehydes, (Fig. 2). More than 75% of the C_6-aldehydes formed by Arabidopsis leaves were unsaturated except for *fad7* leaves, in which hexenals compose only 25% of the total C_6-aldehydes (Fig. 1). In hexenals, *E*-2-hexenal was about 30% except for *fad2* and *fad3*, in which *E*-2-hexenal contents were more than 80% of total hexenals (Fig. 1).

Activities of Enzymes Involved in C_6-Aldehyde Formation

Because there are no reports concerning activities of LOX, HPL, and LAH in Arabidopsis, the pH optima of those three enzymes were investigated. The data showed that Arabidopsis leaf LOX had two pH optima. One was at pH 4.5 and the other was at pH 6.0. This may be due to the presence of two LOXs in Arabidopsis leaves (Bell and Mullet, 1993; Melan et al., 1993). This observation is also consistent with the finding of LOX activity at different pHs in tea leaves by Hatanaka (1993). LOX in tea leaves has the

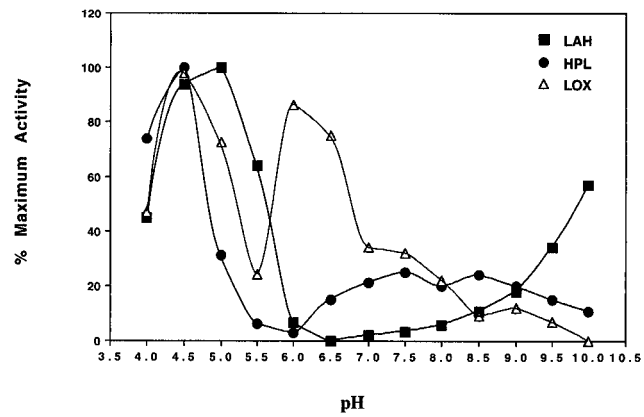

Figure 3. The pH optima of LAH, LOX, and HPL of Arabidopsis leaves. The pH buffers were as follows: sodium acetate was used for pH 4.0 to 5.5, sodium citrate was used for pH 5.0 to 6.5, phosphate was used for pH 6.0 to 8.0, and borate was used for pH 8.5 to 10. Enzyme activities were determined spectrophotometically. The values expressed are the averages of at least two replicates.

Table I. *The activities of LAH, LOX, and HPL in leaves of Arabidopsis fatty acid mutant lines*

Mature Arabidopsis leaves were collected and ground with water for LOX and Hepes buffer for HPL and LAH. LOX, HPL, and LAH activities were determined by measuring spectrophotometric changes in A_{234}, A_{340}, and A_{400}, respectively (Zhuang et al., 1992). Results are means ± SE; $n = 6$.

Mutant Line	Enzyme		
	LAH	LOX	HPL
	relative % activities of enzymes		
WT	100	100	100
act1	95 ± 10	96 ± 21	95 ± 4
fad2	134 ± 26	78 ± 12	107 ± 19
fad3	91 ± 5	83 ± 17	44 ± 13
fad5	116 ± 12	90 ± 6	106 ± 17
fad6	123 ± 21	95 ± 8	95 ± 21
fad7	95 ± 8	88 ± 17	77 ± 10

highest activity at pH 6.3 and 4.5 with 18:2. pH optima of HPL and LAH were approximately 4.5 to 5.0 (Fig. 3).

The relative activities of LOX, HPL, and LAH in Arabidopsis leaves are shown in Table I. No significant difference was found in LAH, LOX, and HPL among the mutants except for HPL activity in *fad3* leaves. The HPL activity in the *fad3* leaf extract was about 50% of HPL in WT leaf extracts.

TFA Composition and Contents

The relative TFA composition of Arabidopsis leaves under our growth conditions showed the same pattern reported by Browse and Somerville (1991). For example, *act1*, *fad5*, and *fad6* contained very low levels (undetectable in our measurement) of both 16:3 and 16:2 compared to WT (Kunst et al., 1988, 1989; Browse et al., 1989). 16:3 in *fad7* leaves was one-tenth of that in WT leaves (Browse et al., 1986). *fad6* and *fad2* contained relatively low 18:2 and high 18:1 (Browse et al., 1989; Lemieux et al., 1990; Miquel and Browse, 1992), and *fad6* and *fad7* had a low content of 18:3 compared to those of WT (Browse et al., 1986, 1989). Contents of TFAs in Arabidopsis leaves were about 0.2% of leaf fresh weight (Table II). In the TFAs, 18:3 was the major component, about 1 mg/g fresh leaves of Arabidopsis. In *fad6* leaves, 18:3 content was 0.60 mg/g fresh leaves. 18:3 of *fad7* leaves was 0.27 mg/g fresh leaves, which was about one-fourth of WT 18:3. The 18:3 contents in *fad5*, *fad2*, and *fad3* were about 0.9 mg/g fresh leaves, which was slightly lower than the control (WT Arabidopsis). The level of 18:2 in *fad7* was about 2-fold that in WT. *fad2*, *fad5*, and *fad6* had lower contents of 18:2 than the WT, especially *fad2*. It contained only one-third of 18:2 compared with that of the WT. *act1*, *fad5*, *fad6*, and *fad7* leaves all contained much lower contents of 16:3 (less than one-tenth of control) and 16:2. In *fad7*, however, the 16:2 concentration in leaves was 10 times higher than that in WT type.

The Relative Level of PUFAs in Different Lipid Fractions

Analysis of the relative fatty acid composition of different lipid fractions, GL, PL, and NL, showed that in the GL fraction *act1* contained the highest relative 18:3 levels (70%) (Fig. 4A). However, the relative 18:3 levels of GLs in *fad6* and especially in *fad7* were much lower than that in the WT (about 30 and 15%, respectively). *fad2* and *fad3* had a level of 18:3 in GLs similar to the WT. In the PL fraction (Fig. 4B), relatively lower levels of 18:3 were found in *act1*, *fad6*, *fad7*, *fad2*, and *fad3* leaves compared to that in WT leaves. The *fad7* line contained only about one-third the level of 18:3 of WT. The 18:3 level in the *fad3* PL fraction was about 60% of 18:3 in the WT PL fraction. In NLs (Fig. 4C), the relative levels of 18:3 in mutant leaves were lower than those of GL fractions but higher compared with those of PL fractions. The distribution patterns among mutants were similar to those in PL fractions. Figure 5 shows the relative levels of 18:2 in the different lipid fractions. In GLs (Fig. 5A), an evidently higher (about 5 times) 18:2 level was observed only in the *fad7* mutant line. In PL (Fig. 5B), only the *fad2* mutant lines showed an apparent reduction in 18:2. *fad7* and *fad3* contained a somewhat higher 18:2 level. Relative 18:2 levels in NL fractions showed the patterns among mutants to be similar to those of GL fractions, with relative higher 18:2 in *fad7* leaves (Fig. 5C).

Table II. *The fatty acid levels of different PUFA mutant lines of A. thaliana (L.)*

The TFAs were measured by a direct transmethylation method (Dahmer et al., 1989) after the lipids were extracted following the procedure of Browse et al. (1986). Results are means ± SE; $n = 4$.

Fatty Acid	Arabidopsis Mutant						
	WT	*act1*	*fad2*	*fad3*	*fad5*	*fad6*	*fad7*
				mg/g fresh wt			
16:0	0.47 ± 0.01	0.32 ± 0.02	0.47 ± 0.02	0.37 ± 0.01	0.52 ± 0.010	0.28 ± 0.03	0.32 ± 0.01
16:1	0.08 ± 0.02	0.06 ± 0.00	0.07 ± 0.00	0.06 ± 0.00	0.07 ± 0.00	0.23 ± 0.04	0.05 ± 0.00
16:2	0.02 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.18 ± 0.00
16:3	0.27 ± 0.01	0.00 ± 0.00	0.34 ± 0.01	0.27 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.02
18:0	0.05 ± 0.00	0.04 ± 0.00	0.02 ± 0.00	0.04 ± 0.00	0.05 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
18:1	0.10 ± 0.01	0.22 ± 0.01	0.61 ± 0.01	0.10 ± 0.00	0.07 ± 0.00	0.44 ± 0.01	0.12 ± 0.01
18:2	0.52 ± 0.03	0.55 ± 0.02	0.16 ± 0.00	0.48 ± 0.00	0.40 ± 0.01	0.37 ± 0.00	1.00 ± 0.01
18:3	1.11 ± 0.01	1.13 ± 0.06	0.94 ± 0.03	0.91 ± 0.01	0.99 ± 0.07	0.61 ± 0.01	0.27 ± 0.03
Total	2.61 ± 0.06	2.31 ± 0.05	2.60 ± 0.04	2.23 ± 0.03	2.10 ± 0.09	1.96 ± 0.01	1.98 ± 0.51

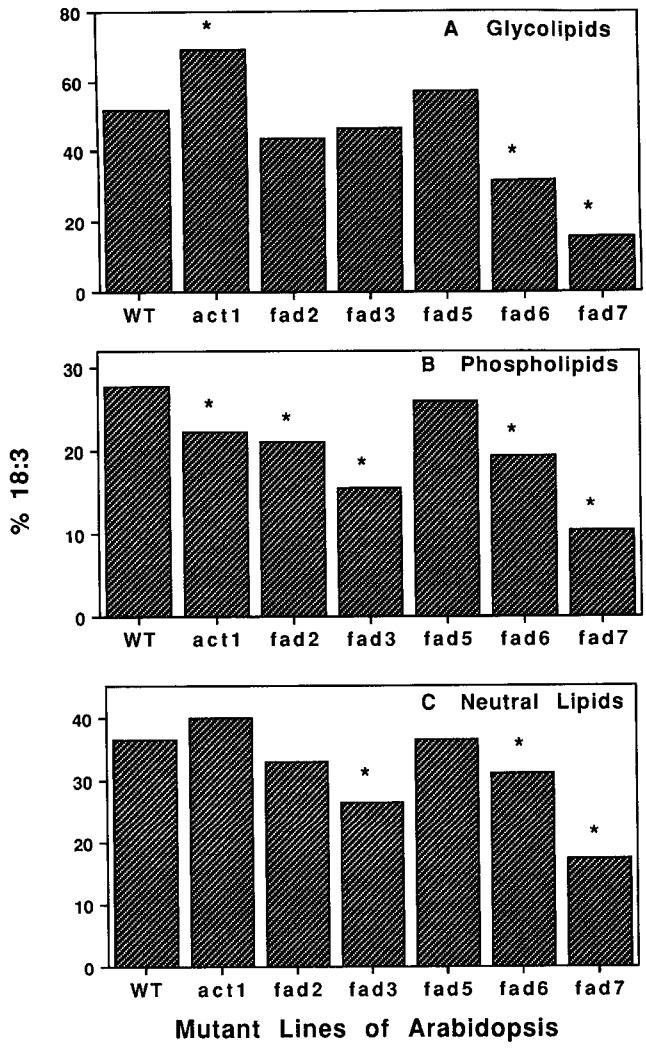

Figure 4. The relative levels of 18:3 of GLs (A), PLs (B), and NLs (C) in Arabidopsis fatty acid mutant lines. The total lipids were extracted following the procedure of Browse et al. (1986). The isolation of different lipid fractions was accomplished using a silica Sep-Pak cartridge (Parkin and Kuo, 1989) or silica TLC plate with hexane: ethyl ether:acetic acid (50:50:2, v/v/v). The identity of each fraction was estimated by adding standard free fatty acids 19:0 and PC-17:0 and TLC analysis with specific stains. Asterisks (*) indicate that the means were significantly different from the WT ($P < 0.05$). Statistical analysis was made by the SAS program.

Relative Level of PUFAs in Chloroplast and Extrachloroplast Cell Fractions

Patterns of relative levels of 18:2 and 18:3 in chloroplast lipids (Fig. 6) were similar to the fatty acid compositions in GLs (Figs. 4A and 5A). Patterns of relative levels of 18:2 and 18:3 in extrachloroplast lipids (Fig. 7) were close to those of PLs (Figs. 4B and 5B). In the chloroplast lipids, relatively higher levels were noted in *act*1, *fad*2, and *fad*3 leaves but lower in *fad*7 and *fad*6 mutant lines (Fig. 6A). The 18:2 level was low in *fad*6; however, it was very high in *fad*7 (Fig. 6B). *fad*3 and *fad*7 had high levels of 18:2 in the extrachloroplast lipids, but *fad*2 contained about one-half of the 18:2 compared with WT (Fig. 7B). *fad*6, *fad*7, *fad*2, and *fad*3 all had lower levels of 18:3 than WT (Fig. 7A). These results are consistent with the results mentioned in the review by Browse and Somerville (1991).

DISCUSSION

The formation of C_6-aldehydes by plant leaves has been noted for more than 80 years (Curtius and Frunzen, 1912). Although the biochemical pathway for C_6-aldehyde production has been elucidated, the mechanism for regulation and control of C_6-aldehyde formation by leaves are still unknown. Hatanaka et al. (1987) reported that C_6-aldehyde formation by tea leaves changed as seasons changed. Zhuang et al. (1992) found that C_6-aldehyde formation showed altered composition as well as quantity during soybean leaf development. The changes in C_6-aldehyde composition have been thought to result from the difference in C_{18} PUFAs present in plant tissues (Selvendran et

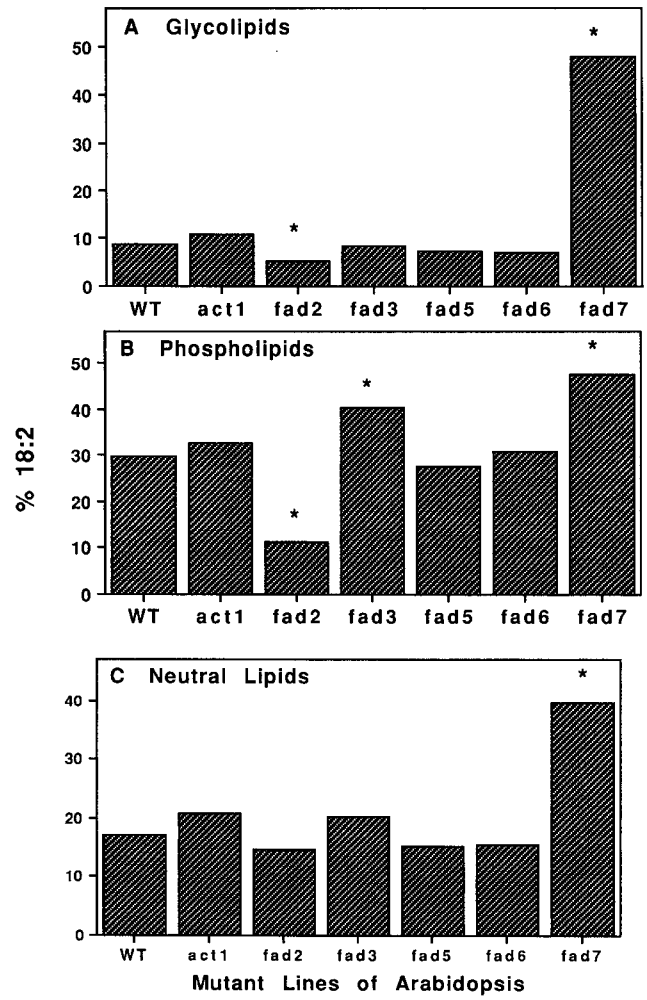

Figure 5. The relative levels of 18:2 of GLs (A), PLs (B), and NLs (C) in Arabidopsis fatty acid mutant lines. The experimental procedure is the same as described in the legend to Figure 4. Asterisks (*) indicate that the means were significantly different from the WT ($P < 0.05$). Statistical analysis was made by the SAS program.

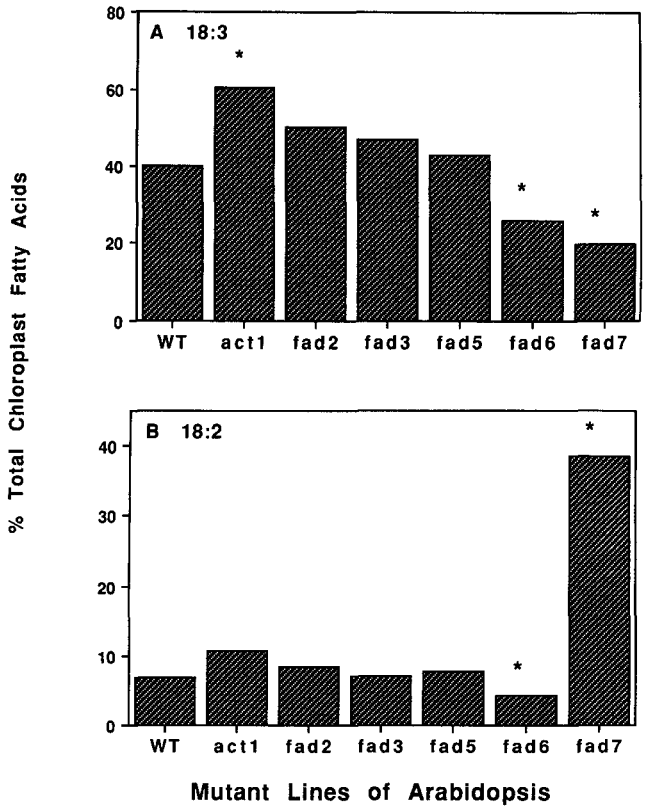

Figure 6. The relative levels of C_{18} PUFAs in chloroplast lipids of leaves of Arabidopsis fatty acid mutant lines. A, Relative percentages of 18:3; B, relative percentages of 18:2. The chloroplast and extrachloroplast fractions of Arabidopsis leaves were prepared as reported by Palmer (1986). The contamination of extrachloroplast lipid fractions by chloroplast lipid was subtracted by using 16:3, 16:2, or 16:1 found in the extrachloroplast lipid fraction as a reference. Asterisks (*) indicate that the means were significantly different from the WT ($P < 0.05$). Statistical analysis was made by the SAS program.

al., 1978; Sekiya et al., 1984; Zhuang et al., 1992). In the current study, this hypothesis was tested using Arabidopsis PUFA mutant lines.

Our investigation showed that alteration of PUFA substrate formation does not always impact both the C_6-aldehyde production and C_6-aldehyde composition. The reduction in only C_{16} PUFAs did not affect C_6-aldehyde formation. However, alteration of C_{18} PUFAs resulted in changes in C_6-aldehyde generation and composition. Investigation of LOX/HPL pathway enzymes indicated that the differences observed in C_6-aldehyde formation by Arabidopsis fatty acid mutants did not likely result from the alteration in LOX/HPL activities (Table I). C_{18} PUFA substrates apparently influence specific product formation. In soybean seed extracts, addition of 18:2 or certain 18:2 ester derivatives resulted in increased hexanal synthesis, and addition of 18:3 resulted in enhanced hexenal formation (Zhuang et al., 1991). During tea fermentation, the addition of free 18:2 or 18:3 to leaf macerates caused an increase in hexanal or hexenals in the volatile fraction (Saijyo and Takeo, 1972). Hatanaka et al. (1990) found that the catalytic activity of soybean seed LOX-1 for C_{16} PUFAs was less than 20% of that for C_{18} PUFA. The catalytic activity of tea leaf HPL for C_{16} PUFAs was also about 10 to 20% of that for C_{18} PUFAs (Hatanaka et al., 1992). In plant leaves, the C_{16} PUFA content is much lower than C_{18} PUFA content (one-fifth in Arabidopsis leaves). In this study, the enhanced formation of hexanal by *fad7* and decreased C_6-aldehyde production in *fad6* leaves apparently resulted from the changes in the C_{18} substrate pool of those mutants. There was no evident effect on C_6-aldehyde formation in the C_{16} PUFA mutant lines *act1* and *fad6*. This could be due to both the low C_{16} PUFA content in Arabidopsis leaves and the poor utilization of C_{16} PUFAs by LOX and/or HPL.

The C_6-aldehyde composition appeared dependent on C_{18} PUFA composition. In most genetic lines used in this research, 18:3 was more than 65% of total C_{18} PUFAs except for *fad7* (Table II). Hexenals were more than 70% of total C_6-aldehydes produced by the lines WT, *act1*, *fad2*, *fad3*, *fad5*, and *fad6* (Fig. 1). The ratio of 18:3 to total C_{18} PUFAs was 0.21 in *fad7* leaves, and the ratio of hexenals to total C_6-aldehydes also was less than 30%. There was, however, no direct relationship between C_6-aldehyde levels and composition and total C_{18} PUFA levels and composition.

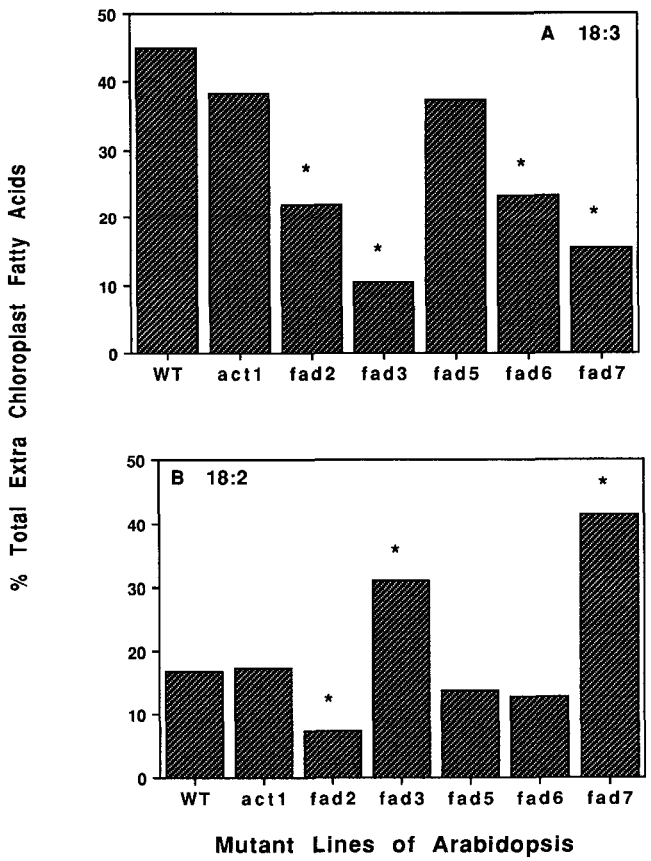

Figure 7. The relative levels of C_{18} PUFAs in extrachloroplast lipids of leaves of Arabidopsis fatty acid mutant lines. A, Relative percentages of 18:3; B, relative percentages of 18:2. The experimental procedure is the same as described in the legend to Figure 6. Asterisks (*) indicate that the means were significantly different from the WT ($P < 0.05$). Statistical analysis was made by the SAS program.

Quantitative and relative levels of 18:3 in *fad*2 and *fad*3 leaves were similar to those in WT leaves; however, *fad*2 and *fad*3 leaves generated much higher levels of C_6-aldehydes compared with WT leaves. In *fad*2 leaves, the ratio of 18:3 to total C_{18} PUFAs was 0.86. The ratio of 18:3 to total C_{18} PUFA in *fad*3 leaves was 0.67 (Table II). However, the values (0.85) of hexenals/total C_6-aldehydes (Fig. 1) were similar in *fad*2 and *fad*3 leaves. These results were consistent with the results for C_6-aldehyde formation by soybean leaves (Zhuang et al., 1992). During soybean leaf aging, the increases in the ratio of 18:3 to 18:2 were accompanied by increased ratios of hexenals to hexanal. However, young soybean leaves produced much more hexanal than hexenals, even though 18:3 levels were much higher than 18:2 levels in the young leaves.

Our results indicate that chloroplast lipids or GLs of chloroplast may be the source or major source of C_6-aldehyde formation in plant leaves. This conclusion can be derived from the following three observations. The mutation that alters 18:2 and 18:3 syntheses in chloroplast, such as *fad*6 and *fad*7, also had an impact on C_6-aldehyde formation in the same patterns. However, the mutation that reduced 18:2 or 18:3 formation in the extrachloroplast fraction, such as *fad*2 or *fad*3, resulted in increased C_6-compound generation (Figs. 1 and 2). Lipid composition in chloroplast is closely associated with C_6-aldehyde composition, but in extrachloroplast lipids, no such relationship was found. For example, *fad*7 that produced a much higher level of hexanal contained a much elevated content of 18:2 in chloroplast lipids compared to that from extrachloroplast lipids. In the extrachloroplast lipid fraction, *fad*7 and *fad*3 mutant leaves showed similar high levels of 18:2 compared with WT leaves (Fig. 7B). However, *fad*7 produced 4-fold more hexanal than *fad*3 (Fig. 2A). The correlation coefficient between hexenals/total C_6-aldehydes and 18:3/C_{18} PUFAs in chloroplast lipid fractions was 0.97, and the coefficient was 0.48 in extrachloroplast lipid fractions (data not presented). Comparison of C_{18} PUFA composition in different lipid fractions to C_6-aldehyde formation showed that the best correlation was found with the GL fraction. The correlation coefficient between hexenals/total C_6-aldehydes and 18:3/C_{18} PUFAs in GL fractions was 0.99; however, the coefficient was 0.67 in PL fractions and 0.87 in NL fractions (data not presented). Previous studies indicated that LAH, LOX, and HPL, needed for C_6-aldehyde production in vivo, are localized in the chloroplasts of plant leaves. LAH was found in leaf chloroplasts of runner bean (Sastry and Kates, 1964), spinach (Anderson et al., 1974), and potato (Matsuda and Hirayama, 1979). LOX appeared to be bound to chloroplastic lamella or to be soluble in the chloroplast stroma in wheat shoots (Douillard and Bergeron, 1978) and pea shoots (Douillard and Bergeron, 1981; Douillard et al., 1982). In tea leaves (Hatanaka et al., 1982), most LOX and HPL activities were found in chloroplast fragments (lamella-rich fraction). In soybean leaves, HPL activity appears to be predominantly in chloroplasts (Gardner et al., 1991). In the current study, we further demonstrate that C_6-aldehydes are generated primarily from chloroplast lipids.

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