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Identification of an Allele Attributable to Formation of Cucumber-like Flavor in Wild Tomato Species (*Solanum pennellii*) That Was Inactivated during Domestication

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Carbon 6 (C6)-aldehydes formed by fatty acid 13-hydroperoxide lyase (13HPL) specific to fatty acid 13-hydroperoxides (13-HPO) are important flavor constituents in fresh tomato fruits. C9-aldehydes are usually formed by 9/13HPL showing dual specificity to 9- and 13-HPOs and are scarcely found in tomato fruits. Mature red fruits of one of the introgression lines, IL1−4, generated by hybridization of a cultivated tomato (*Solanum lycopersicon*) to its wild relative *Solanum pennellii*, form high amounts of C9-aldehydes upon homogenization. The IL1−4 fruits showed high 9/13HPL activity. One of the genes isolated from IL1−4 showed a high similarity to plant *9/13HPL*s. Recombinant proteins expressed in *Escherichia coli* showed 9/13HPL activity. Cleaved amplified polymorphic sequence analyses indicated that the gene was specific to IL1−4 and *S. pennellii. S. lycopersicon* had a gene having high similarity to the *S. pennellii* gene. It was absent in IL1−4. Among the differences of amino acid residues found between the two genes, a Cys to Ser exchange may be responsible for the inactivation of resultant protein product of *S. lycopersicon* gene because the Cys is an essential amino acid residue for HPL activity. From these observations, it could be assumed that a tomato gene corresponding to *S. pennellii 9/13HPL* gene had been inactivated through domestication of tomatoes.

KEYWORDS: Tomato; *Solanum lycopersicon*; *Solanum pennellii*; fatty acid 9/13-hydroperoxide lyase; flavor; (*E*)-2-nonenal

INTRODUCTION

Tomatoes (*Solanum lycopersicon*; formerly known as *Lycopersicon esculentum*, Mill.) are widely cultivated fruits worldwide. In the year 2005, 91.2 million tons of tomatoes were produced in 138 countries according to FAOSTAT (http://faostat.fao.org/). Fruit flavor in tomatoes is affected mainly by the amounts of sugars and acids and their ratio, and aroma, and there is a particular interest in understanding and improving the composition of the latter. Among more than 400 volatile compounds identified so far (*1*), those derived from fatty acid through the oxylipin pathway are known to be the most important constituents (*2*). These are carbon six (C6) compounds, such as *n*-hexanal, (*E*)-2-hexenal, (*Z*)-3-hexenal, *n*-hexan-1-ol, or (*Z*)-3-hexen-1-ol. Linoleic or linolenic acid is oxidized by lipoxygenase (LOX) to form 13-hydroperoxide (HPO) derivatives, which in turn are cleaved by the fatty acid 13-hydroperoxide lyase (13HPL)—a unique cytochrome P450 enzyme—to form a C6-aldehyde and a C12-oxo acid (12-oxo-(*Z*)-9-dodecenoic acid) (**Figure 1**) (*3*). The aldehydes formed can be reduced to the corresponding alcohols. If isomerase or isomerization factor would act on (*Z*)-3-hexenal, then (*E*)-2-hexenal would be formed.

In some plants, such as cucumbers or melons, C9-compounds are important flavor compounds. C9-compounds are formed in the oxylipin pathway using different substrate/product specificities of LOX and HPL than in the C6-compound-forming pathway (**Figure 1**). In the former case, 9LOX introduces the HPO group to the 9-position of linoleic or linolenic acid, and then C9-aldehyde and a C9-oxo acid (9-oxononanoic acid) are formed by the action of a HPL that can act on 9-HPO of fatty acids. HPL that is involved in C9-compound formation can usually act on either 9- or 13-HPO with a preference to the former; therefore, this type of HPL is named 9/13HPL (*4*). In general, except cucumber, melon, and some other fruits (*5*, *6*), C9-compounds are regarded as off-flavor in food materials (*7*, *8*).

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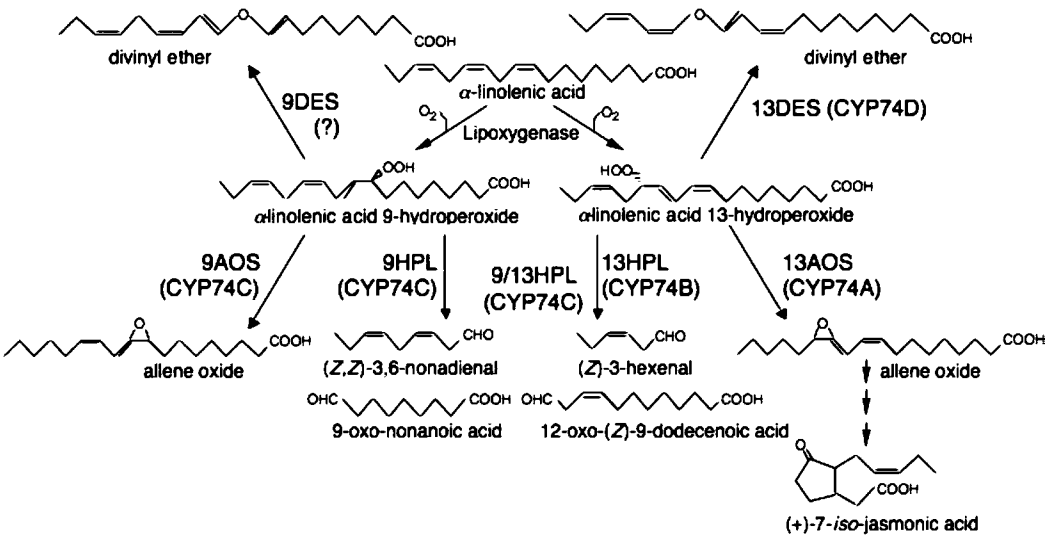


Figure 1. Part of oxylipin pathway in plants. Polyunsaturated C18 fatty acids are oxygenated to form corresponding 9- or 13-hydroperoxides (HPOs). They are further converted to various oxylipins by cytochrome P450 enzymes. HPL, fatty acid hydroperoxide lyase; AOS, allene oxide synthase; DES, divinylether synthase. With 9/13HPL both 9- and 13-HPOs can be its substrate. This pathway can start from linoleic acid, and in this case, the final products lack one double bond.

Other than the C6- and C9-branches of the oxylipin pathway, another branch to form allene oxide and divinylether have been characterized in plants (*9*, *10*). Unusual cytochrome P450 enzymes, such as allene oxide synthase (AOS) and divinylether synthase (DES), catalyze the formation of these derivatives from fatty acid HPOs. These two enzymes and HPL do not require any reducing equivalents and act on HPO instead of molecular oxygen. They share distinct homology with each other, and then, they grouped into a family of CYP74. Based on their catalytic properties and sequences, fatty acid 13-HPO-specific AOS (13AOS), 13HPL, 9/13HPL, and DES were categorized as CYP74A, CYP74B, CYP74C, and CYP74D, respectively (*9*, *10*). In *Arabidopsis* a single gene for CYP74A or CYP74B has been identified, as compared with several genes for CYP74A, CYP74B, or CYP74D in tomatoes. Recently, a novel CYP74 in tomatoes, which could be categorized into CYP74C but has fatty acid 9-HPO-specific AOS activity instead of 9/13HPL activity, was reported (*11*). There has been no report on cloning of *9/13HPL* in tomatoes, and consequently, little formation of C9-compounds in tomato fruits has been reported.

In general, only a very small fraction of genetic variation that is available in related wild species is found in cultivated crops (*12*). This is also the case with cultivated tomatoes. Its wild relatives, such as *Solanum pennellii* or *Solanum hirsutum*, differ significantly in their phenotypic and agronomic traits. A series of introgression lines (ILs), each containing a single defined chromosome segment from a wild species in the genetic background of the cultivated tomato, has been developed. Through comprehensive analyses of phenotypes of the lines, various agronomically important loci have been identified (*13*, *14*). As flavor of tomato fruits is one of most important traits, these ILs were also used to map loci that influence the flavor properties. For example, the *malodorous* locus, which is involved in intensive formation of the off-flavor compound phenylacetaldehyde, has been identified, and a gene corresponding to the locus has been clarified (*15*, *16*). It was assumed that the locus had been modified during domestication of tomatoes. Recently, we found that fruits of one of the *S. pennellii* ILs of *S. lycopersicon* produce large amounts of C9-volatiles, suggesting that the IL and the corresponding wild species carry a gene accountable to the expression of 9/13HPL activity. The present study resulted in identification of a gene encoding for 9/13HPL that appeared to be inactivated during domestication of the tomato.

MATERIALS AND METHODS

Plant Material. The *S. pennellii* introgression line IL1−4 was previously described for their marker data (*13*). IL1−4, together with cultivated tomato (*S. lycopersicon*. cv M82), and wild species (*S. pennellii*) were obtained from Tomato Genetics Resource Center (http://tgrc.ucdavis.edu/). They were grown on the experimental field in Yamaguchi University. Red-ripe fruits were harvested in August and stored at −80 °C until use.

Volatile Analyses. Tomato fruits (20 g) were homogenized by a Polytron homogenizer (PT10-35, Kinematica, Littau, Switzerland) on ice and were incubated at 25 °C for 20 min in a tightly closed vessel. Subsequently, 20 mL of saturated $CaCl_2$ solution was added to the homogenate and mixed vigorously, and 4.08 pmol of *n*-undecane was added as an internal standard. The resulting homogenate (10 mL) was put into a glass tube closed tightly with a cap equipped with a Teflon liner, and then solid-phase micro extraction fiber (SPME Fiber Assembly 50/30 μm DVB/Carboxen/PDMS StableFlex, Supelco, Bellefonte, PA) was exposed to the headspace of the tube for 30 min with heating at 80 °C. The volatiles on the fiber were desorbed at 240 °C for 1 min in the injection port of QP-5050A GC-MS system (Shimadzu, Kyoto, Japan) with a DB-WAX column (J&W Scientific, Folsom, CA, 60 m × 0.25 mm i.d., 0.25 μm film thickness). The injection port was operated in split less mode with a constant He flow of 2.0 mL min^{-1}. The initial oven temperature was 40 °C, held for 5 min, ramped at 5 °C min^{-1} to 200 °C, and held for 5 min. MS was operated in the electron ionization mode at 70 eV and a source temperature of 230 °C with a continuous scan from *m*/*z* 40 to 350. Each volatile was identified by comparing the retention time and MS profile of an authentic specimen.

Enzyme Assay. IL1−4 fruits were homogenized in a chilled mortar with an equal volume of 100 mM MOPS−KOH (pH 7.6), supplemented with 7% sucrose and 10 mM EGTA, and the resultant homogenate was centrifuged at 2500 rpm (RPR 20-2 rotor, Hitachi, Tokyo, Japan) for 3 min to remove cell debris. The resultant supernatant was used as crude enzyme solution. 9 and 13HPL activities were determined with 200 nM each of 9-HPOD and 13-HPOD, respectively, in 100 mM MES-KOH (pH 6.0). The HPO substrates were prepared as earlier described (*17*). The reaction proceeded for 10 min at 25 °C, and 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, Japan) using a solvent system of acetonitrile/tetrahydrofuran/water (74:1:25, v/v/v) at a flow rate of 1 mL/min. Detection was performed at 350 nm.

HPO decomposing activity was determined spectrophotometrically by following the decrease in absorption at 234 nm with 16.5 μM 9-HPOD or 13.3 μM 13-HPOD substrate in 100 mM sodium phosphate buffer, pH 7.5 at 25 °C. The initial velocity of the decrease was followed for 1 min to estimate the activity. An absorption coefficient of 25 mM^{-1} cm^{-1} was used.

RT-PCR Cloning. Total RNA from IL1−4 fruits was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA was treated with DNase I (Ambion, Austin, CA) to remove remaining genomic DNA. Complementary DNA (cDNA) was synthesized in a 10 μL reaction with 3.6 μg of the DNA-free RNA with the Thermoscript RT-PCR System using oligo $(dT)_{20}$ as a primer (Invitrogen). Degenerate primers were designed based on the highly conserved regions among 9/13HPLs reported so far (5′-AAYATGCCNCCNGGNCCNTT-3′, 5′-AAYATGCCNCCNGGNC-CNTTYAT-3′, and 5′-YTNGAYGGNACNTWYATGCC-3′ as sense primers, and 5′- TYYTTNCCNGGRCAYTGYTTRTT-3′ as an antisense primer). Sequences used were those from *Medicago truncatula* (CAC86899 and CAC86898), *Prunus dulcis* (CAE18065), *Cucumis melo* (AAK54282), *Cucumis sativas* (AAF64041), and *Oryza sativa* (Q6Z6K9 and Q6Z6L1). PCR was performed with IL1−4 fruit cDNA as a template using any possible combination of the primers. The amplified DNAs were cloned into pGEM-T (Easy) vector (Promega, Madison, WI) and sequenced. The cDNA showing high homology to *9/13HPL* (named *Sp9/13HPL*) was chosen for further analyses.

Thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) (*18*) was performed to obtain additional sequences at the 5′- and 3′-ends of the *Sp9/13HPL* gene. IL1−4 genomic DNA was used as a template. As short arbitrary degenerate primer, 5′-NGTCGASWGA-NAWGAA-3′ was used. To obtain 5′-end of the gene, gene-specific primers, 5′-CACAAGGACGATATCCACCAAAG-3′, 5′-TCCAC-CAAAGAAATCAGTTGATGGC-3′, and 5′-TTTGGAATCAAGAA-GAGCGATGACG-3′ were used subsequently. For 3′-end, 5′-TCCAT-CACCGATCAAGTGGGTCG-3′, 5′-GGTGGGTCCATCATCACTCTA-TCAGC-3′, and 5′-TTCCGTTCCAGTACGGTAAGGCC-3′ were used.

Expression of Recombinant *SpHPL*. PCR was performed on *Sp9/13HPL* cDNA with forward primer (5′-GGATCCATGTCTTCATTTT-GCTCCAAATC-3′) and reverse primer (5′- CTGCAGTCATGTCGT-TTTTTTGTCCAGAG-3′) in order to introduce *BamH*I and *Pst*I restriction sites, respectively (underlined). The PCR product was cloned into the pQE30 expression vector (Qiagen, Valencia, CA), and then *Escherichia coli* host strain M15 [pREP4] was transformed with it. An overnight culture (15 mL) of the transformant was inoculated into 300 mL of fresh TB medium supplemented with 100 μg/mL ampicillin and 25 μg/mL kanamycin. Bacteria were grown at 37 °C to reach OD_{600} of 0.6. The cultures were chilled to 16 °C, and isopropyl-thio-β-D-galactopyranoside was added to a final concentration of 0.1 mM. The induced cultures were further grown for 24 h at 16 °C with gentle shaking (120 rpm). The cells were collected by centrifugation and disrupted with a French press (Aminco, Rochester, NY) in 50 mM Tris-HCl (pH 8.0) buffer containing 1 mM EDTA, 100 mM NaCl, 0.4 mM PMSF, and 0.27 mg/mL lysozyme. The resultant lysate was used for the enzyme assay as shown above.

Cleaved Amplified Polymorphic Sequences (CAPS) Analysis. Based on the sequences of *Sp9/13HPL* and an EST clone found in fruit pericarp of *S. lycopersicon*, *cLEG16G15* (GenBank acc. no. BE434321), a forward primer (5′-GCTTATGGTGGGATGAAAGTT-3′) and a reverse primer (5′-CTCTGCAATAAACTCTTCTGG-3′) were designed. These two primers fit completely to the two sequences; however, only the product with *cLEG16G15* has a *Hinc*II-restriction site. PCR was performed with genomic DNA isolated from *S. pennellii*, *S. lycopersicon* (M82), and IL1−4 for 2 min at 94 °C, followed by 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 45 s for 30 cycles, followed by a final 72 °C extension for 10 min. Following amplification, a portion of the DNA was digested with *Hinc*II. The product was analyzed by electrophoresis on 8% polyacrylamide gel.

Expression Analyses. Total RNA was extracted from leaves, roots, and stem of 2 month old tomato plants and from available mature red fruit pericarp (100 mg FW) and converted to cDNA. Fruits of *S. pennellii* were not available as no setting of the wild tomato occurred under the growing conditions employed here. RT-PCR was performed with the primers of 5′-AGTGTTAAGAATTGAACCACCAATT-3′ (forward) and 5′-TCTTGAACGTTACTGATGATCCAGC-3′ (reverse) for *Sp9/13HPL*, 5′-AGTATTGAGAATTGAACCTCCATTG-3′ (forward) and 5′-TCTTGAACGTTACTGATGATCCAAA-3′ (reverse) for *cLEG16G15*, 5′-TATGAGCTCCCAGATGGGCAGGTGA-3′ (forward) and 5′-TCATGCTGCTAGGAGCCAATGCAGT-3′ (reverse) for *S. lycopersicon actin*, and 5′-CGTGGTTACTCATTTACCACTACAG-3′ (forward) and 5′-AAGTACGATGTTTCCATAGAGATCC-3′ (reverse) for *S. pennellii actin*. RT-PCR was performed by using Takara ExTaq polymerase (Takara Biomedicals, Otsu, Japan) on a Perkin-Elmer 9700 (Perkin-Elmer Applied Biosystems, Foster City, CA): 94 °C for 2 min followed by 94 °C for 45 s, 57 °C for 45 s, and 72 °C for 45 s for 20−27 cycles, followed by a final 72 °C extension for 8 min (*19*).

Figure 2. Fully matured red fruits of *S. lycopersicon* (cv. M82) and IL1−4. The fruits were collected at the same time from the same age (3 months) of plants. A bar = 1 cm.

RESULTS

IL1−4 Had High 9HPL Activities. It has been shown that IL1−4 fruits grown in Rehovot, Israel, can form C9-volatiles in addition to C6-volatiles (Ben-Oliel et al., in preparation). In Yamaguchi, Japan, IL1−4 and M82 grew similarly from spring to summer and set fruit at almost the same time, and their fruits ripened concomitantly (**Figure 2**). SPME-GC-MS analyses on the volatiles formed in the homogenate of mature red fruits of IL1−4 grown in Japan confirmed that (*E*)-2-nonenal and (*E,Z*)-2,6-nonadienal, which could be hardly detected in M82 fruits, could be detected as major volatile constituents besides the volatiles usually found in commonly cultivated tomatoes, such as (*E*)-2-hexenal, (*E*)-2-heptenal, 6-methyl-5-heptene-2-one, (*Z*)-3-hexen-1-ol, 2-isobutylthiazole, and methyl salicylate (*2*). We therefore conclude that the ability of IL1−4 fruits to form C9-volatiles is strictly dependent on genetic factors and less influenced by growth conditions.

It has been reported that 13HPL activity was high, while little formation of C9-aldehydes from 9-HPOs could be detected in cultivated tomato fruits (cv UC82B) (*17*). When 13-HPOD was used as a substrate with crude enzyme solution prepared from IL1−4 fruits, significant 13HPL activity to form *n*-hexanal could be detected (**Figure 3**). When 9-HPOD was used instead, 9HPL activity to form (Z)-3-nonenal was 2 times higher than 13HPL activity. This activity was lost after heat denaturation at 100 °C for 10 min, which suggested that the activity was attributable to an enzyme.

Molecular Cloning of 9/13HPL. The above results suggest that IL1−4 possessed a HPL gene responsible for the formation of C9-aldehydes from 9-HPOs of fatty acids. Based on highly conserved regions of amino acid sequences of 9/13HPLs reported so far, degenerate primers were designed, and then RT-PCR cloning was performed with RNA isolated from red ripe fruits of IL1−4. Two clones could be obtained, and partial sequencing revealed that one had a known sequence identical to *LeAOS2* (*LeCYP74A*, AAF67141) (*20*), and the other had a

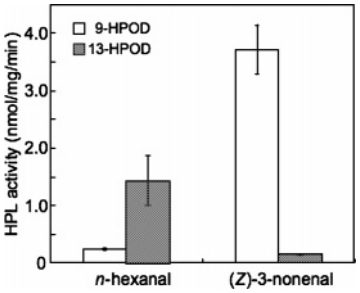


Figure 3. 9- and 13HPL activities in crude homogenate prepared from mature red fruits of IL1–4. The amounts of *n*-hexanal and (*Z*)-3-nonenal formed from 9- (white bar) or 13-HPOD (gray bar) are shown. Values are the mean and SD of three replicates for each fruit.

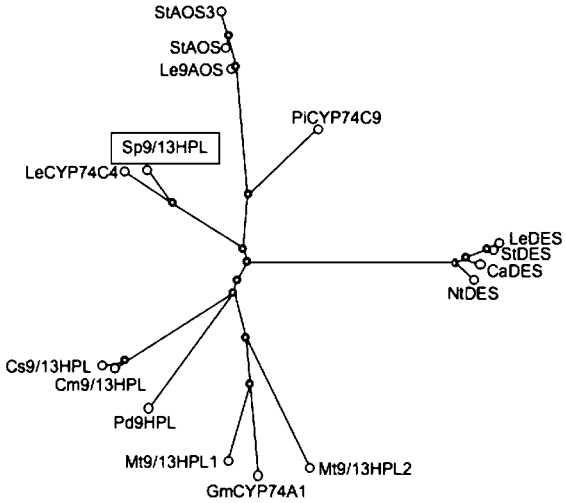
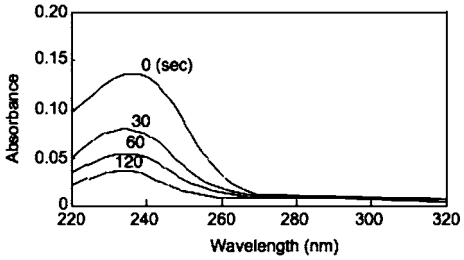


Figure 4. A phylogenetic tree made with amino acid sequences of various CYP74s. Sequences used are Mt9/13HPL1 and 2 from *Medicago truncatula* (CAC86899 and CAC86898), Pd9HPL from *Prunus dulcis* (CAE18065), Cm9/13HPL from *Cucumis melo* (AAK54282), Cs9/13HPL from *Cucumis sativas* (AAF64041), GmCYP74A1 from *Glycin max* (ABC68416), NtDES from *Nicotiana tabacum* (AAL40900), CaDES from *Capsicum annuum* (ABH03632), StDES from *Solanum tuberosum* (CAC28152), LeDES from *Solanum lycopersicon* (AAG42261), PiCYP74C9 from *Petunia inflata* (ABC75839), StAOS3 from *Solanum tuberosum* (CAI30876), StAOS from *Solanum tuberosum* (AAU21293), Le9AOS from *Solanum lycopersicon* (AAN76867), LeCYP74C4 from *Solanum lycopersicon* (AAL86702), and Sp9/13HPL from *Solanum pennellii* (ABI93819). With the sequences whose enzymatic activities are known, the corresponding enzyme names are given, while those without such confirmation, CYP names are given.

novel sequence having high sequence similarity with *LeAOS4* (*LeCYP74C4*, AAL86702) and StAOS3 (CAI30876) (*11*, *21*). A full-length sequence of the latter gene was obtained with TAIL PCR (GenBank acc. no. DQ914831). It consists of an ORF of 1464 bp. The gene encodes a protein of 488 amino acids with Mr of 55.5 kDa. The deduced amino acid sequence showed highest homology with LeAOS4 (84% identity) among CYP74s reported so far. PSORT analysis (http://wolfpsort.seq.cbrc.jp/) showed that the deduced protein located in the cytoplasm.

BLAST search and subsequent Tree view analysis with an algorithm of Fast Minimum Evolution (http://www.ncbi.nlm.nih.gov/BLAST/) showed that the novel sequence could be categorized into the subfamily consisting of 9/13HPL (CYP74C), DES (CYP74D), and 9AOS (CYP74C) (**Figure 4**). 13HPL (CYP74B) and 13AOS (CYP74A) were allocated into a distinct subfamily within the phylogenetic tree made based on the



	HPL activity (nKat/mg)	
	9-HPOD	13-HPOD
Sp9/13HPL	9.66 ± 0.57	3.46 ± 0.21
Cs9/13HPL	13.3 ± 0.41	4.23 ± 0.53

Figure 5. Enzymatic activity of recombinant Sp9/13HPL. Recombinant Sp9/13HPL was reacted with 9-HPOD and the changes in the spectrum of the substrate were monitored (upper figure). Time (s) after addition of the recombinant Sp9/13HPL is shown. The HPL activity was determined with 9- or 13-HPOD as substrate (lower table). As a positive control, the activities of recombinant cucumber 9/13HPL (Cs9/13HPL) (*4*) are also shown. Values are the mean and SD of three replicates.

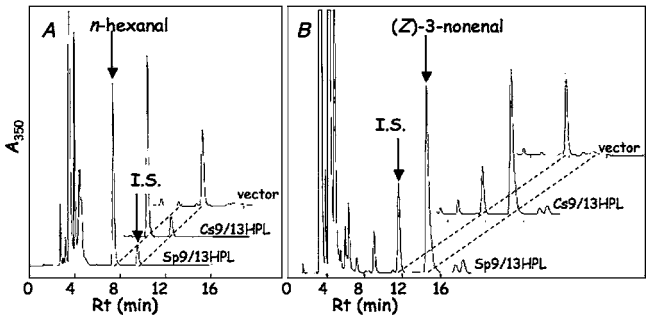


Figure 6. HPLC analyses of products formed from 13-HPOD (**A**) and 9-HPOD (**B**) with recombinant Sp9/13HPL. The results with recombinant Cs9/13HPL (*4*) as a positive control and vector control are also shown.

algorithm, respectively. In that tree, the novel sequence identified here was allocated distantly from CYP74Ds (DES), from which it could be concluded that it belonged to the CYP74C subfamily. It was suggested that 9/13HPLs and 9AOSs could be further divided into distinct subfamilies within CYP74Cs. The newly identified sequence from IL1−4 was allocated between them and located close to LeCYP74C4, whose enzymatic properties has not been indentified yet (*10*).

When the gene was expressed in *E. coli*, the resultant recombinant enzyme showed activity to decompose 9-HPOD (**Figure 5**). During the decomposition of the chromophore derived from 1-hydroperoxy-2,4-diene moiety of 9-HPOD, no increase in the other chromophore was observed, such as a chromophore peaking at 252 nm derived from divinylether (*19*). The recombinant enzyme could decompose both 9- and 13-HPOD; however, it preferred the former. The substrate preference was similar to that found with recombinant cucumber 9/13HPL (Cs9/13HPL) (**Figure 5**) (*4*). From 9-HPOD, (*Z*)-3-nonenal was formed predominantly, while from 13-HPOD, *n*-hexanal was formed as recombinant Cs9/13HPL did (**Figure 6**). Taken together, it could be concluded that the gene harbored in IL1−4 encoded 9/13HPL (named *Sp9/13HPL* thereafter).

***S. lycopersicon* Has an Inactive Counterpart of *Sp9/13HPL* on Chromosome 1.** A database search for *Sp9/13HPL* with an EST database in the SOL Genomics Network (http://www.sgn.cornell.edu/) showed that one of the genes expressed in fruit pericarp of *S. lycopersicon*, SGN-U338232 (isolated as an EST clone, *cLEG16G15*), had the highest similarity to *Sp9/13HPL*.

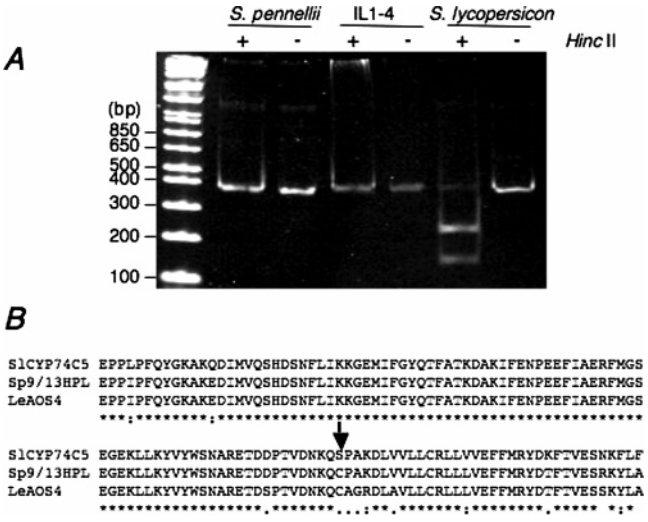


Figure 7. CAPS analyses of *Sp9/13HPL* gene and *SlCYP74C5* gene in *S. pennellii*, IL1–4, and *S. lycopersicon*. The PCR products were divided into two parts, and one of them was digested with *Hinc*II. They were separated with 8% acrylamide gel and stained with ethidium bromide (**A**). A part of deduced amino acid sequence alignment with SlCYP74C5, Sp9/13HPL, and LeAOS4 are shown in (**B**). (*) The amino acid conserved with all three proteins; (:) the amino acid similar to the three proteins. The Cys/Ser residue, which is thought to be a ligand for heme, is shown with an arrow.

DNA sequencing of the insert of the EST clone (GenBank acc no., DQ914832) showed that it had 92.3% identity at a nucleotide level and 92.1% identity at an amino acid level with *Sp9/13HPL* (named *SlCYP74C5* thereafter, **Figure 7B**). We designed a CAPS marker based on those sequences. Accordingly, *Sp9/13HPL* could be detected only with *S. pennellii* and IL1−4, while with *S. lycopersicon* only *SlCYP74C5* could be detected instead of *Sp9/13HPL* (**Figure 7A**). From this, it could be concluded that the chromosomal region where *SlCYP74C5* is located in *S. lycopersicon* was replaced with the chromosomal region harboring *Sp9/13HPL* originated from *S. pennellii* in IL1−4. Interestingly, the Cys residue that was shown to be essential to HPL activity (*23*) was exchanged to Ser residue within the amino acid sequence deduced from *SlCYP74C5* sequence. Thus, it could be assumed that *SlCYP74C5* in *S. lycoperosicon* might have no HPL activity even if it would be appropriately expressed.

Expression of Sp9/13HPL. RT-PCR analyses of leaves, roots, or stems from 2 month old tomato plants and of fruits (when available) showed that the expression of *Sp9/13HPL* gene could be found only in fruits of IL1−4 (**Figure 8**). Expression of *SlCYP74C5* was undetectable under the experimental condition employed here. When primers for *Sp9/13HPL* were used for *S. lycopersicon*, or vice versa, no signal could be detected with RT-PCR.

DISCUSSION

Identification of a Wild Species' Allele Affecting Tomato Flavor as *9/13HPL*. We found that one of the ILs developed from a cross between the wild green-fruited species *S. pennellii* and the cultivated tomato *S. lycopersicon* can form significant amounts of C9-aldehydes in homogenates of ripened fruits (Ben-Oliel et al., in preparation). Several lines of evidence shown here indicated that the wild species, and the relevant introgression line, IL1−4, had an active *9/13HPL* gene; however, the gene might be inactivated during the tomato domestication processes.

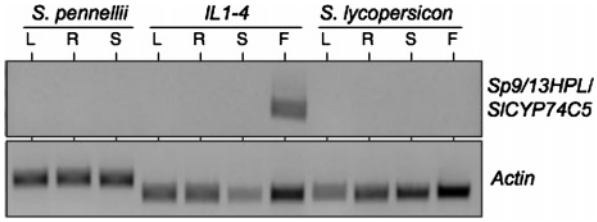


Figure 8. RT-PCR analyses of *Sp9/13HPL* and *SlCYP74C5* with leaves (L), roots (R), stems (S), or mature red fruits (F) of *S. pennellii*, IL1–4, or *S. lycopersicon* M82. For *S. pennellii* and IL1–4, primers for *Sp9/13HPL* were used and for *S. lycopersicon* M82, those for *SlCYP74C5* were used. Expression of *actin* gene of *S. pennellii* and *S. lycopersicon* are shown to verify equal loading of the template.

C9-aldehydes and alcohols are widespread in the *Cucurbitaceae* family and have distinct cucumber- or melon-like flavor properties. 9HPL activity to form C9-aldehydes from 9-HPOs was originally reported in cucumber (*24*). Since then, it had been believed that 9/13HPL was restricted to plants belonging to the *Cucurbitaceae*. Here we report that wild species of the *Solanaceae*, *S. pennellii*, possess an active 9/13HPL. This might not only be the case with *S. pennellii* since a specific line (LA3995) of the *L. hirsutum*-derived introgression lines is also capable of forming C9-volatiles [Tomato Metabolite Database (http://tomet.bti.cornell.edu/)]. LA3995 includes an introgression section in chromosome 1 close to the location of the introgression of *S. pennellii* in IL1−4. Therefore, it could be assumed that the two *Solanum* species possess an active *9/13HPL* gene at the same allocation on the bottom of choromosome 1. Recently, it has been found that *Fabaceae* (alfalfa), *Gramineae* (rice), and *Rosaceae* (almond) also have genes encoding *9/13HPL* or *9HPL* (in the case of almond) (*23*, *24*, *26*). Even a non-flowering plant, the moss *Physcomitrella patens*, has been shown to have *9/13HPL* gene (*27*). Taken together, it can be concluded that *9/13HPL* gene is not restricted to *Cucurbitaceae*, but rather widespread in the plant kingdom even though there is an exception such as *Arabidopsis*, where only one *13HPL* and one *13AOS* could be found in its genome. Functional divergence of *9/13HPL* gene from the other CYP74 genes might have occurred prior to the establishment of plant families.

It has been reported that C9-aldehydes have toxicity against various microbes including phytopathogenic ones (*28*). (*E*)-2-Nonenal can be an insect repellent and insecticide as well (*29*). Some plants form a potent genotoxic compound, 4-hydroxy-(*E*)-2-nonenal, from the primary product of 9HPL ((*Z*)-3-nonenal) (*30*, *31*). Thus, 9HPL might be involved in the interaction between the wild tomato species and pathogens or herbivores. Several reports showed that C6-volatiles could exert a direct deterring function toward pests and/or pathogens (*3*). Since C9-aldehydes are less volatile than C6-aldehydes, they might have different spectra of toxicity against microbes and insects, which might be the reason for the specific significance of C9-aldehydes (*32*). Thus, it could be beneficial for plants to have 9/13HPL activity in addition to 13HPL activity. It has also been reported that C9-aldehydes and alcohols are pheromones, attractants, or kairomones for various insects (*33*−*35*), which would also emphasize the specific function of C9-volatiles in a specified ecosystem that cannot be attributable to C6-counterparts.

Our study shows that *9/13HPL* originally existed in wild tomato species and has probably been inactivated during domestication. Among several amino acid exchanges found in the amino acid sequence deduced from *SlCYP74C5*, Cys to Ser exchange (corresponding to Cys441 in Sp9/13HPL) must be

the most crucial for the inactivation since the Cys residue is known to be a ligand for heme that is essential for the activity of HPLs (*23*). Because C9-volatiles are largely off-flavor for tomatoes, breeders invested efforts in silencing 9HPL activity, which in turn resulted in inactivation of the corresponding gene during domestication. The dominant nature of this trait (Ben-Oliel et al., in preparation) further facilitated the elimination of this trait.

It has been assumed that the *malodorous* allele, all which is located on the short arm of chromosome 8, was also selected against during domestication (*15*). Accordingly, some traits relating to defense against disease and herbivores should be left over; however, this unfavorable agronomic trait appeared to be overcome by improved management of cultivation or became insignificant through changing the cultivated region.

Altered Metabolic Flow in the Fruits of IL1−4. Fruits of cultivated tomatoes have high activity of LOX that specifically forms 9-HPOs from fatty acids. Nonetheless, there are apparently little or no 9HPL or other enzyme activities to act on 9-HPOs (*36*). In this context, it might be reasonable to expect formation of significant amounts of C9-aldehydes after introduction of active *9/13HPL* into the cultivated tomatoes. However, when we introduced cucumber *9/13HPL* into cultivated tomatoes by transformation, only a little increase in the amount of C9-aldehydes was evident despite the high 9HPL activity in the transgenic tomato fruits (*17*). On the other hand, when *Sp9/13HPL* was introduced into the cultivated tomatoes, significant formation of C9-aldehydes could be observed as shown in this study. Two possible explanations for this apparent discrepancy might be the following: (1) Among five LOX isozymes, TomloxA to E, found in tomato fruits, only one, TomloxC, was shown to be involved in the generation of C6-volatiles (*37*). TomloxC shows high homology with potato 13-LOX H1, which was also ascribed as an enzyme supplying the substrates for HPL (*38*). From these observations, it is now believed that there is a LOX specific to the HPL branch of the oxylipin pathway even though the other LOXs can form the same products. This specificity cannot be explained only by compartmentalization because especially in tomatoes this specificity could be observed even after maceration of the tissues. Although a molecular basis for the specificity between LOX and HPL has not been clarified, Cs9/13HPL might not be compatible with tomato LOX while Sp9/13HPL might be.

(2) Another explanation is the possible incorporation of a gene encoding a "specific" LOX from *S. pennellii* with *Sp9/13HPL* in IL1−4. If this is the case, the counterpart gene in *S. lycopersicon* should also be located at the same region of chromosome 1, but should be inactive. However, it is doubtful whether simultaneous inactivation of two different genes can happen. To address these two possibilities, further study is now underway in our laboratory.

Here we could provide a possible evolutionary explanation as to why the trait was left out during tomato domestication. Also, it was shown that the use of introgression lines, especially, IL1−4, would provide further insights into the metabolic flow of C6- and C9-volatiles that are detrimental to flavor quality of tomato fruits.

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