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Watermelon (*Citrullus lanatus*) Hydroperoxide Lyase Greatly Increases C_6 Aldehyde Formation in Transgenic Leaves

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Fatty acid hydroperoxide lyase (HL) is the key enzyme for the production of the "green note" compounds, leaf aldehyde [(2*E*)-hexenal] and leaf alcohol [(3*Z*)-hexenol], in plant tissues. A cDNA encoding HL was cloned from leaves of watermelon (*Citrullus lanatus*) and expressed in *Nicotiana tabacum*. The enzyme is 3 times more active with 13-hydroperoxylinolenic acid than with 13-hydroperoxylinoleic acid. The activity against 9-hydroperoxides of polyunsaturated fatty acids is minimal. Enzyme activity of the watermelon HL in the transgenic leaves was ~50 times higher than endogenous HL activity in the wild-type *N. tabacum* plants. When compared with *Arabidopsis* HL also expressed in *N. tabacum*, the highest HL activity is 10 times higher in watermelon HL overexpressing leaves than in *Arabidopsis* HL overexpressers.

KEYWORDS: Hydroperoxide lyase; (2*E*)-hexenal; (3*Z*)-hexenal; *Citrullus lanatus*; *Nicotiana tabacum*; oxylipins

INTRODUCTION

When leaves of nearly any plant species are crushed, a large amount of six-carbon (C_6) aldehydes and alcohols is generated, which are characteristics of the "green notes" of leaves. These C_6 volatile compounds such as leaf aldehyde [(2*E*)-hexenal] and leaf alcohol [(3*Z*)-hexenol] are also important components of the aroma and flavor of fruits and vegetables and are widely used as flavors in foods and beverages. The enzyme responsible for the production of these compounds is a fatty acid hydroperoxide lyase (HL), which forms very unstable hemiacetals from hydroperoxides of polyunsaturated fatty acids, generating aldehydes and aldehyde enols by spontaneous dissociation (*1*). This reaction mechanism was proposed (*2*, *3*) and recently elucidated (*1*). Some of the C_6 aldehydes and alcohols have been known to have antimicrobial and antifungal properties (*4*−*8*) as well as negative effects on arthropods directly and indirectly (*9*−*11*). Recently, these volatiles were also reported to induce defense-related genes in plants (*12*, *13*) as well as the emission of other volatiles (*14*, *15*).

There are possibly three types of HLs depending on the substrate specificity. 13-HLs, such as *Arabidopsis* HL and watermelon HL, have a strong preference for 13(*S*)-hydroperoxides over 9(*S*)-hydroperoxides, whereas 9-HLs such as pear HL (*16*) have the opposite preference. 9/13-HLs can act on both 9- and 13-hydroperoxides somewhat equally. So far, no 9-HL has been cloned. HLs belong to a novel family of cytochrome P450s, CYP74, which catalyze the conversion of hydroperoxides of polyunsaturated fatty acids without molecular oxygen or cofactors (*17*−*19*). The CYP74 family is divided into four subfamilies based on sequence identity: CYP74A [allene oxide synthases (AOSs)], CYP74B (13-HLs), CYP74C (9/13-HLs), and CYP74D [divinyl ether synthases (DES)], all of which synthesize oxylipins, bioactive oxygenated fatty acid derivatives such as jasmonic acid (*20*−*22*).

A gene encoding HL was first cloned and characterized from green pepper (*17*), followed by *Arabidopsis* (*23*, *24*), alfalfa (*25*), tomato (*26*, *27*), guava (*28*), cucumber (*29*), melon (*30*), and potato (*11*). However, until now, this gene has not been isolated from watermelon (*Citrullus lanatus*), the leaves of which are reported to be one of the highest C_6 aldehyde producers among plant tissues (*31*) and also used as the source of industrial production of leaf aldehyde and alcohol (*32*). Here, we report the cloning of the HL gene from watermelon leaves and confirm its high activity.

MATERIALS AND METHODS

Chemicals. All of the chemicals, unless otherwise noted, were obtained from Sigma (St. Louis, MO). 13(*S*)- or 9(*S*)-hydroperoxides of linolenic acid or linoleic acid were prepared using soybean LOX (*33*) or tomato LOX (*34*).

Plant Material. Watermelon (*C. lanatus*) cv. Sugar Baby was grown in soil in a greenhouse maintained with a 16 h light/8 h dark photoperiod, a 25 °C day/21 °C night temperature setting, and a light intensity >800 μEinstein m^{-2} s^{-1} with supplemental high-pressure sodium bulbs. *Nicotiana tabacum* cv. Petite Havana and KY14 for transformation were grown aseptically in a growth room maintained at 25 ± 3 °C with a 23 h light/1 h dark photoperiod and a light intensity of 60 μEinstein m^{-2} s^{-1}.

cDNA Cloning of Watermelon HL. Total RNA was isolated from young but fully expanded leaves of watermelon plants using the TRIzol reagent (Life Technologies, Gaithersburg, MD). Messenger RNA was partially purified using oligo(dT)-cellulose. Several pairs of degenerate primers were designed on the basis of conserved regions of published sequences of several HL and AOS genes and obtained from Integrated

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DNA Technologies (Coralville, IA). A product of the expected size was obtained using the following primer pair: HL5B (GGNTTYAAYGCNTWYGGNGG) and HL3B (CYTTNGCNGCRCAYTGYTGYTTRTT). The sequence of the HL cDNA was determined by a RACE using a SMART cDNA amplification kit (Clontech, Palo Alto, CA). From the sequence obtained, the following primer pair was designed for the cloning of the full-length coding region of watermelon HL: CLHL5 (CGCACTAGTATGAAGGTCACCATGACCTC) and CLHL3 (GGTAAGCTTCAGTTGGTCCTTTGAAAAGC). The 5′-end primer was designed to contain the *Spe*I recognition site just before the start codon, whereas the 3′-end primer contained a *Hin*dIII recognition site just after the stop codon. The PCR product obtained using this primer pair was ligated into the pGEM-T Easy cloning vector (Promega, Madison, WI).

cDNA Cloning of *Arabidopsis* HL. Total RNA was isolated from wounded, young rosette leaves of *Arabidopsis thaliana* with the TRIzol reagent. The messenger RNAs were partially purified using oligo(dT)-cellulose. The following pair of primers was designed from a published sequence of the *Arabidopsis* HL gene: AtHL5d (ACACTAGTATGTCACAGCTTCCCCTCCGTA) and AtHL3b (CACTAGTCGGCGACCGTTGAAGATTGATTA). These primers were designed to contain the *Spe*I recognition sites flanking the coding region. The PCR product obtained using this primer pair was ligated into the pGEM-T Easy cloning vector.

Phylogenetic Analysis. Amino acid sequence alignment and phylogenetic tree construction were performed with Vector NTI Advance 9 (InforMax, Frederick, MD) using ClustalW algorithm (*35*) and the neighbor-joining method (*36*).

Plant Transformation. The cloned cDNA of watermelon or *Arabidopsis* HL was inserted in a CMV 35S promoter-driven binary vector pKYLX71:35S2 (*37*) (<http://www.uky.edu/~aghunt00/kylx.html>). The cDNA was digested with *Spe*I and ligated into the vector digested with *Xba*I, which produces a compatible 5′-extension. The binary vector was then transferred into *Agrobacterium tumefaciens* strain GV3850 using the freeze−thaw method, and integrity was confirmed by PCR. *N. tabacum* plants Petite Havana and KY14 were transformed by the leaf disk method with *Agrobacterium* containing the watermelon HL and *Arabidopsis* HL, respectively, and the transgenic lines were selected for kanamycin resistance.

Enzyme Extraction and Assay. The crude enzyme extracts were prepared from leaves. Two volumes of an extraction buffer [100 mM Tris HCl, pH 8.5, 3 mM EDTA, 3 mM dithiothreitol, 1% (w/v) protease inhibitor cocktail, 0.5% (w/v) Triton X-100, and 5 g L^{-1} polyvinylpolypyrrolidone (PVPP)] was used for the extraction. The slurries were filtered through a layer of Miracloth (EMD Biosciences, San Diego, CA) and briefly centrifuged at 4 °C to remove the remaining PVPP. The enzyme activity was measured using 13(*S*)-hydroperoxylinolenic acid (13-HPOT) in the presence of yeast alcohol dehydrogenase and NADH, where the production of aldehydes was measured by the reduction of NADH consumed by ADH converting aldehyde to alcohol spectrophotometrically (*38*). The reaction buffer consisted of 25 mM PIPES buffer, pH 6.5, 0.1 mM 13-HPOT, 0.1 mM NADH, 57 units mL^{-1} yeast alcohol dehydrogenase, and 0.01% (w/v) Tween 20. The loss of absorbance at 340 nm wavelength was measured.

RESULTS

Cloning and Sequence Analysis of the Watermelon HL Gene. Poly(A)$^+$ RNA isolated from leaves was used as a template for RT-PCR using degenerated primers designed from conserved amino acid sequences of several HLs and AOSs cloned thus far. RT-PCR yielded a single clone with high similarity with other HLs. RACE was attempted to obtain the full-length cDNA for the putative watermelon HL. The sequence of the putative coding region of this enzyme was obtained (**Figure 1**), but very little 5′-untranslated region was sequenced, possibly due to the secondary structure in this region. This has been deposited in GenBank, accession no. AY703450. The open reading frame encoded a deduced protein of 481 amino acids, which had 87.5% identity with a hypothetical 13-HL of

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   1  ATGAAGGTCACCATGACCTCCGGCGGAATGCCTTCCATACCTTCATCGATTTCGCCACCG
   1   M  K  V  T  M  T  S  G  G  M  P  S  I  P  S  S  I  S  P  P

  61  CCGGTCACTTTACCGCTCAGAAATATCCCCGGCAGCTACGGTTTGCCGCTGTTCGGATCC
  21   P  V  T  L  P  L  R  N  I  P  G  S  Y  G  L  P  L  F  G  S

 121  ATCGGTGACCGGCTGGATTACTTCTGGTTTCAAGGACCCGAGAAGTTCTTCAGGTCTCGG
  41   I  G  D  R  L  D  Y  F  W  F  Q  G  P  E  K  F  F  R  S  R

 181  ATGGAGAAGAATCAAAGTACGGTTTTCAGAACGAATGTTCCTCCGTCGTTCCCTTTCTTC
  61   M  E  K  N  Q  S  T  V  F  R  T  N  V  P  P  S  F  P  F  F

 241  TTCACCGATCCGAGAGTGATTGCGGTTCTGGATTGCAAGTCGTTTGCGCATCTATTCGAC
  81   F  T  D  P  R  V  I  A  V  L  D  C  K  S  F  A  H  L  F  D

 301  ATGGAAATCGTGGAGAAGAAGAATGTTCTGGTCGGTGATTTCATGCCGAGCACAAGTTTC
 101   M  E  I  V  E  K  K  N  V  L  V  G  D  F  M  P  S  T  S  F

 361  ACCGGAAATATGAGAGTCTGTGCGTATTTGGATACGTCGGAATCTCAACACTCGAAGATA
 121   T  G  N  M  R  V  C  A  Y  L  D  T  S  E  S  Q  H  S  K  I

 421  AAAAACTTCGTCATGGACGTTCTGCGGCGGAGCTCGAGGATTTGGATACAGGAGTTGGAA
 141   K  N  F  V  M  D  V  L  R  R  S  S  R  I  W  I  Q  E  L  E

 481  TCGAACCTATCGACGATGTGGGACAGCATAGAATCCGAAATCGCAAAGGACACAAAATCC
 161   S  N  L  S  T  M  W  D  S  I  E  S  E  I  A  K  D  T  K  S

 541  AGCTTCAGAAACCATCTCCAACCAACTCTTTTCAATTTCTTCTCCAAAACCCTGGCCGGC
 181   S  F  R  N  H  L  Q  P  T  L  F  N  F  F  S  K  T  L  A  G

 601  GCCGACACTGCAAAATCACCGGAAGTGGCTAAATCCGGCTACATCGACGTCATAATTTGG
 201   A  D  T  A  K  S  P  E  V  A  K  S  G  Y  I  D  V  I  I  W

 661  CTGGGGCTCCAGCTGGTCCCCACCATCCACATCGGCATTCTCCAACCCCTGGAAGAAATA
 221   L  G  L  Q  L  V  P  T  I  H  I  G  I  L  Q  P  L  E  E  I

 721  TTCCTCCACTCTTTCCGATTACCCTTCTTCCCCATCGCCTCTCGCTACCAAAGACTCTAC
 241   F  L  H  S  F  R  L  P  F  F  P  I  A  S  R  Y  Q  R  L  Y

 781  GATTTCATCCAAAAAGAAGGGGAAGAAGTGGTTGAGCGAGGCGTTTCGGAGTTCGGGTTG
 261   D  F  I  Q  K  E  G  E  E  V  V  E  R  G  V  S  E  F  G  L

 841  ACGAAGGATGAAGCAATTCACAATCTCATCTTCACCATGGGATTCAACGCCTACGGTGGT
 281   T  K  D  E  A  I  H  N  L  I  F  T  M  G  F  N  A  Y  G  G

 901  TTCAGTCTCTTCTTCCCGGTTCTACTCGATCGGATACTCAACGACAAAACCGGTTTACAA
 301   F  S  L  F  F  P  V  L  L  D  R  I  L  N  D  K  T  G  L  Q

 961  CAGAGAATCCTCGAGGAAGTCAAGGCAAAAACCGGCTCCGGTCTGACATTCGAGTCGGTC
 321   Q  R  I  L  E  E  V  K  A  K  T  G  S  G  L  T  F  E  S  V

1021  AAGGAGATGGATCTCATCTACTCCGTCGTTTACGAGACACTCCGGCTTGACCCGCCGGTT
 341   K  E  M  D  L  I  Y  S  V  V  Y  E  T  L  R  L  D  P  P  V

1081  CCAACCCAGTACGCGAGAGCCAGAAAGGATTTCAAGCTAAGTTCCTACGATTCAGCGTAT
 361   P  T  Q  Y  A  R  A  R  K  D  F  K  L  S  S  Y  D  S  A  Y

1141  AGCATCAAGAAAGGGGAGCTGCTTTGTGGGTATCAGCCGCTGGTGATGAGAGACCCGAAG
 381   S  I  K  K  G  E  L  L  C  G  Y  Q  P  L  V  M  R  D  P  K

1201  GTGTTCAATAAACCGAAGACGTTTAATCCGGGCCGGTTCCGGGGAGAGAAGGGGGCGGCG
 401   V  F  N  K  P  K  T  F  N  P  G  R  F  R  G  E  K  G  A  A

1261  CTGCTGGATTATTTGTTCTGGTCGAACGGGCCGCAGACGGGACTACCGAGCGAGCATAAC
 421   L  L  D  Y  L  F  W  S  N  G  P  Q  T  G  L  P  S  E  H  N

1321  AAGCAGTGCGCCGGGAAGGATTTGGTGGTGCTGACGGCAGTGGTGTTCGTGGCTTACATA
 441   K  Q  C  A  G  K  D  L  V  V  L  T  A  V  V  F  V  A  Y  I

1381  TTTCGAAGGTATGATTGGATTGCAGGGGAAGGAGGTTCGATTACAGCTTTTCAAAGGACC
 461   F  R  R  Y  D  W  I  A  G  E  G  G  S  I  T  A  F  Q  R  T

1441  AACTGAAGTGAAATATATATATATATGTAGATTGAGAACTGCAGCTTTTTTTGTTCATGG
 481   N  *

1501  CTTCTTTTTTATGTATGAGTGTGGAGCCCAAATGAAAAAAATTGGAAAAATTAATCAATA

1561  AAATTAAGATTCCATTTAAAAAAAAAAAAAAAAAAAAAAAGCAAAAAAAAAAAAAAAAA

1621  AAAAAAAAAAA
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Figure 1. Nucleotide and deduced amino acid sequences of the watermelon HL gene. Absolutely conserved amino acid residues among cytochrome P450s (Glu352, Arg355, Arg412, and Cys443) are underlined. GenBank accession no. AY703450.

cucumber (*29*). Like several other HLs cloned, the N-terminal sequence lacked the typical features of a transit peptide and thus failed to be identified (PSORT <http://psort.nibb.ac.jp>; TargetP 1.0 <http://www.cbs.dtu.dk>) or was predicted to be longer than expected (SignalP 3.0 <http://www.cbs.dtu.dk>; ChloroP 1.1 <http://www.cbs.dtu.dk>). The phylogenetic analysis of amino acid sequences of 25 HL genes showed the clustering of HLs within plant families except for the diversion between 13-HL and 9/13-HL (**Figure 2**).

Cytochrome P450s have a conserved structural fold even though their sequences are diverse and have low identity (*39*, *40*). These proteins consist of two domains: an α-domain rich in α-helices and a β-domain rich in β-strands. Bundles of four helices, D, E, I, and LL, and helices J and K compose the structural core of the α-domain. Although overall sequence identity is low among P450s, there are several consensus

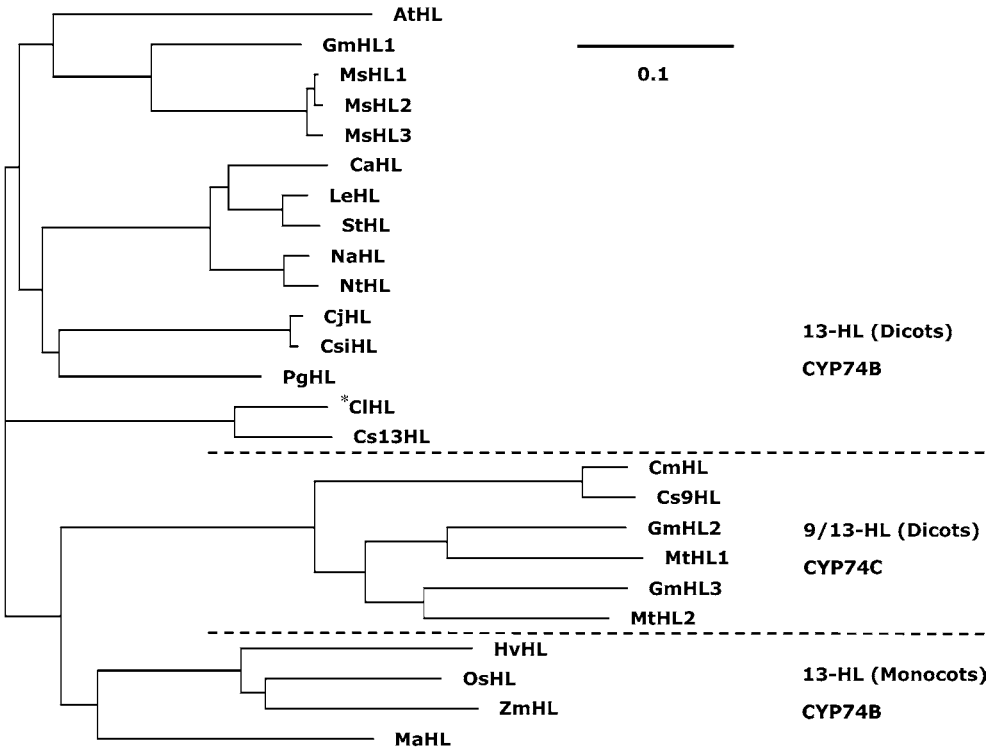

Figure 2. Phylogenetic tree of the HLs. The phylogenetic tree was constructed using the neighbor-joining method in Vector NTI Suite 9 (Informax) from the deduced amino acid sequences of HL genes. The organism names and GenBank accession numbers, if available, are as follows: AtHL (*Arabidopsis thaliana*), AF087932; CaHL (*Capsicum annuum*), U51674; CjHL (*Citrus jambhiri*), AB077765; *ClHL (*Citrullus lanatus*), this paper; CmHL (*Cucumis melo*), AF081955; Cs13HL (*C. sativus*), AF229812; Cs9HL (*C. sativus*), AF229811; CsiHL (*Citrus sinensis*), AY242385; GmHL1, 2, and 3 (*Glycine max*), U.S. Patent Application 0040010822; HvHL (*Hordeum vulgare*), AJ318870; LeHL (*Lycopersicon esculentum*), AF230372; MaHL (*Musa* sp.), A65873; MsHL1 (*Medicago sativa*), AJ249245; MsHL2 (*M. sativa*), AJ249246; MsHL3 (*M. sativa*), AJ249247; MtHL1 (*M. truncatula*), AJ316562; MtHL2 (*M. truncatula*), AJ316563; NaHL (*Nicotiana attenuata*), AJ414400; NtHL (*N. tabacum*), our unpublished data; OsHL (*Oryza sativa*), AY340220; PgHL (*Psidium guajava*), AF239670; StHL (*Solanum tuberosum*), AJ310520; and ZmHL (*Zea mays*), AAN88707. The scale bar indicates the expected number of amino acid substitutions per amino acid.

sequences known. One is Phe-X-X-Gly-X-Arg-X-Cys-X-Gly located just before the L helix, with the absolutely conserved cysteine that serves as the fifth ligand to the heme iron. The second is the ERR triad consisting of the Glu-X-X-Arg motif in the K helix and the Pro-Glu-Arg-Phe motif found near the so-called "meander" region near the L helix. This set of motifs is considered to stabilize the core structure. These four amino acids (Cys, Glu, Arg, and Arg underlined) are absolutely conserved among P450s. The third is Ala/Gly-Gly-X-Asp/Glu-Thr-Thr/Ser in the I helix, which is thought to be involved in oxygen binding. There is also the Trp/His-X-X-X-Arg motif found in the C helix, which coordinates one of the heme propionates. All of the plant P450s characterized thus far are membrane-bound proteins (*19*). The membrane interaction of mitochondrial and microsomal P450s is considered to involve a hydrophobic helix near the N-terminal and/or the F−G loop between the F and G helices (*41*−*43*). When the multiple sequence alignment was expanded to include other members of CYP74 genes, several interesting findings emerged (**Figure 3**). First, the consensus sequence around the heme-binding site near the L helix appears to be Trp-Ser-Asn-Gly/Glu-Pro/Arg-Glu/Gln-Thr-Glu/Gly-X-Pro-X-X-X-Asn-Lys-Gln-Cys-Ala/Pro-Gly/Ala-Lys-Asp/Asn-X-Val, where Arg, two residues ahead of the heme-binding Cys, is replaced with Lys. The fifth residue from Trp is Pro for13-HLs and 13-AOS, whereas a charged/polar residue, mainly Arg, replaces Pro in 9/13-HLs, 9-DESs, and 9/13-AOSs. Second, in the Thr/His-X-X-X-Arg motif in the C helix, Arg is replaced by Lys, and His and Lys are absolutely conserved among CYP74s sequenced thus far. Third, the length of the F−G loop is two to three amino acids longer among 9/13-HLs with an extra charged residue (Lys) compared to other CYP74s. 9-DESs, LeDES, and NtDES, which also act on 9-hydroperoxides, have a shorter loop, but they also contain two extra charged residues (Lys). This region is also flanked by two of the possible six substrate recognition sites (*44*). How the Lys here affects the substrate specificity and/or the interaction with the membrane is not clear and requires mutation experiments. The highly conserved Thr in the oxygen binding motif in the I helix is not seen in CYP74s because these enzymes do not require molecular oxygen for the reaction. The C-terminal end of 13-HLs past the L helix, which is considered to form two to four β-strands and one of the substrate recognition sites, is much shorter than other CYP74s.

Characterization of Watermelon HL Expressed in Transgenic Plants. The full-length coding region of the watermelon HL was expressed in *N. tabacum* leaves behind the enhanced 35S promoter. The highest expresser line 16, which had three or more copies of the gene, had ∼50 times more HL activity than that of wild-type *N. tabacum* (**Figure 4**) or a vector control (**Figure 5**), and the main volatile product from 13-hydroperoxylinolenic acid (HPOT) was confirmed as (3*Z*)-hexenal by GC-MS (data not shown). Some of the third and fourth generations of line 16 plants had HL activities as low as that in nontransformed *N. tabacum* (data not shown). It is likely that the transgenes were cosuppressed due to the high number of copies and/or transcripts. Moreover, the highest activities in the third and fourth generations among different lines, whether they contained single or multiple copies of the gene, rarely reached

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13-HL (Dicots)
ClHL     -HSKIK-  -WLGLQLVPTIHIGIL   QPLEE-  -GGFSLF-  -VYETLRLDP-  -FNPDRF-  -WSNGPQTGLPSEHNKQCAGKDIV-  -RYDWIAGEGG        SITAFQRTN
LeHL     -HAQIK-  -WLAIQLAPTVSIGVL   QPLEE-  -GGFSIF-  -VYETLRLSP-  -FVLERF-  -WSNGPQTGRPTESNKQCAAKDMV-  -KYDSVSFSSG        SLTSVKKAS
MsHL2    -HAKAK-  -WLAVQLLPTVSVGTI   QPLEE-  -GGFSIF-  -VYETLRMNP-  -FKPERF-  -WSNGPQTGSPTVSNKQCAGKDIV-  -RYDLIKGDGS        SITALRKAK

13-HL (Monocots)
MaHL     -HARVK-  -WLALQLLPTVKVGAIP  QPLEE-  -GGFSVF-  -VYEVLRLNP-  -FAPERF-  -WSNGPETGTPTPANKQCAAKDYV-  -RYDEFVCADD        AISVTKLDRAREWE
OsHL     -HARTK-  -WLALQLVPTQKVGVIP  QPLEE-  -GGFSVF-  -VYEVLRMQP-  -FAPERF-  -WSNGPETGEPSPGNKQCAAKEVV-  -RYDDFECDGT        SFTKLDKRELTPS

9/13-HL
CmHL     -HSVLK-  -WLVFQLAPLASIGLPKIFSVFED-  -GGMKVL-  -VYEALRIEP-  -FVGDRF-  -WSNERETVEPTPENKQCPGKNLV-  -RYDTFTVEVADLPLGPAVKFKSLTRATDMV
MtHL2    -HALIK-  -WLLFQLAPLATLGPPKIFNYLED-  -GGLKNQ-  -VYEAMRIEP-  -FVAKRF-  -WSNGKETEEPSVGNKQCPGKNLV-  -RYDTFENETKNNAFGAAVSITSLTKASSV

9-DES
LeDES    -HAALK-  -WLFPQLIPSLSAKKLP  NIIED-  -AGLNAF-  -VYETLRLRP-  -FVPDRF-  -WSNGRETESPAPDNKQCPGKDLV-  -RYDTFTLEITPLFRAPNVAFNTLTKASK
NtDES    -HAALK-  -WLFPQLIPSLSARKLP  SFIED-  -AGLNAF-  -VYETLRLRP-  -FIPDRF-  -WSNGRETENPAPDNKQCAGKDLV-  -RYDTFTVEITPLFRAPNVAIKTLTKAT

9/13-AOS
HvAOS1   -HTKVK-  -WLIFQLHPLVTLGLP   MILEE-  -GGLKVL-  -VWEALRLDP-  -FVGDRF-  -WSNGRETESPSVHNKQCPGKNLV-  -RYDTFTAKVGLDLLGTKVEPTGVTKATSGVADAV
OsAOS    -HAKIK-  -WLLWQLAPLTTLGLP   MIIED-  -GGFKLL-  -VWEALRLDP-  -FVGDRF-  -WSNGRETENPSVDNKQCPGKNLV-  -RYDTFTAEAG      KKVVITGVTKASTSAVNRTA

13-AOS
LeAOS2   -HEKLK-  -WILLQLHPVITLGLP   KFLDD-  -GGMKIF-  -VYEALRVDP-  -FVADRF-  -WSNGPETESPTVGNKQCAGKDFV-  -RYGTLNVDVGTSALGSSITITSLKKA
MtAOS    -HEQLK-  -WVLFQLGPVLKLGLP   KFVED-  -GGMKLF-  -VYEAFRIDP-  -FVADRF-  -WSNGPESQSPTVGNKQCAGKDFT-  -RYDSFEIQVGNSPLGPSITLTSLKRSSF

CYP74     HXXXK    WLXXQLXPXXXXG       LEE    GGXXXX    VYEXXRXXP    FXXDRF    WSNGPETEXPXXXNKQCAGKDXV
Motifs                                  I D    A         W            E         ERQ G         PA N

P450      WXXXR                                AGXDTT      EXXR          PERF              FXXGXRXCXG
Motifs    H                                    G    S                                          H

          C-Helix          F-G Loop            I-Helix    K-Helix              (Heme-binding Site)  L-Helix    β3/β4-Sheets
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Figure 3. Multiple alignment of conserved amino acid sequences of CYP74. Two to three sequences were chosen from each of dicot 13-HLs, monocot 13-HLs, 9/13-HLs, 9-DESs, 9/13-AOSs, and 13-AOSs for the alignment, although the actual comparison was conducted using 44 published plant CYP74s excluding the ones from a moss. The four absolutely conserved amino acid residues (Glu, Arg, Arg, and Cys) are underlined in the CYP74 motifs. The charged residues, Arg and Lys, in the F–G loop and near the heme-binding motif are in bold. The organism names and GenBank accession numbers are as follows: HvAOS1 (*H. vulgare*), AJ250864; LeAOS2 (*L. esculentum*), AF230371; LeDES (*L. esculentum*), AF317515; MtAOS (*M. truncatula*), AJ316561; NtDES (*N. tabacum*), AF070976; and OsAOS (*Oryza sativa*), AY055775. For HLs, see the caption of **Figure 2**.

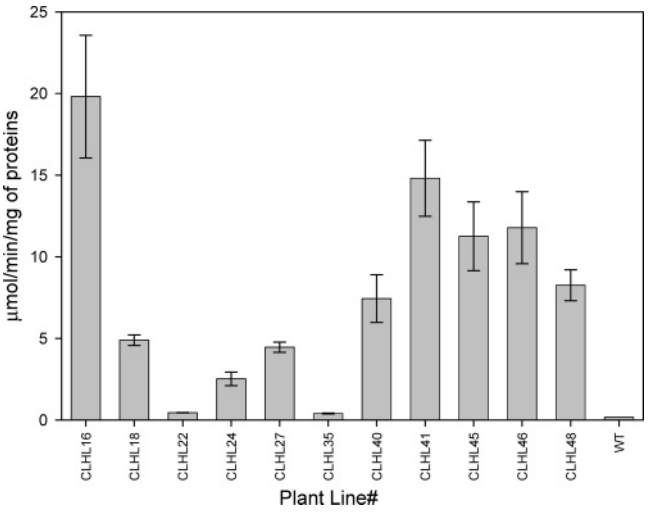

Figure 4. HL activity among transgenic lines (T_1 generation) of *N. tabacum* plants overexpressing watermelon HL. HL activity was measured at pH 6.5 using 13-HPOT as a substrate. Each bar represents the mean and standard error of three or four replicates. CLHL, watermelon HL overexpressing line; WT, wild-type *N. tabacum*.

above 15 μmol min^{-1} mg^{-1} of protein or ~75% of the highest level seen in the T_1 generation, indicating the activity levels were regulated. It was not clear if the regulation is at DNA, RNA, and/or protein levels.

The substrate specificity of watermelon HL is shown in **Table 1**. It has very little activity with 9-hydroperoxides and is ~3 times more active toward 13-HPOT than13-hydroperoxylinoleic acid (HPOD). The specificity toward 13-HPOT was not as specific as that of guava HL (10 times) (*28*), green pepper HL (12 times) (*45*), or sunflower leaf HL (16 times) (*46*), but more specific than that of alfalfa HLs (<1.5 times) (*25*) or sunflower hypocotyl HLs (~2 times) (*46*). The pH profile of the enzyme activity was very similar to the data from the original report with its optimum at pH 6.2 (*47*) (data not shown).

Comparison of HL Activity in Transgenic Plants. To compare the watermelon HL activity with another HL gene,

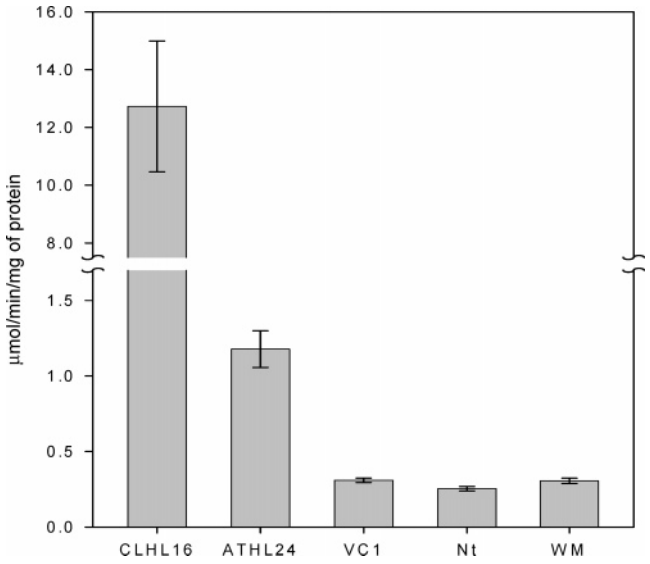

Figure 5. Comparison of HL activity among leaves of transgenic *N. tabacum* plants (T_3 generation) overexpressing watermelon HL or *Arabidopsis* HL, vector control, nontransgenic *N. tabacum* and watermelon. See the caption of **Figure 4** for the activity measurement. CLHL, watermelon HL overexpressing line; ATHL, *Arabidopsis* HL overexpressing line; VC, vector control; Nt, nontransgenic *N. tabacum* Petite Havana; WM, watermelon.

HL cDNA was isolated from *Arabidopsis* (*Arabidopsis thaliana*) and expressed in *N. tabacum* plants using the same binary vector. The results shown in **Figure 5** demonstrated 10 times more activity from watermelon HL than from the *Arabidopsis* HL transgenic line with the highest activity among 22 transformed plants screened.

DISCUSSION

The HL in watermelon plants was first purified from germinating seedlings and characterized as a 13-HL by Vick and Zimmerman (*47*). Watermelon leaves were also noted as

Table 1. Substrate Specificity of Watermelon HL Expressed in *N. tabacum* Leaves

substrate	activity[a] (μmol min^{-1} mg^{-1} of protein)	relative activity (%)
13(*S*)-hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid	4.13 ± 0.13	100.0
13(*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid	1.47 ± 0.015	35.6
9(*S*)-hydroperoxy-(10*E*,12*Z*,15*Z*)-octadecatrienoic acid	0.0131 ± 0.0043	0.32
9(*S*)-hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid	0.0143 ± 0.0028	0.35

[a] Average and standard error of three readings.

one of the high C_6 aldehyde producers among 28 plant species by Sekiya et al. (*31*), apparently due to the high activity of both HL and lipoxygenase (LOX). Currently, watermelon leaves are used in the industrial production of C_6 aldehydes (*32*). The watermelon HL cDNA cloned from mature leaves in this study shows an 87.5% amino acid identity with the hypothetical 13-HL from cucumber, another member of the Cucurbitaceae family to which watermelon belongs. Its amino acid identity dropped to 63% with the next closest known peptide sequence of guava HL, indicating the highly diverse nature of CYP74 sequences, which is typical for cytochrome P450s.

All of the plant cytochrome P450s characterized so far are considered to be membrane-bound proteins (*19*). The majority of HLs were found in membrane fractions from tea leaves (*48*) as well as green pepper fruits (*45*), and solubilizing HLs from leaf tissues requires a high detergent concentration in sunflower (*46*). Although many of the HLs cloned thus far lack a typical chloroplast targeting transit peptide, tomato HL proteins were shown to be incorporated into the outer membrane of chloroplasts (*49*). These data suggest that HLs are bound to membranes of chloroplasts in green tissues. On the other hand, HLs were extracted with a buffer without any detergent from hypocotyl−root sections of etiolated watermelon seedlings (*47*). Similarly, about a half of the HL enzymes were extracted from etiolated seedlings of sunflower without detergent (*46*). Also, HL activity was mostly associated with the soluble fractions of the extracts from alfalfa seedlings (*25*). These data indicate that the HL proteins are either soluble or loosely associated with membranes in hypocotyls, where few mature chloroplasts are present. Although there is a possibility that two or more isozymes of HLs exist and are expressed differently in different tissues and/or different developmental stages, it is also possible that the difference in the subcellular localizations in different tissues could be due to the post-translational modification of the protein, or the lack of targeting mechanisms and/or destination (chloroplasts) in etiolated seedlings. Tijet el. (*28*) speculated partial proteolysis or glycosylation to be a cause of two HL forms of different molecular weights in guava fruits. Further experiments are required to confirm this.

Watermelon HL overexpressing *N. tabacum* leaves had ~10 times higher HL activity than *Arabidopsis* HL overexpressing leaves and almost ~40 times higher activity than watermelon leaves. This clearly shows the strong activity of watermelon HLs, which was reported as 423 μmol min^{-1} mg^{-1} as partially purified forms (*47*). The activity is slightly lower than the 644 μmol min^{-1} mg^{-1} of purified guava HL, the highest activity among HLs characterized thus far (*28*). Studies are under way to explore the potential use of these transgenic plants as a source of HL for industrial production of green note compounds.

ABBREVIATIONS USED

ADH, alcohol dehydrogenase; AOS, allene oxide synthase; C_6, six-carbon; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GC-MS, gas chromatography−mass spectrometry; HL, hydroperoxide lyase, 13-HPOT, 13(*S*)-hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic (linolenic) acid; 13-HPOD, 13(*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic (linoleic) acid; LOX, lipoxygenase; NADH, β-nicotinamide adenine dinucleotide (reduced form); PCR, Polymerase Chain Reaction; PIPES, piperazine-*N*,*N*′-bis(ethanesulfonic acid); PVPP, polyvinylpolypyrrolidone; RACE, rapid amplification of cDNA ends.

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