Effect of Overexpression of Fatty Acid 9-Hydroperoxide Lyase in Tomatoes (*Lycopersicon esculentum* Mill.)

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To modify the flavor properties of tomato fruits, cucumber fatty acid hydroperoxide lyase (HPL), which can act on 9-hydroperoxides of fatty acids to form volatile C9-aldehydes, was introduced to tomato plants. Through enzyme assay, high activity of the introduced HPL could be found in either the leaves or fruits of transgenic tomatoes; however, the composition of volatile short-chain aldehydes and alcohols in the transgenic tomato fruits was little modified. This was unexpected because tomato fruits have high lipoxygenase activity to form 9-hydroperoxides. When linoleic acid was added to a crude homogenate prepared from the transgenic tomato fruits, a high amount of C9-aldehyde was formed, but the amount of C6-aldehyde was almost equivalent to that in nontransgenic tomatoes. Through quantification of fatty acid hydroperoxides, it has been revealed that 13-hydroperoxides of fatty acids are preferably formed from endogenous substrate, whereas 9-hydroperoxides are formed from fatty acids added exogenously. From these observations, possible mechanisms to regulate metabolic flow of the lyase pathway are discussed.

Keywords: *Tomato (Lycopersicon esculentum Mill.); volatile aldehydes; fatty acid hydroperoxide lyase; lipoxygenase*

INTRODUCTION

Tomatoes are widely consumed fruits worldwide because of their peculiar flavor. It has been known that one of the most important constituents in tomato flavor is short-chain aldehydes and alcohols (*1*). In tomato fruits, C6 compounds, such as *n*-hexanal, (*Z*)-3-hexenal, (*E*)-2-hexenal, and (*Z*)-3-hexen-1-ol, are most abundant, whereas the amounts of C9 compounds, such as (*Z*)-3-nonenal and (*Z,Z*)-3,6-nonadienal, are scarce. These short-chain aldehydes and alcohols are formed via a sequential action of enzymes consisting of hydrolysis of lipids by lipolytic acyl hydrolase (LAH, or nonspecific lipase) to form free fatty acids, oxygenization of free fatty acids by lipoxygenase (LOX) to form fatty acid hydroperoxides (HPOs), and cleavage of HPOs to form short-chain aldehydes and oxo-acids by fatty acid hydroperoxide lyase (HPL) (Figure 1). The other enzymes involved in oxylipin formation, such as allene oxide synthase, or divinyl ether synthase, may act on the HPOs, however, there is no report on these activities in tomato fruits. The fate of fatty acids in this enzyme system is determined by the substrate and product specificities of LOX and HPL. HPLs can be grouped into two types (*2*). One type of HPLs (13-HPLs) found in tea leaves, bell pepper fruits, *Arabidopsis* leaves, and so on, specifically acts on 13-HPOs to form C6-aldehydes, whereas the other type (9-HPL) can act on both 9- and 13-HPOs with a preference to the former. 9-HPL was found in cucumber (*3*) and melon (*4*). In tomato fruits, only 13-HPL can be found so far (*5*), and no 9-HPL activity could be found. A recombinant protein expressed in *E. coli* harboring tomato fruit HPL cDNA has very high activity to 13-HPOs, but 9-HPOs show activity of less than 1% of that with 13-isomers (*6*). There are also two types of LOX. One specifically forms 9-HPOs (9-LOX) and the other specifically forms 13-HPOs (13-LOX). There are at least four LOX genes in tomato, namely, *tomLoxA, B, C,* and *D* (*7, 8*). Among these, three genes (*tomLoxA* to *C*) express in the fruits but in different fashions. For example, *tomLoxA* is expressed at highest level during the breaker stage of ripening fruits, but expression of *tomLoxB* is highest in ripe fruits. From biochemical analyses, it is known that the most abundant LOX in tomato fruits has high specificity to form 9-HPOs (*5*). There is apparently little or no activity to act on 9-HPOs in tomato fruits, and this property is widely utilized to prepare 9-HPOs in a simple manner (*9*).

Until now, there have been at least three attempts to modify flavor constituents in tomato fruits. Wang et al. (*10*) expressed yeast Δ-9 desaturase gene in tomato, and succeeded in changing the fatty acid composition in tomato fruits, which resulted in modification of their flavor profile. Genetic manipulation of alcohol dehydrogenase in the fruits shifted the balance of short-chain aldehydes and alcohols and resulted in a more intense "ripe fruit" flavor (*11*). Antisense-suppression of lipoxygenase in tomatoes resulted in extensively lower LOX activity, however, it resulted in little changes in the flavor profiles (*12*). Recently, we successfully isolated cDNA (*Cs9HPL*) encoding 9-HPL in cucumber hypocotyls (*3*). A recombinant protein expressed in *E. coli* showed higher activity to 9-HPOs. As the fourth attempt, in this study, we tried to express 9-HPL in

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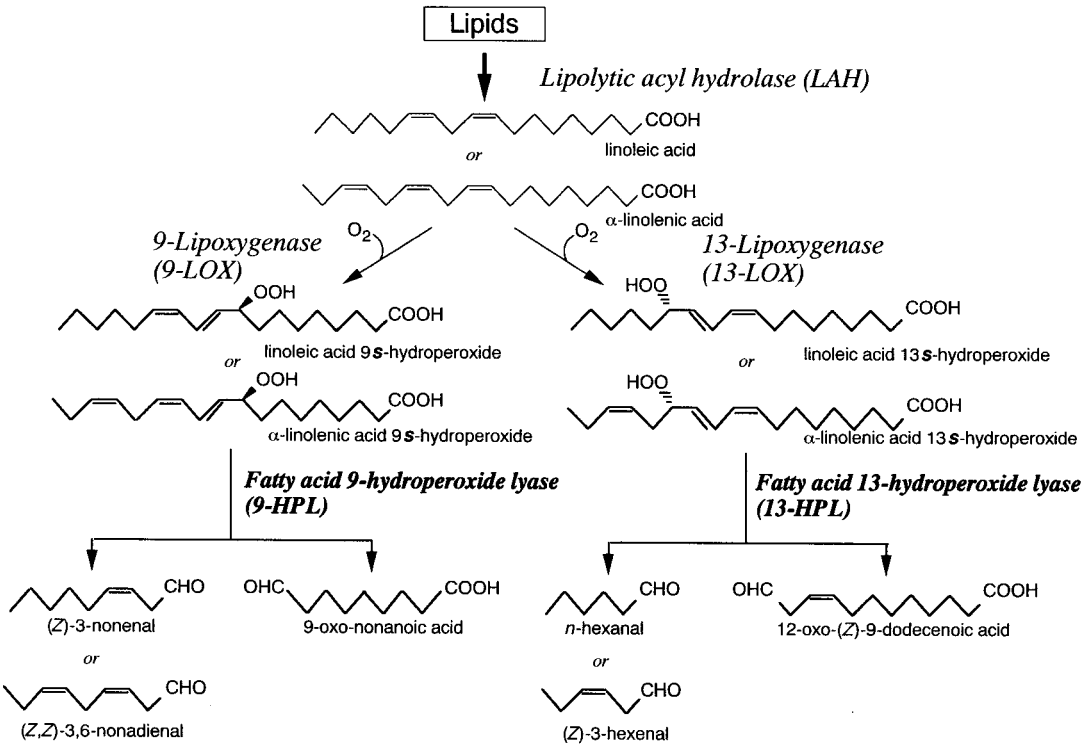

Figure 1. Short-chain aldehyde forming system in plants.

tomato fruits. As described above, tomato fruits have high 9-LOX activity but no activity to consume 9-HPOs. Therefore, it was expected that overexpression of cucumber 9-HPL in tomato fruits could modify the metabolic pathway to form volatile aldehydes.

MATERIALS AND METHODS

Tomato (*Lycopersicon esculentum* Mill. cv UC82B) plants were grown under controlled conditions in a greenhouse. Harvesting of fruits was randomized so that environmental or positional effects were minimized. In most experiments at least three independent fruits were used for one assay to exclude fruit to fruit variation.

Construction of *Cs9HPL* Transgene. Almost the full-length of *Cs9HPL* cDNA was ligated into *Kpn* I site of pCGN8059, which contains the enhanced promoter of cauliflower mosaic virus 35S ribosomal RNA promoter (*p-e35S*), leader sequence of HSP70, and the nopaline synthase 3′ terminator sequences (*13*). The DNA ranging from the promoter to the terminator was then ligated into *Not* I site of the binary vector pCGN 5138. The pCGN5138 vector contains the *nptII* gene between CaMV35S promoter and tml 3′ terminator sequences (*13*). The construct, named pCGN8337, was transferred to *Agrobacterium tumefaciens* LBA4404. Cotyledon segments obtained from tomato seedlings (cv. UC82B) were co-cultivated with the *Agrobacterium*, then the transformed cells were selected with kanamycin (*14*). After regeneration, the plantlets were grown in a greenhouse. Segregation of the transformants in terms of kanamycin-resistance was determined by kanamycin spray onto the seedlings. Genomic DNA was extracted from young leaves by the method of Murray and Thompson (*15*), and was digested with *Bgl* II. After the digestion, the DNAs were fractionated by electrophoresis on 0.4% agarose gel. The DNA was transferred onto nylon membranes (Hybond N^+, Amersham) as described by the manufacturer. The filter was hybridized with ^{32}P-labeled probes corresponding to the *Cs9HPL* region of the transgene. Hybridization was carried out at 65 °C for 15 h, and the filter was washed twice with 2 × SSC, 0.1% SDS at 65 °C for 15 min each, followed by a wash with 0.1 × SSC, 0.1% SDS at 65 °C for 15 min. The membrane was exposed to an X-ray film (RX-U, Fuji Photo Film, Tokyo) overnight.

Enzyme Assay. Leaves were homogenized in 4 vol of 100 mM MOPS−KOH (pH 7.6) supplemented with 7% sucrose in a chilled mortar, and the resultant homogenate was centrifuged at 2000 rpm (RPR 20-2 rotor, Hitachi, Tokyo) for 3 min to remove cell debris. Fruits were homogenized in an equal volume of the same buffer containing 10 mM EGTA with a Polytron mixer on ice, and centrifuged as above. In either of the cases, the resultant supernatant was used as the crude enzyme solution. 9- and 13-HPL activities were determined with 80 μM each of linoleic acid 9-HPO and 13-HPO, respectively, in 100 mM MES−KOH (pH 6.0) essentially according to the method described previously (*3*). The substrates were prepared as described previously using partially purified tomato LOX and soybean LOX-1. The reaction proceeded for 10 min at 25 °C, then the formed aldehydes were converted to their 2,4-dinitrophenylhydrazone derivatives. The hydrazones were quantified with HPLC (Purospher RP-18, 4.6 mm × 250 mm, Kanto Chemical, Tokyo) with a solvent system of acetonitrile/tetrahydrofuran/water (74:1:25, v/v) at a flow rate of 1 mL/min. Detection was performed at 350 nm. To evaluate short-chain aldehyde forming activity from free fatty acids, 0.4 mM linoleic acid was added to the crude enzyme solution. For quantification of fatty acid HPOs, tomato fruits were homogenized in 4 vol of 100 mM potassium phosphate buffer (pH 6.3) with a Polytron mixer, and the homogenate was incubated for 30 min at 25 °C with or without 0.4 mM linoleic acid. Lipids were extracted from the homogenate with the method of Folch. To evaluate the endogenous level of fatty acid HPO in nondisrupted, intact fruits, the tissue was first homogenized with 3.3 vol of methanol, and lipids were extracted immediately. The crude lipids were reduced by adding 0.1 mM triphenylphosphine under nitrogen atmosphere. Fatty acid HPOs were analyzed with a straight-phase HPLC system equipped with a Mightysil Si60 column (4.6 mm × 250 mm, Kanto Chemical, Tokyo) with a solvent system of *n*-hexane/2-propanol/acetic acid (94:5.5:0.5, v/v/v) at a flow rate of 1 mL/min. Detection was done at 234 nm.

Volatile Analyses. Tomato fruits (100 g) were homogenized with a Polytron mixer on ice, and were incubated at 25 °C for 30 min in a tightly closed vessel. Subsequently, 100 mL of saturated $CaCl_2$ solution was added to the homogenate and mixed vigorously, and 34 μg of *n*-undecane was added as an internal standard. Activated carbon fiber (10 mg) was used to

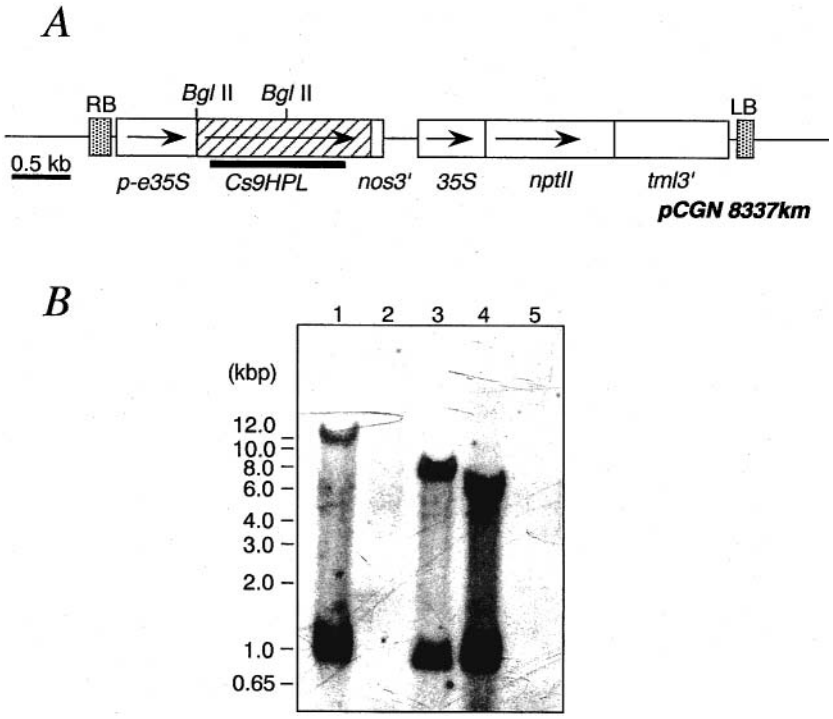

Figure 2. A, Structure of the 9-HPL constructs. The region corresponding to that used for a probe is shown in a black bar. *Bgl* II sites in the T-DNA region are also shown. Abbreviations: p-e35S, enhanced promoter of cauliflower mosaic virus 35S ribosomal RNA; Cs9HPL, cucumber fatty acid 9-hydroperoxide lyase; nos3′, nopaline synthase 3′ terminator; nptII, kanamycin selectable marker; tml3′, *tml* terminator. B, Southern blot of the transformed plants. Genomic DNA digested with *Bgl* II was separated, transferred to the nylon membrane, and then probed with ^{32}P-labeled DNA. Lanes 1, 3326; 2, 3356; 3, 3361; 4, 3406; and 5, UC82B.

adsorb volatile compounds in a closed-loop stripping apparatus (*16*) with circulating air from a magnetic pump (PMD-111B, Sanso Electronics, Tokyo). After the air circulated for 2 h, the fiber was recovered, and adsorbed compounds were eluted with 100 μL of dichloromethane. The solution was concentrated by hand warming to 2 μL, and then the compositions were analyzed by GC and GC−MS. GC was performed with a GLC 10A (Shimadzu, Kyoto) equipped with a DB-WAX column (60 m × 0.25 mm, J & W Scientific, Folsom, CA). The column temperature was held at 40 °C for 5 min and programmed to increase at 5 °C/min to 220 °C. MS analyses were performed on a GCMS-QP 5050A (Shimadzu) with a detector gain of 1.10 kV. For a similarity search of the spectrum, NIST107 and NIST21 (Shimadzu) were used as libraries. Most peaks were identified by MS profile and retention time of authentic specimens.

RESULTS

Explants prepared from cotyledons of tomato seedlings were transformed with constructs having the cucumber fatty acid 9-HPL cDNA (*Cs9HPL*) as shown in Figure 2A. The cDNA was incorporated at the downstream of enhanced CaMV 35S promoter, which provides constitutive expression of the cDNA. The transformants were selected on kanamycin, then regenerated as T_0 plants. The seeds obtained from T_0 fruits (T_1 generation) were germinated, and kanamycin was sprayed onto them. By counting kanamycin-resistant and -sensitive plants, it was shown that they were segregated into a three-to-one ratio, which indicates that they have a single copy of the gene. This was also indicated by the genomic Southern analysis as shown in Figure 2B. Because *Bgl* II cuts at a position within and at the outside of the *Cs9HPL* sequence corresponding to that used as a probe, there must be two bands, one of which has 0.9 kbp and the other of which has various length depending on the adjacent sequence of tomato genome. This analysis indicates that transgenic tomato lines 3326, 3361, and 3406 have only single copies of the foreign gene, whereas 3356 does not have *Cs9HPL* cDNA. Because even 3356 shows resistance against kanamycin, it is suggested that integration of the T-DNA was only partial, and the left-half (including *Cs9HPL* cDNA) was deleted out during integration of the T-DNA into the tomato genome.

HPL activities in the transgenic tomatoes were measured in mature leaves and in fruits at the mature red stage. As shown in Figure 3A, only 13-HPL activity could be detected in leaves of the nontransgenic tomato, UC82B as reported (*17*). No 9-HPL activity could be detected. With 3326, 3361, and 3406, high 9-HPL activity could be detected, which indicates that the introduced *Cs9HPL* is transcribed and translated to an active enzyme in these transgenic lines. As expected from the substrate and product specificities of Cs9HPL, 13-HPL activity was also increased. The ratio of 9- and 13-HPL activity is almost the same as that of recombinant Cs9HPL expressed in *E. coli* (*3*). With 3356, both the activities were very low, and almost the same levels as those of the control tomatoes. This is in good accordance with the result of the genomic Southern blot analysis. With red mature fruits of these lines, almost the same profiles of the HPL activities could be seen as shown in Figure 3B, however, both the 9- and 13-HPL activities in fruits of line 3406 were very small, and almost the same level as those in the UC82B control. The difference in the HPL activities of line 3356 in leaves and fruits might account for position-effect of the integration of the gene in the tomato genome.

As we succeeded to overexpress 9-HPL activity in tomato fruits, subsequentlythe volatile compositions of

Table 1. Composition of Volatile Compounds in Mature Fruits from Transformed Plants (reported in μg/100 g fr. wt.)

compound	8337-3326	8337-3361	8337-3406	UC82B
n-pentanal	n.d.[a]	14.28	n.d.	12.92
1-penten-3-one	15.98	5.78	9.52	9.86
2-butanol	n.d.	1.70	n.d.	2.04
2-methyl-3-buten-2-ol	n.d.	2.04	n.d.	n.d.
n-hexanal	298.18	131.24	135.66	123.76
2-methyl-1-propanol	7.82	5.78	n.d.	9.18
(*E*)-2-pentenal	20.06	n.d.	n.d.	12.58
2-methyl-4-pentenal	n.d.	n.d.	n.d.	2.38
1-butanol	n.d.	2.72	n.d.	1.70
1-penten-2-ol	12.58	5.78	7.48	7.48
n-heptanal	0.34	1.70	n.d.	2.38
3-methyl-2-butenal	n.d.	4.08	n.d.	n.d.
3-methyl-1-butanol	24.82	18.02	21.08	n.d.
(*E*)-2-hexenal	192.44	66.98	83.98	62.90
5-ethyl-2-methyl-octane	n.d.	n.d.	n.d.	n.d.
1-pentanol	26.86	9.86	11.22	17.34
1-octen-3-one	n.d.	n.d.	n.d.	3.06
(*Z*)-2-penten-1-ol	0.68	5.78	9.86	n.d.
(*E*)-2-heptenal	7.82	3.06	n.d.	5.10
6-methyl-5-hepten-2-one	130.90	100.64	83.98	12.24
1-hexanol	61.54	61.20	24.48	51.00
(*E*)-3-hexen-1-ol	5.78	3.74	n.d.	5.78
(*Z*)-3-hexen-1-ol	137.02	93.16	50.66	118.32
n-hexadecane	17.00	n.d.	9.86	7.14
n-nonan-1-ol	n.d.	n.d.	n.d.	1.36
furfural	n.d.	n.d.	n.d.	n.d.
5-methyl-5-octen-2-ol	n.d.	n.d.	9.86	n.d.
n-nonadecane	n.d.	n.d.	n.d.	1.36
benzaldehyde	n.d.	n.d.	n.d.	5.44
(*E*)-2-nonenal	n.d.	2.38	n.d.	n.d.

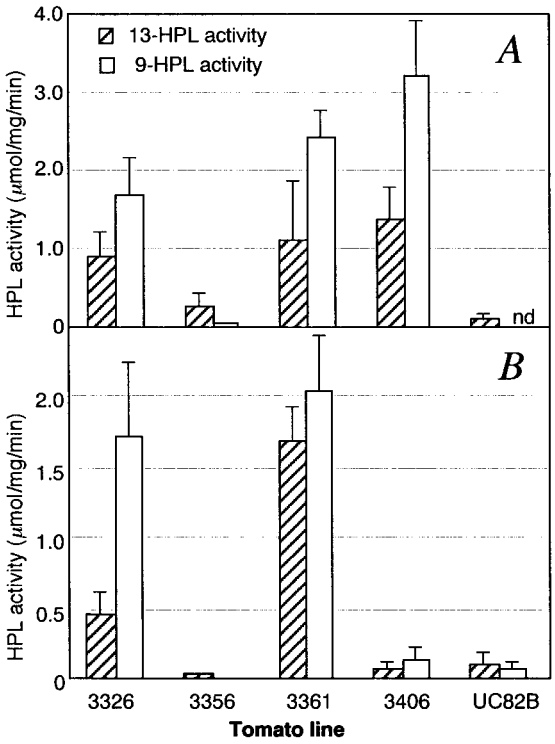

Figure 3. HPL activities in leaves (A) and fruits (B) of transformed tomatoes. Values represent means ± SE of replicates ($n = 3-4$).

these fruits were analyzed. Short-chain aldehydes and alcohols are known to be formed rapidly upon tissue disruption of tomato fruits (*18*). Therefore, in this study, we made juice from mature red fruits of respective transgenic tomato and incubated the juice to facilitate formation of the aldehydes. Volatiles thus formed were adsorbed on active charcoal in a closed-loop stripping system (*16*). As shown in Table 1, C6 compounds, such as *n*-hexanal, (*E*)-2-hexenal, and (*Z*)-3-hexen-1-ol, were

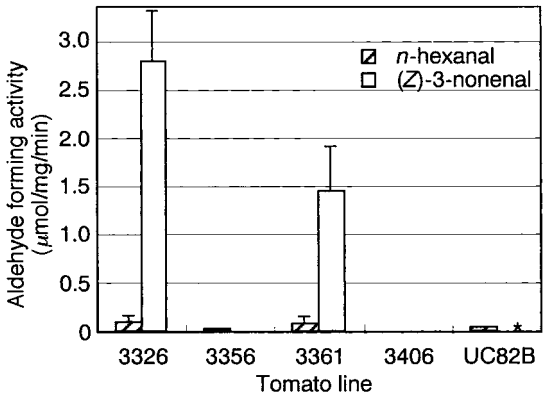

Figure 4. Aldehyde forming activity from linoleic acid exogenously added to the crude enzyme solution prepared from the fruits of respective transformed tomatoes. Values represent means ± SE of replicates ($n = 3-4$).

the most abundant constituents in tomato juice prepared from the control tomato of UC82B. The amounts of C9-aldehydes and C9-alcohols were very low. With transgenic tomatoes, the amount of C6-aldehyde increased a little, but the increase was not significant. Unexpectedly, the amount of C9-aldehyde was little changed, and they were almost not detected.

The fact that the amounts of C9-compounds in the transgenic fruits did not increase led us to check whether 9-LOX in these fruits can serve fatty acid 9-HPOs to 9-HPL. To this end, linoleic acid was added to the juice prepared from the respective fruits, and a corresponding C9-aldehyde, namely (*Z*)-3-nonenal, was quantified (Figure 4). From this analysis, it was evident that a significant amount of (*Z*)-3-nonenal was formed from linoleic acid added exogenously to the juice prepared from fruits of lines 3326 and 3361. It must be noticed that with these two lines, (*Z*)-3-nonenal was formed preferentially, whereas the formation of *n*-hexanal was almost the same as that in nontransgenic

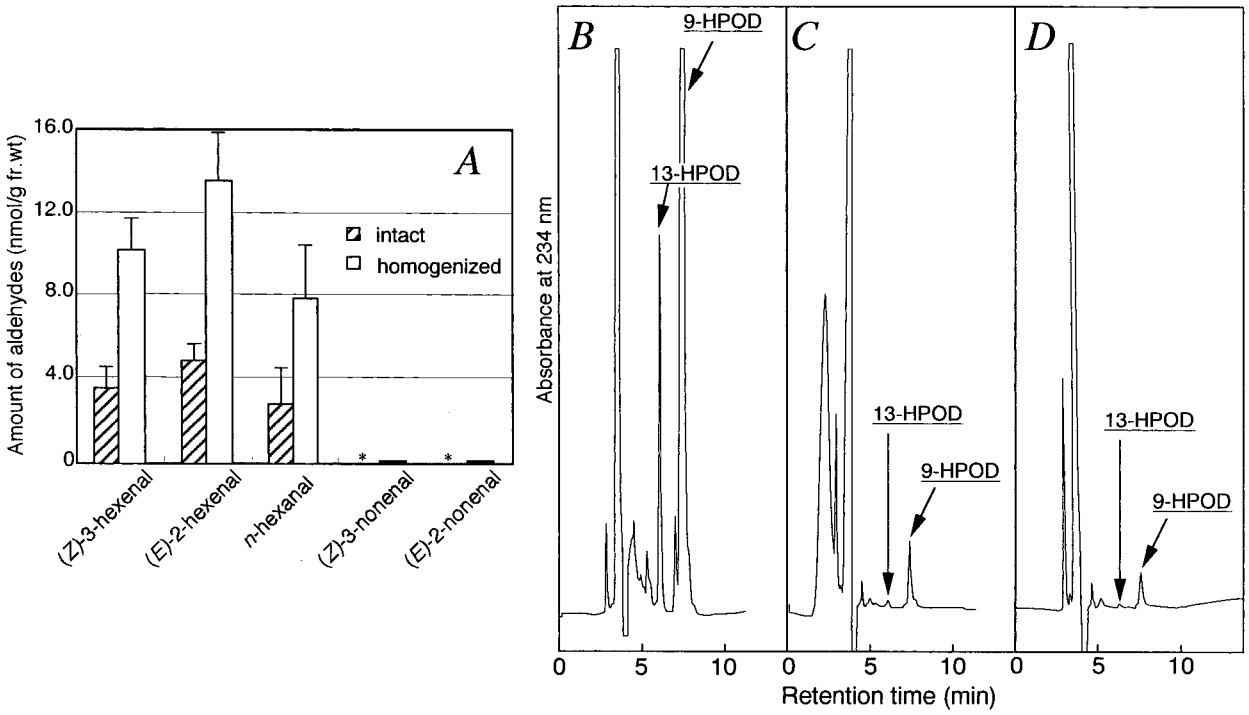

Figure 5. A, Amounts of short-chain aldehydes in intact and homogenized tomato fruits. Nontransgenic tomatoes were used in this study. "*" indicates not detected. Values represent means ± SE of replicates (n = 3−4). B, Chromatograms showing the formation of linoleic acid 9-hydroperoxide (indicated with arrows). Lipid fraction extracted from tomato ftuits was treated with triphenylphosphine and diazomethane, and then separated with SP−HPLC with following absorbance at 234 nm. Trace a shows a chromatogram of the extract prepared from homogenate after addition of linoleic acid. Traces b and c show those of the extracts prepared from homogenized and intact tomato fruits (only part of the chromatograms are shown).

UC82B, or in transgenic plants showing low 9-HPL activities, such as 3356 or 3406. This indicates that 9-LOX in tomato fruits can serve 9-HPOs to 9-HPL. The activity of 9-LOX is high enough to support high activity of 9-HPL.

When fruits of nontransgenic tomatoes were homogenized, the amounts of C6-aldehydes increased (Figure 5A). This indicates that upon tissue disruption, lipids in tomato cells were hydrolyzed to form free fatty acids, and then free linoleic and linolenic acids thus formed were converted to C6-aldehydes through the sequential reaction catalyzed by 13-LOX and 13-HPL in the homogenate. The lipid-hydrolysis step catalyzed by LAH is thought to be a key step accountable for this rapid formation of C6-aldehydes (*19*). Because active 9-LOX is abundant in the tomato homogenate, linoleic and linolenic acids formed by LAH might be converted into the corresponding 9-HPOs, however, the amount of C9-aldehydes scarcely increased upon tissue disruption as shown in Figure 5A. From these it can be expected that accumulation of 9-HPOs in the homogenates occurs. To confirm this, the amounts of 9-HPOs, as well as 13-HPOs, in tomato fruits before and after homogenization were determined by quantitative HPLC analysis (Figure 5B). The amount of 9-HPOs in intact tomato fruits was only 0.77 ± 0.36 nmol/g fr wt (n = 3), and it increased a little to 4.35 ± 1.32 nmol/g fr wt after homogenization. The amounts of 13-HPOs were almost comparable with those of 9-HPOs (0.88 ± 0.41 nmol/g fr wt in intact fruits and 2.18 ± 0.89 nmol/g fr wt after homogenization). Approximately 20 nmol/g fr wt of C6-aldehydes were formed upon homogenization (see Figure 5A), which means an equivalent amount of 13-HPOs was consumed by 13-HPL. Therefore, the total amount of 13-HPOs formed after homogenization should be more than 22 nmol/g fr wt. When linoleic acid was added to the homogenate, high accumulation of 9-HPO (329.3 ± 89.0 nmol/g fr wt) was observed. The amount of 13-HPO increased was 27.6 ± 10.4 nmol/g fr wt and the amount of C6-aldehydes formed after addition of linoleic acid was 132 nmol/g fr wt; thus, the total amount of 13-HPO formed from linoleic acid accounts to approximately 150 nmol/g fr wt. These indicated that free fatty acids formed from endogenous substrates were converted to 13-HPOs by 13-LOX more preferentially than they were converted to the 9-isomers by 9-LOX. However, if free fatty acid was supplied exogenously, 9-LOX could act on it to form a higher amount of 9-HPOs. If substantial activity of 9-HPL would exist as in the transgenic fruits used here, most 9-HPOs can be further converted into C9-aldehydes as shown in Figure 4. Thus, one of the reasons for the low formation of C9-aldehydes in transgenic tomatoes as shown above is thought to be lower capacity to form 9-HPOs from an endogenous substrate.

DISCUSSION

In this study, we aimed to modify the flavor properties of tomato fruits through genetic engineering of HPL which is involved in the formation of short-chain aldehydes and alcohols. By introducing cucumber 9-HPL cDNA, we succeeded in overexpressing 9-HPL in tomato fruits; however, only a little change in the compositions of volatile compounds was observed. This is unexpected because in tomato fruits 9-LOX activity, which can form 9-HPOs of fatty acids, is abundant (*9, 20*). It has been reported that there exist at least four *LOX* genes in tomato, and three of these genes, namely, *tomLoxA, B,* and *C,* have been shown to be expressed during fruit ripening (*7, 8*). From biochemical analyses, it has been noted that 9-LOX is the major LOX in mature tomato fruits (*9, 20*) although no assignment of each gene to

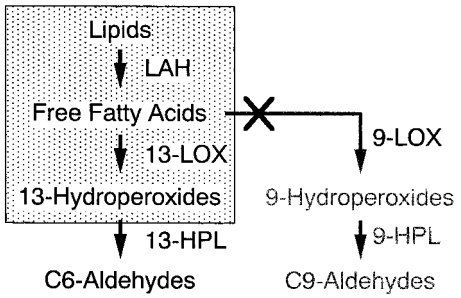

Figure 6. Metabolic flow of the lyase pathway in tomato fruits. Upon disruption of tomato fruit tissues, LAH (or nonspecidic lipase) starts to hydrolyze lipids to form free fatty acids. Free fatty acids thus formed are preferentially oxygenized by 13-LOX to form 13-HPOs, which are further converted to C6-aldehydes by 13-HPL. Although there is active 9-LOX abundant in the tissues, this enzyme cannot act on free fatty acid formed in situ by LAH.

the 9-LOX activity has yet been established. Recently, Griffiths et al. (*12*) showed that antisense-suppression of all three fruit-specific LOXs in tomato fruits to an undetectable level caused no significant changes in the content of flavor volatiles. From this finding, they postulated that either a very low level of LOXs is sufficient for the generation of C6-aldehydes and alcohols, or that a specific isoform is responsible for the production of these compounds. These results indicate that the major forms of LOX in mature tomato fruits may not contribute to the formation of flavor volatiles in the fruits. Our study showed that this is also the case even if 9-HPL is introduced into the enzyme system. Considering all of this evidence raises questions regarding the purpose of the 9-LOX in the fruits and about what reaction it carries out. LOX genes express highly in the outer pericarp of the fruits, and some of them are induced by jasmonates (*21*). Furthermore, our study showed that 9-LOX can accept fatty acids added exogenously, and can accumulate 9-HPOs, which are toxic to most organisms. From these lines of evidence, it is assumed that the major LOX in tomato may be involved in resistance response against biotic challenge through the toxicity of the HPOs.

This study evoked another question. Because C6-aldehydes are formed upon disruption of tomato fruits, liberation of free linoleic and linolenic acids must proceed in the homogenate (*19*). At least under the conditions employed here, free linoleic and linolenic acids thus formed are predominantly consumed by 13-LOX; however, 9-LOX forms a lower amount of 9-HPOs. Because we disrupted the tissue completely under the hypotonic condition, compartmentation cannot be taken into account to explain this preference. This result suggests that free fatty acids formed by the LAH action can escape from 9-LOX action and, thus, there might be a mechanism to supply liberated fatty acids preferentially to 13-LOX (Figure 6). Concentration of free fatty acids formed endogenously is thought to be low, while the initial concentration of linoleic acid added exogenously was as high as 0.4 mM in our experimental condition. *Km* value of the major and soluble LOX in tomato fruits, which is assumed to form 9-HPOs, is reported to be 3.8 μM (*22*). If affinity of 9-LOX to the lower concentration of fatty acid is less than that of 13-LOX, 9-LOX can form only lower amount of 9-HPOs. It is not known which form of LOX is the 13-LOX contributing C6-aldehyde formation, thus, direct comparison of the *Km* value is impossible. Anyway, differences in enzymatic properties of 9-LOX and 13-LOX, such as kinetic parameters and the effects of physicochemical properties of substrates (*23*), are thought to play a crucial role in determining the fate of free fatty acids in tomato fruits. Further study is needed to confirm this possibility. As one of the other possibilities, co-localization of LAH and a LOX involved in C6-aldehyde formation can also be postulated. In human neutrophils, it has been reported that phospholipase A2 and 5-lipoxygenase translocate onto the nuclear membrane after cell stimulation with a calcium ionophore (*24*). If such tight interaction between LAH and the specific LOX would exist in tomato fruits, free fatty acids formed could escape from 9-LOX action.

ABBREVIATIONS USED

HPL, fatty acid hydroperoxide lyase; HPO, fatty acid hydroperoxide; LAH, lipolytic acyl hydrolase; LOX, lipoxygenase.

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