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cis-3-Hexenal production in tobacco is stimulated by 16-carbon monounsaturated fatty acids

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Abstract

Transgenic tobacco plants O9 and T16 expressing the yeast acyl-CoA Δ9 desaturase and an insect acyl-CoA Δ11 desaturase, respectively, displayed altered profiles of fatty acids compared to wild-type tobacco plants and marked increases in *cis*-3-hexenal, a major leaf volatile derived from α-linolenic acid (18:3). As expected, O9 and T16 plants had increased levels of the major unsaturated fatty acid products formed by the transgenic desaturases they expressed, viz., palmitoleic acid ($16{:}1^{\Delta 9}$) and palmitvaccenic acid ($16{:}1^{\Delta 11}$), respectively. In addition, levels of 18:3 lipid declined slightly and the pool of free 18:3, which accounts for about 30% of free fatty acids in wild-type plants, disappeared completely in both transgenics. Both O9 and T16 plants were found to have a two-fold increase in 13-lipoxygenase (13-LOX) activity, which catalyzes the first of two steps leading to hexenal production from 18:3. In O9 and T16 plants, the activity of 9-lipoxygenase and hydroperoxide lyase, the latter catalyzing the formation of *cis*-3-hexenal from α-linolenic acid hydroperoxide, was significantly different from that of the wild-type plants. Although $16{:}1^{\Delta 9}$ and $16{:}1^{\Delta 11}$ had no direct effects on 13-LOX activity in vitro, *cis*-3-hexenal production increased in tobacco leaves treated with these fatty acids, suggesting that they may act in vivo by stimulating 13-LOX gene expression.

Keywords: *Cis*-3-hexenal; Palmitoleic acid; Palmitraccenic acid; Hexanal; Lipoxygenase

1. Introduction

The volatile compound *cis*-3-hexenal, which is found in most plant leaves, is known to possess biological activities involved in disease and insect resistance (Bisignano et al., 2001). This compound, which is also a valuable flavor component of the food industry, is formed in vivo by degradation of the polyunsaturated fatty acid α-linolenic acid (18:3) via the sequential action of 13-lipoxygenase and 13-hydroperoxide lyase (Hatanaka et al., 1977, 1986). Thus, the activities of these two enzymes and the level of 18:3 precursor are predicted to be the proximal determinants of *cis*-3-hexenal levels in plants. Effectors regulating lipoxygenase expression in plants include jasmonic acid (Creelman and Mullet, 1997), abscisic acid (Melan et al., 1993), wounding (Porta et al., 1999), and pathogen attack (Melan et al., 1993).

Transgenic tomato plants (*Lycopersicon esculentum* Mill.) expressing the acyl-CoA Δ9 desaturase gene from the yeast *Saccharomyces cerevisiae* (Stuckey et al., 1989) have several fold more *cis*-3-hexenal than wild-type (Wang et al., 1996, 2001). These transgenic plants have altered fatty acid profiles with sharp increases in $16{:}1^{\Delta 9}$ and $18{:}1^{\Delta 9}$, resulting from the expression of their respective transgenes, and reduced amounts of 18:3. The latter observation suggests that the level of the 18:3 precursor is not the limiting factor for *cis*-3-hexenal production in wild-type plants. These changes are associated with enhanced resistance against powdery mildew caused by *Erysiphe polygon* (Wang et al., 2000). Interestingly, an endogenous plant stearoyl-ACP desaturase of *Arabidopsis thaliana* has been shown to play a critical role in modulating defense signaling pathways (Kachroo et al., 2001).

In light of the evidence suggesting that monounsaturated fatty acids can directly or indirectly

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influence levels of *cis*-3-hexenal and other defense-related compounds in plants, we examined the effects of the expression of two desaturase transgenes, the yeast acyl-CoA Δ9 desaturase (Stuckey et al., 1989) and an acyl-CoA Δ11 desaturase from the cabbage looper moth *Trichoplusia ni* (Knipple et al., 1998), on lipid profiles and levels of enzyme activity involved in the production of *cis*-3-hexenal in tobacco plants. The results reported here show that expression of these transgenes causes significant changes in the profile of fatty acids and their degradation products, including greatly increased levels of *cis*-3-hexenal and enhanced expression of 13-lipoxygenase. These effects are likely attributable to the predominant monounsaturated fatty acid products formed by these transgenic desaturases, $16{:}1^{\Delta 9}$ and $16{:}1^{\Delta 11}$, respectively, as direct application of these compounds to the leaves of wild-type tobacco plants also stimulates *cis*-3-hexenal production.

2. Results

2.1. Changes of fatty acid composition in transgenic plants

Independently regenerated kanamycin-resistant plantlets from transformation experiments were subjected to Southern blot and fatty acid analyses. Significant changes in fatty acids were found in the plants transformed with the yeast Δ9 desaturase gene as well as the plants containing the moth Δ11 desaturase gene; 20 transformants were recovered from transformation with each gene. Changes in 16:1 fatty acids were most dramatic, albeit the degree of changes varied with individual primary transformants (data not shown). Only minute amounts of 16:1 (less than 0.3%) were found in non-transformed, wild-type plants. Transformants expressing the yeast Δ9 desaturase gene showed a 2- to 17-fold increase in $16{:}1^{\Delta 9}$, and the transformants expressing the moth Δ11 desaturase gene produced a novel fatty acid, $16{:}1^{\Delta 11}$. Since $16{:}1^{\Delta 9}$ and $16{:}1^{\Delta 11}$ have similar mass spectra, the double bond position was determined by mass spectral analysis of the dimethyl disulfide (DMDS) adducts as described by Shibahara and coworkers (1986) (Fig. 1). Not all Southern-positive plants displayed changes in fatty acids, but all plants that exhibited altered fatty acid composition were shown to contain the transgene by Southern blot analysis (data not shown). One Δ9-desaturase gene transformant, O9, and one Δ11 desaturase gene transformant, T16, were arbitrarily selected for subsequent investigation.

Fig. 2 shows the profiles of fatty acids in total lipid extracts of the control and the two transgenic plants, O9 and T16. Levels of polyunsaturated fatty acids 18:2 and 18:3 were similar in the transformants and the control tobacco plants, and together comprised over 60% of the total fatty acids. In contrast, the levels of monounsaturated fatty acids were markedly different between the transformants and control tobacco plants. Expression of the yeast Δ9 desaturase transgene resulted in a sharp increase in $16{:}1^{\Delta 9}$ (>17-fold) and expression of the moth Δ11 desaturase transgene led to the appearance of appreciable amount of $16{:}1^{\Delta 11}$, which is absent in the wild-type plants. Another monounsaturated fatty acid, 18:1, which was present in negligible quantities in wild-type tobacco, was present in significant amounts in O9 and T16 transgenic plants.

The profiles of fatty acids in total lipids (Fig. 2) were very different from those of free fatty acids (Fig. 3). Several free monounsaturated fatty acids were increased, apparently influenced by the expression of the Δ9 and Δ11 desaturase genes: viz. $16{:}1^{\Delta 9}$, $18{:}1^{\Delta 9}$, and $18{:}1^{\Delta 11}$ in O9, and $18{:}1^{\Delta 9}$ and $18{:}1^{\Delta 11}$ in T16. The trends of these changes corresponded with those of total fatty acids. In contrast, although free $16{:}1^{\Delta 11}$ was present in T16, it was detected only at a relatively low level and, strikingly, free 18:3 was completely absent in both O9 and T16, although it constitutes approximately 30% of free fatty acids in wild-type plants and is abundant in total lipids in all three plant types (Fig. 2). Similarly, free 16:3 was not detected in either of the transgenics.

2.2. Change in profiles of volatile compounds in the transformants

Table 1 shows the identities of the major volatile compounds and their concentrations. The fatty acid precursors of some of these compounds (Frankel, 1985; Gardner, 1989) are also indicated. In wild-type tobacco leaves, several aldehydes derived from fatty acids constitute approximately 60% of the detected volatiles, of which nonanal, hexenal, and hexanal are the three most abundant (Table 1). Aldehyde content in the leaves of both transformants was significantly higher (~80%) than in wild-type leaves, mostly due to increased hexenal (approximately two-fold in O9 and over three-fold in T16). We examined the volatiles in leaves from three additional independent transformants expressing the yeast Δ9-desaturase transgene and three more expressing the moth Δ11-desaturase transgene. Although minor variations were observed, the profiles within the same transgenic group were similar, and they all showed appreciable increases in hexenal (data not shown). On a percentage basis, *trans*-2, 4-hexadienal was increased the most in O9 and T16 plants; however, the baseline level of this compound was relatively low in wild-type plants. Thus, in absolute quantities, its increase was less significant than that of hexenal. Not all aldehydes were increased in the transgenic plants; rather the levels of several declined. Most striking was the marked (~90%)

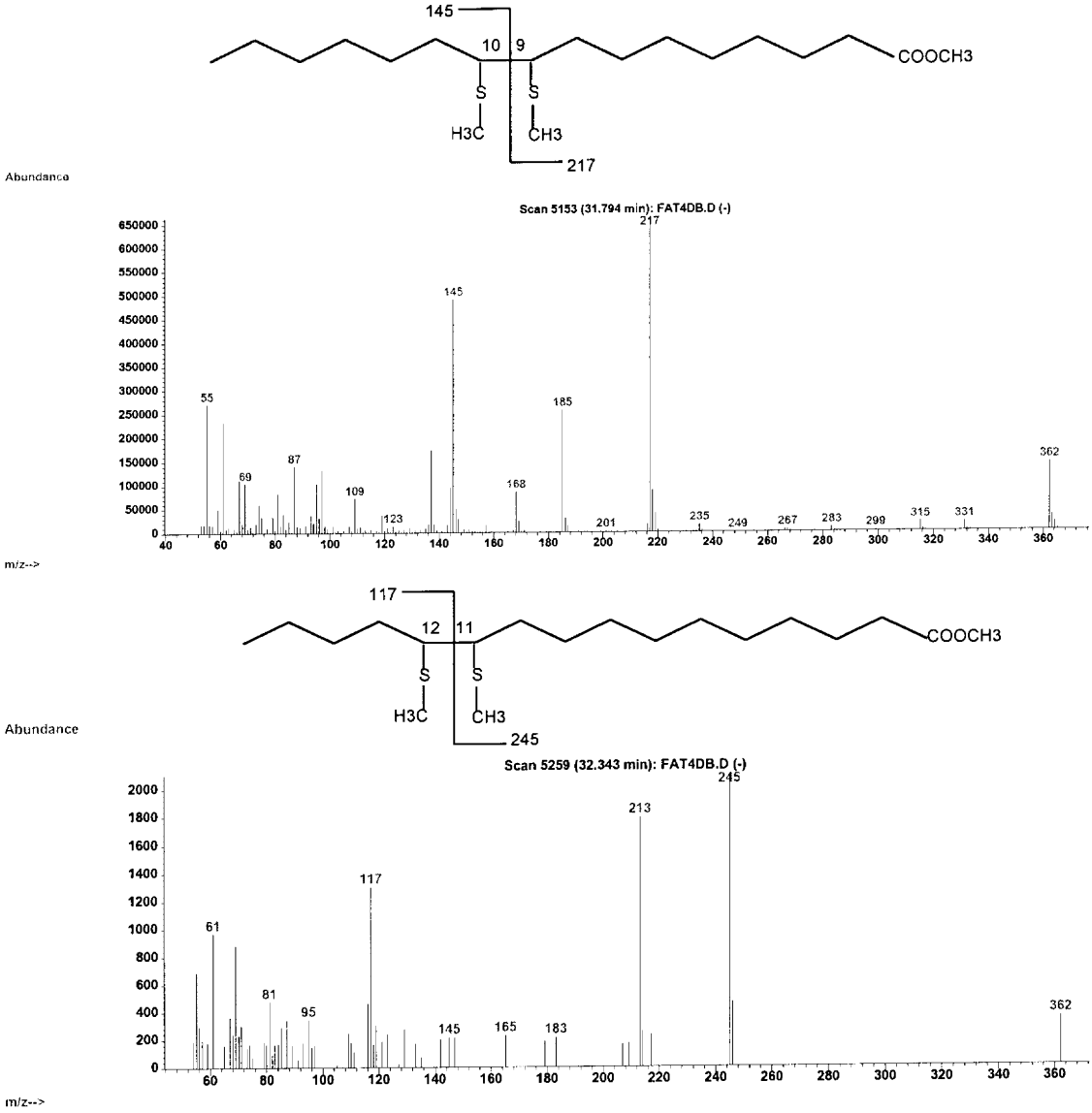

Fig. 1. Splitting patterns and mass spectra of $16{:}1^{\Delta 9}$ and $16{:}1^{\Delta 11}$ fatty acid methyl esters. The dimethyl disulfide (DMDS) adduct method as described by Shibahara et al. (1986) was used to identify the double bond position of 16:1Δ9 and 16:1Δ11 fatty methyl esters.

decline in T16 of nonanal, which was the most abundant aldehyde in the wild-type plants.

2.3. Lipoxygenase and lipid hydroperoxide lyase activities

The relative enzymatic activities of 9-lipoxygenase and 13-lipoxygenase were measured in extracts of wild-type, O9, and T16 tobacco leaves. In wild-type plants, 9-LOX activity was approximately 10 times higher than that of 13-LOX. The activities of both 9-LOX and 13-LOX increased in O9 and T16 plants relative to the wild-type control (Figs. 4 and 5). 13-LOX activity was similar in O9 and T16 plants, and it was nearly twice that of wild-type (Fig. 5). 13-Hydroperoxide lyase activity was also measured and found to be marginally higher in O9 and T16 plants compared to wild-type (Fig. 6).

2.4. Effect of fatty acid application on hexenal production in wild-type plants

Fig. 7 shows the time course of the effect of foliar application of $16{:}1^{\Delta 9}$ on hexenal and hexanal production. The level of hexenal increased rapidly after treatment and reached a maximum within about 2 h after application. In contrast, the increase in hexanal was more gradual and reached a peak within about 3 h of treatment. Thereafter, levels of hexenal and hexanal declined, but by 6 h the latter remained approximately 50% higher than its pretreatment level (Fig. 7).

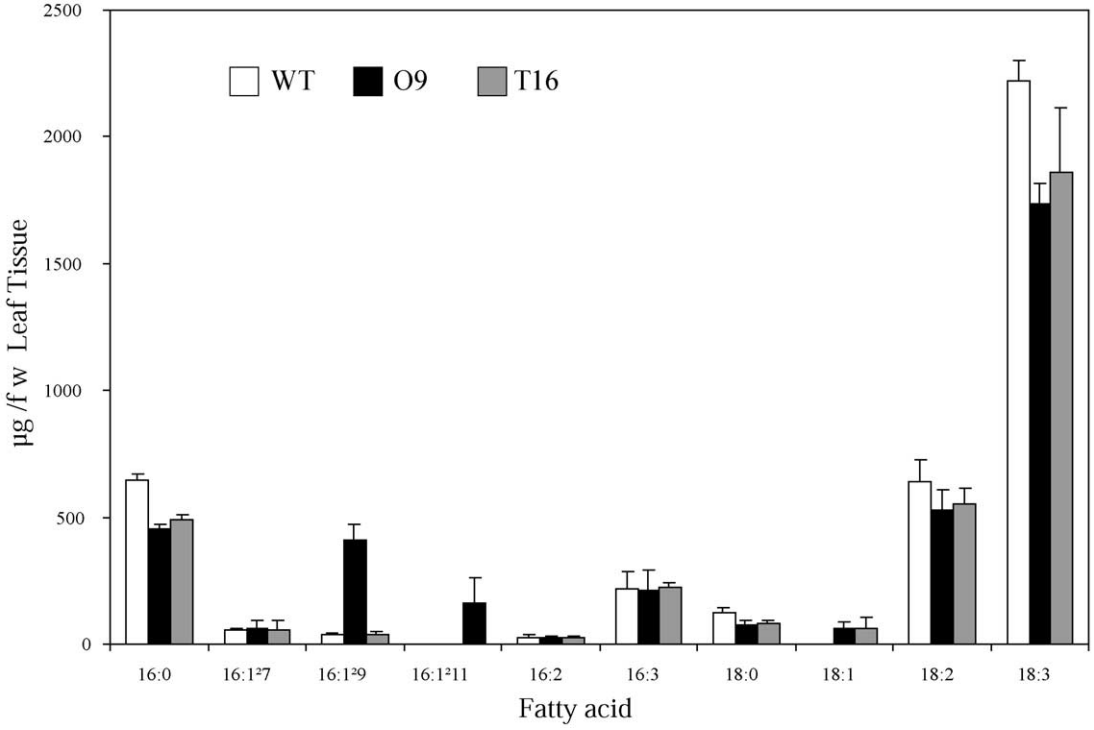

Fig. 2. Total fatty acids in wild-type, O9 and T16 tobacco leaves.

Table 1
Volatile compounds in wild-type (WT), O9, and T16 leaves

	WT (%)	O9 (%)	O9/WT	T16 (%)	T16/WT	Fatty acid precursor
Alcohol						
Butanol	0	2.21	<<	6.42	<<	
1-Butanol, 3-methyl	0.50	0.49	97	0	0	
1-Pentanol	0	0.92	<<	0	*	
1-Penten-3-ol	0	0	0	1.59	<<	
1-Pentanol, 3-methyl	0	0	0	0.5	<<	
cis-3-Hexen-1-ol	1.06	1.78	168	0	0	18-1
Aldehyde						
Hexanal	13.18	16.82	128	14.89	113	16-1, 18-2
Trans-2-hexenal	16.49	32.49	197	57.37	348	18-3
Heptanal	3.21	6.26	195	0.65	20	16-1
Trans-2,4-hexadienal	0.40	1.75	437	1.99	496	
Octanal	2.50	3.69	148	0.57	23	
Nonanal	23.89	21.33	89	2.3	10	18-1
Decanal	2.78	0.63	23	1.15	41	
Hydrocarbon						
Cyclohexane,methyl-	0	0	0	3.18	<<	
1,5-Heptadien-3-yne	0	0.28	<<	0	*	
Octane	8.65	0.57	7	0	0	
1-Heptene, 6-methyl	0	1.30	<<	0	*	
Nonane	1.07	1.73	162	0	0	
Decane	7.45	7.62	102	1.57	21	
Dodecane	10.26	4.20	41	0.95	9	
Tetradecane	3.12	0	0	0.28	9	
Aromatic compounds						
Benzene,1,3-dimethyl	1.48	1.06	72	3.02	204.52	
Benzaldehyde	1.43	1.38	96	0.84	58	
Furan, 2-pentyl	1.56	2.27	145	2.42	155	18-2
Ketone						
1-Penten-3-one	0	1.10	<<	1.59	<<	
2-Cyclopentene-1-one	1.49	0.93	62	0.24	16	

*Not detected in both WT and T16.

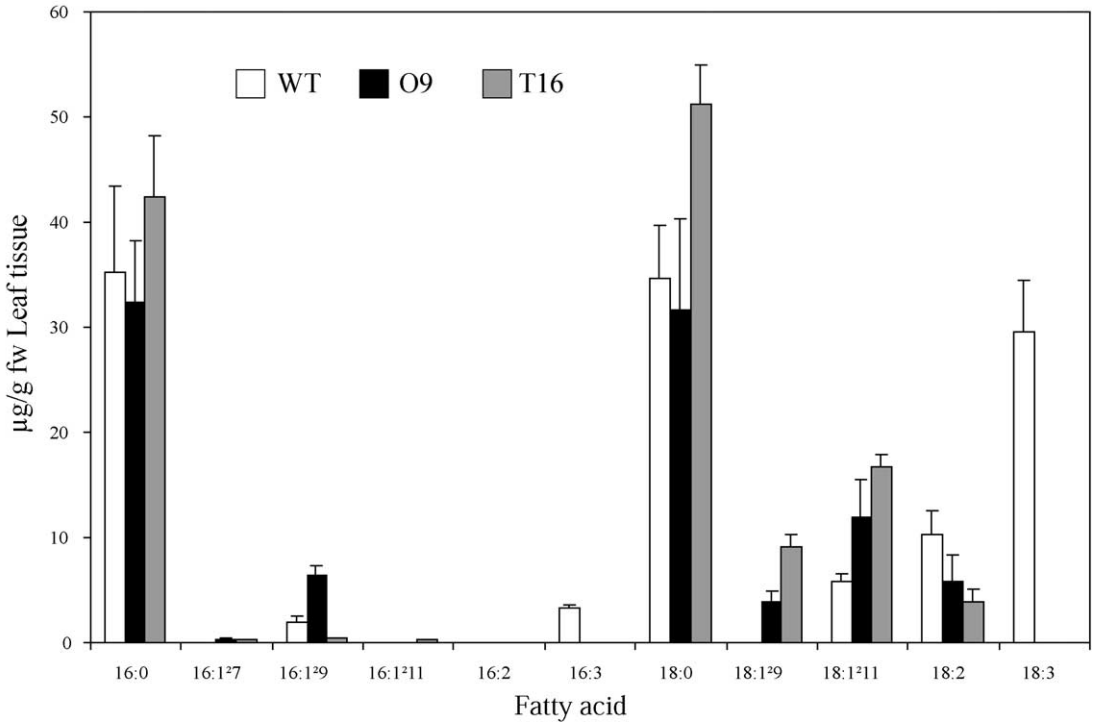

Fig. 3. Free fatty acids in total lipids in wild-type, O9 and T16 tobacco leaves.

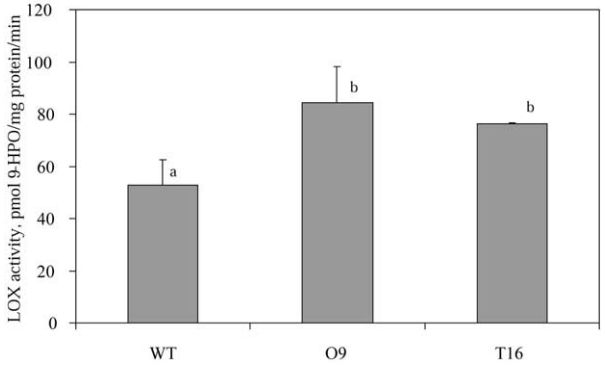

Fig. 4. 9-LOX activity in wild-type, O9 and T16 tobacco leaves. The product of 9-LOX activity, 9-hydroperoxide, was determined by HPLC. Means marked by different letters are significantly different from each other.

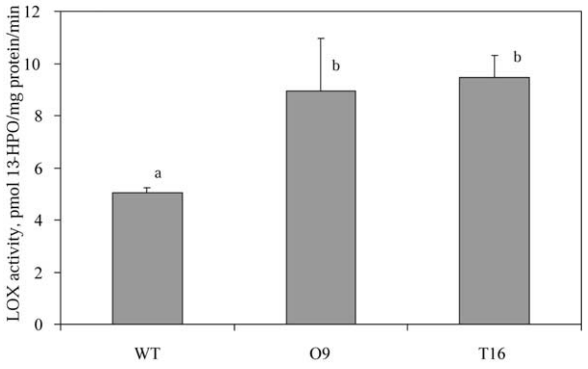

Fig. 5. 13-LOX activity in wild-type, O9 and T16 tobacco leaves. The product of 13-LOX activity, 13-hydroperoxide, was determined by HPLC. Means marked by different letters are significantly different from each other.

The effects of foliar application of several other fatty acids on hexenal production in wild-type tobacco plants were also examined. Clear differences among the fatty acids tested were observed: $16:1^{\Delta 9}$, $16:1^{\Delta 11}$ and 18:3, but not 16:0 and 18:1, stimulated the production of hexenal (Fig. 8).

2.5. Effects of 16:1 on 13-LOX transcript abundance

Foliar application of $16:1^{\Delta 9}$ on wild-type tobacco leaves led to an increase in 13-LOX transcript abundance, as shown in the northern blot (Fig. 9). There was subsequently a decline to pre-application levels.

3. Discussion

Aldehydes are the predominant volatiles found in tobacco leaves and most are derived from fatty acid precursors. Thus, changes in the ratios and levels of specific fatty acids would be expected to influence the levels of plant volatiles. Indeed, in this study we show that altered fatty acid profiles, resulting from the expression of transgenes encoding yeast Δ9 desaturase and moth Δ11 desaturase, are accompanied by a dramatic increase in hexenal, a major leaf volatile.

Although this compound is identified as *trans*-2-hexenal by GC analysis, we interpret this as corresponding to a mixture of *cis*-3-hexenal and *trans*-2-hexenal in the plant, since *cis*-3-hexenal is enzymatically derived in vivo from α-linolenic acid (Hatanaka, 1993) and is efficiently converted to *trans*-2-hexenal during thermal desorption in GC analysis (Karmas et al., 1993). Thus, the *trans*-2-hexenal we have detected was most likely derived from non-enzymatic isomerization of *cis*-3-hexenal (Hatanaka et al., 1977).

Because of the precursor-product relationship of α-linolenic acid and hexenal, we were particularly interested in examining the effects of desaturase transgene expression on the relative levels of these two compounds. We found that levels of 18:3 in total lipids (mostly phospholipids and glycerolipids) of O9 and T16 plants actually declined slightly relative to wild-type. Thus, increases in hexenal in O9 and T16 cannot be attributed to higher levels of its precursor, 18:3, in total lipids. Because hexenal is formed from free 18:3 released from lipid reservoirs by lipases, we also examined free fatty acid profiles. Remarkably, free 18:3, which constitutes approximately 30% of free fatty acids in control plants, was undetectable in O9 and T16. The complete absence of free 18:3 and the significantly higher levels of hexenal in O9 and T16 indicate that these plants convert 18:3 to hexenal much more efficiently than do wild-type tobacco plants. These results also suggest that the availability of free 18:3 is not the limiting factor for hexenal production in wild-type plants.

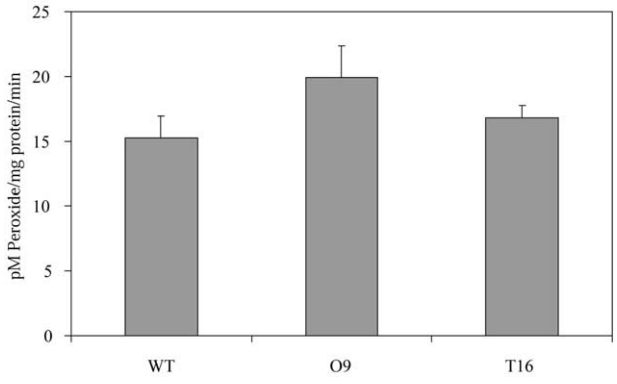

Fig. 6. Hydroperoxide lyase activity in wild-type, O9 and T16 tobacco leaves. Activity was measured spectrophotometrically at 234 nm by the disappearance of the substrate, linoleic acid (13S)-hydroperoxide.

As O9 and T16 have respectively higher $16{:}1^{\Delta 9}$ and $16{:}1^{\Delta 11}$, we examined whether these two 16-carbon fatty acids could be involved in enhanced hexenal production. Foliar application of $16{:}1^{\Delta 9}$ and $16{:}1^{\Delta 11}$ significantly increased the hexenal levels of wild-type tobacco leaves (Figs. 7 and 8). The association of $16{:}1^{\Delta 9}$ or $16{:}1^{\Delta 11}$, whether endogenous (effected by the transgenic expression) or exogenously applied, with elevated hexenal production suggests that these two fatty acids are the cause of the elevated hexenal levels. Not all fatty acids were effective in increasing hexenal levels (Fig. 8). Among the fatty acids tested, only $16{:}1^{\Delta 9}$, $16{:}1^{\Delta 11}$, and 18:3 were effective. The stimulatory effect of exogenously applied 18:3 can be readily explained by the fact that 18:3 is a natural substrate of 13-LOX. Since neither $16{:}1^{\Delta 9}$ nor $16{:}1^{\Delta 11}$ is a substrate of any enzyme in the

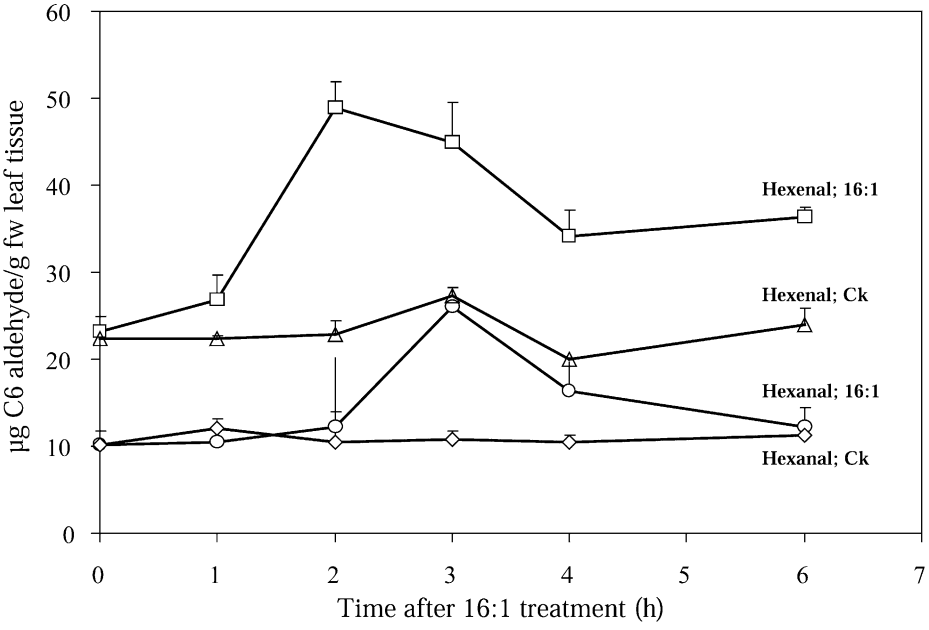

Fig. 7. Effect of foliar application of $16{:}1^{\Delta 9}$ on production of C6 aldehydes. Thirty microliters of 1 mM $16{:}1^{\Delta 9}$ in 0.1% Triton X-100 was applied to the surface of wild-type tobacco leaves of approximately 0.5 g in fresh weight. Control leaves (Ck) were treated similarly with 0.1% Triton X-100. C_6 aldehydes were measured according to Avdiushko et al. (1995).

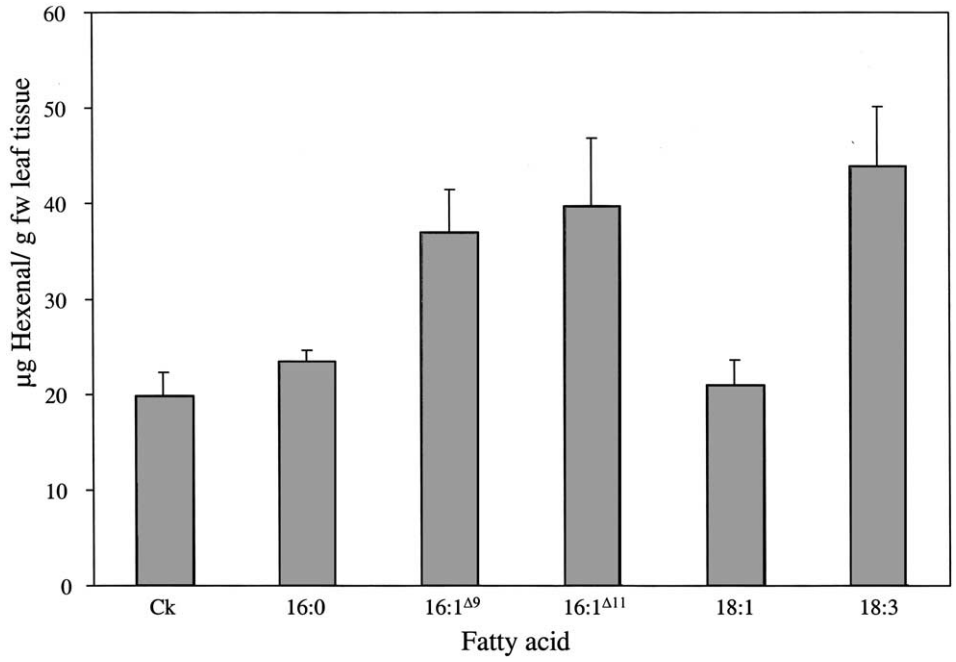

Fig. 8. Effect of foliar application of various fatty acids on hexenal production in wild-type tobacco. Thirty microliters of 1 mM fatty acid in 0.1% Triton X-100 was applied to the surface of a leaf of approximately 0.5 g in fresh weight. Control treatment (Ck) received 30 µl of 0.1% Triton X-100. Hexenal production was determined according to Avdiushko et al. (1995).

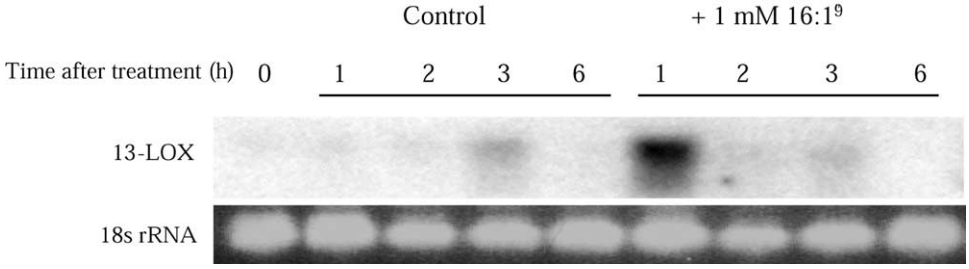

Fig. 9. Time course of steady-state transcript levels of 13-LOX in wild-type tobacco leaf tissue sprayed with either H_2O (control) or 1 mM $16:1^{\Delta 9}$, as analyzed by Northern blot. Equal loading of RNA was assessed by ethidium bromide staining of the gel prior to transfer.

hexenal degradative pathway, their effects must involve another mechanism.

The production of hexenal from free 18:3 is mediated by 13-LOX and 13-hydroperoxide lyase. Whereas the enzyme activity of the latter was not significantly different in wild-type, O9, and T16 plants, 13-LOX activity was much higher in both types of transgenic plants relative to wild-type. These data suggest a direct causal relationship between 13-LOX activity and hexenal levels in tobacco leaves. Neither $16:1^{\Delta 9}$ nor $16:1^{\Delta 11}$ had an effect on the in vitro rate of conversion of α-linolenic acid to linolenic acid 13-hydroperoxide (data not shown). On the other hand, application of $16:1^{\Delta 9}$ to wild-type leaves led to a transitory increase in 13-LOX transcript abundance (Fig. 9). These data indicate that 16:1 likely exerts its effect via activation of 13-LOX expression. This interpretation is in agreement with the observation that both O9 and T16 plants have higher 13-LOX activity.

The common structural features associated with the stimulatory effects of $16:1^{\Delta 9}$ and $16:1^{\Delta 11}$ on hexenal production in tobacco plants are 16-carbon chain length and monounsaturation. The double bond position does not appear to be critical, although additional positional isomers must be evaluated to establish the generality of this conclusion. Since free 16-carbon monounsaturated fatty acids are typically present at extremely low levels in tobacco and many other plants, we suggest that their observed effect on the production of hexenal, a potent inhibitor of microorganisms (Bisignano et al., 2001), may reflect a specific plant defense response elicited by pathogens that contain them. Expression of the yeast Δ9 desaturase in tomato and eggplant confers enhanced resistance to tomato powdery mildew caused by *Erysiphe polygoni* and eggplant Verticillium wilt caused by *Verticillium dahliae*, respectively (Wang et al., 2000; Xing and Chin, 2000). While these resistant plants also have increased hexenal levels, additional defense

mechanisms could be involved, since endogenous fatty acid-derived signals are known to play a critical role in modulating distinct defense signaling pathways in *Arabidopsis thaliana* (Kachroo et al., 2001). At the least, the present study supports the conclusion that high endogenous levels of 16:1 or exogenous treatment with $16{:}1^{\Delta 9}$ and $16{:}1^{\Delta 11}$ results in elevated hexenal levels as a consequence of increased 13-LOX activity.

4. Experimental

4.1. Plant transformation

Nicotiana tabacum var. Samsun NN was used in these studies. *Agrobacterium tumefaciens* LBA4404 was transformed with pBI101 containing either the yeast Δ9 desaturase coding sequence (Polashock et al., 1992) or with pBI121 containing the cabbage looper moth Δ11 desaturase coding sequence (Knipple et al., 1998). Each of the binary vectors contained the neomycin phosphotransferase II gene sequence for selection of transformants. The expression of the desaturases was controlled by the CaMV 35S promoter. These *Agrobacterium* strains were used to infect tobacco leaf discs using the leaf disc transformation method (Horsch et al., 1985). Kanamycin-resistant plantlets were regenerated on MS medium (Murashige and Skoog, 1962) containing 30 g/l sucrose, 1 μM naphthalene acetic acid, 10 μM 6-benzylaminopurine, 200 μg/ml kanamycin sulfate, and 500 μg/ml carbenicillin. Transgenic plantlets were multiplied in vitro on 1/2 strength MS medium containing 30 g/l sucrose and were subsequently transplanted to potting mix (vermiculite/peat, 1:1) and grown in a greenhouse. Tobacco plants regenerated from uninoculated leaf discs were used as control plants.

4.2. Analysis of volatiles

Leaves 3, 5 and 7 (8 g) from the top were harvested from 7 to 8 week old greenhouse-grown tobacco plants, flash frozen in liquid nitrogen, and stored at −80 °C. The frozen leaves were ground with a mortar and pestle in liquid nitrogen to a fine powder. The thawed leaf slurry was then poured into a 250-ml Wheaton purge and trap apparatus, and tridecane (5 μg) was added as an internal standard. Volatile compounds were trapped onto a Tenax-Carbotrap tube (Scientific Instrument Services, Inc. Ringoes, NJ) with a nitrogen gas flow of 40 ml/min at 55–60 °C for 1 h. The trap was thermally desorbed at 250 °C for 5 min with a TD-4 short-path thermal desorption system (Scientific Instrument Services, Inc.) and injected into a GC–MS system (HP 6890 GC and HP 5973 MS). A 60-m HP-5MS capillary column with an ID of 0.25 mm was used. The injector temperature was set at 250 °C, and the detector temperature at 280 °C. Column temperature was programmed at 30 °C for 10 min, followed by a 2 °C/min ramp until 220 °C.

4.3. Fatty acid analysis

4.3.1. Total fatty acids

Leaf three from the apex of several plants was excised, and extracts were prepared according to Browse and coworkers (1986) with minor modifications (Wang et al., 1996). Fatty acids were separated and identified with a HP GC–MS (HP 6890 GC and HP 5973 MS). A 60-m HP-5MS capillary column with an ID of 0.25 mm was used. The GC was programmed to begin at 170 °C for 10 min, followed by a 10 °C/min ramp until 220 °C, at a flow rate of 1 ml/min. More than one 16:1 fatty acid were detected. To identify the double bond position of different 16:1 fatty acids, the dimethyl disulfide (DMDS) adduct method as described by Shibahara and coworkers (1986) was used. Briefly, the hexane phase (300 μl) containing the methylated fatty acids was dried with a stream of nitrogen, and then dimethyl disulfide (200 μl) containing 15 mg/ml I_2 was added. The samples were incubated at 35 °C for 30 min. Excess I_2 was reduced with a saturated solution of $NaHSO_3$. Hexane/ether (1:1, v/v, 100 μl) was then added, mixed by vortexing, and was then centrifuged at 250 g for 10 min. One to two microliters of the upper phase was injected into the GC–MS.

4.3.2. Free fatty acids

Fatty acids were extracted from leaves using the method described by Kates (1986) as modified by Conconi et al. (1996). 17:0 was added to the extract as an internal standard. Free fatty acids were separated from other lipids by one-dimensional TLC on silica gel G developed in hexane:ether:acetic acid (80:20:2). Lipids were visualized with iodine vapor. Authentic 18:3 was used as a reference to locate the free fatty acids. The free fatty acids were collected from the gel and methylated with methanol in 1 N H_2SO_4.

4.4. Assay of lipoxygenase and hydroperoxide lyase enzymatic activity

Partially purified lipoxygenase and hydroperoxide lyase were prepared from tobacco leaves from greenhouse-grown plants according to the protocol of Vick (1991). Assay of lipoxygenase activity was based on production of 18:3-hydroperoxides (Iacazio et al., 1990); 18.3 was used as a substrate. The 10 ml reaction mixture contained 200 mg 18:3 and 4 mg of the partially purified lipoxygenase extract. The reaction was carried out at 2–4 °C for 40 min under a constant flow of oxygen. The hydroperoxides produced by the reaction were reduced to hydroxy fatty acids using 100 mg $NaBH_4$. Linoleic

acid (13S)-hydroperoxide, prepared with commercial soybean lipoxygenase-1 from Sigma was used as a standard. Reaction mixture containing the hydroxy- and hydroperoxide products were extracted with equal volume of ethyl ether and subjected to analysis by HPLC (Shimadzu LC-10AT HPLC; LUNA C18, particle size 3 μm, 150×4.6 mm reversed phase column). The column was eluted with CH_3CN:H_2O (50:50) at 0.9 ml/min, and the fatty acid hydroperoxides and hydroxy fatty acids were detected at 234 nm.

Determination of hydroperoxide lyase activity was based on the method of Vick (1991). Linoleic acid (13*S*)-hydroperoxide, prepared with commercial soybean lipoxygenase-1, was used as a substrate.

4.5. Production of C_6-aldehydes

C_6 aldehydes were measured according to Avdiushko et al. (1995). Briefly, tobacco leaves were collected and stored at −80 °C. Prior to measurement, tridecane (1 μg) was added as internal standard to the vial, which was then placed in a water bath for 20 min at 30 °C, followed by 5 min incubation at 80 °C. Then headspace gas (500 μl) was withdrawn using a 500 μl gas-tight syringe (SUPELCO) and analyzed with a HP GC–MS (HP 6890 GC and HP 5973 MS) equipped with a 60-m HP-5MS capillary column (ID of 0.25 mm). The GC was programmed for an initial temperature of 10 °C, followed by a 5 °C/min ramp until 150 °C, then a 10 °C / min ramp to 220 °C, which was finally held at 220 °C for 5 min. The flow rate was 0.7 ml/min.

4.6. RNA gel blot analysis

Total RNA was extracted from the leaf tissue using Quantum Prep Master Blaster RNA kit (BioRad) following the manufacturer's specifications. Denatured RNA (20 μg) was separated on a 1.2% agarose/formaldehyde denaturing gel, transferred by capillary action to a Duralon membrane (Stratagene) and cross-linked to the membrane by use of a UV Stratalinker (Stratagene).

The cDNA probe used in the hybridization was obtained from tomato leaf tissue. Oligonucleotide primers were designed to amplify tomato LOXD mRNA (Heitz et al., 1997); the forward and reverse primer sequences were respectively 5′-TGTCGCGCTAGAACTCAT-3′ and 5′-GGGGAGGCTAAGTTCAAT-3′ and were synthesized by InVitrogen. RT–PCR was performed using QIAGEN's One-Step RT–PCR system according to the manufacturer's protocol. The DNA probe was radiolabeled by random-prime labeling (InVitrogen) using the manufacturer's specifications.

Membrane hybridization and washing were performed using standard methods (Church and Gilbert, 1984).

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