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Recombinant Watermelon (*Citrullus Lanatus*) Hydroperoxide Lyase and Uses Thereof

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(54) **RECOMBINANT WATERMELON
(CITRULLUS LANATUS) HYDROPEROXIDE
LYASE AND USES THEREOF**

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C12P 9/88 (2006.01)
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435/320.1; 435/252.33; 536/23.2

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435/232, 69.1, 320.1, 252.33, 419; 536/23.2
See application file for complete search history.

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Primary Examiner—Rebecca E. Prouty

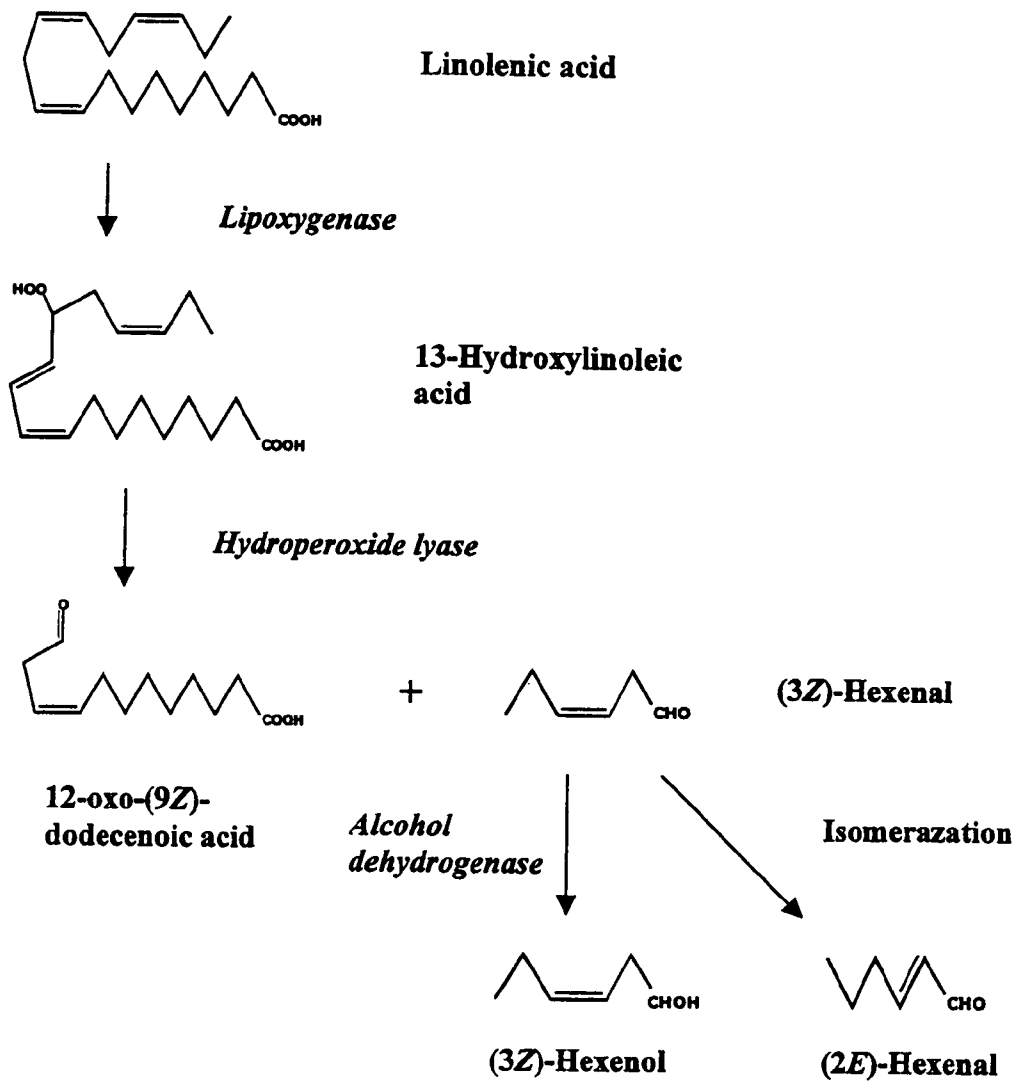
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(57) **ABSTRACT**

Recombinant watermelon (*Citrullus lanatus*) hydroperoxide lyase protein, DNA sequences encoding the protein, vectors containing the DNA sequences and hosts containing the vectors are provided, together with methods for recombinantly producing watermelon hydroperoxide lyase, DNA sequences, vectors and hosts.

5 Claims, 5 Drawing Sheets



Hydroperoxide Lyase Pathway

Figure 1

Citrullus lanatus Hydroperoxide Lyase Peptide Sequence

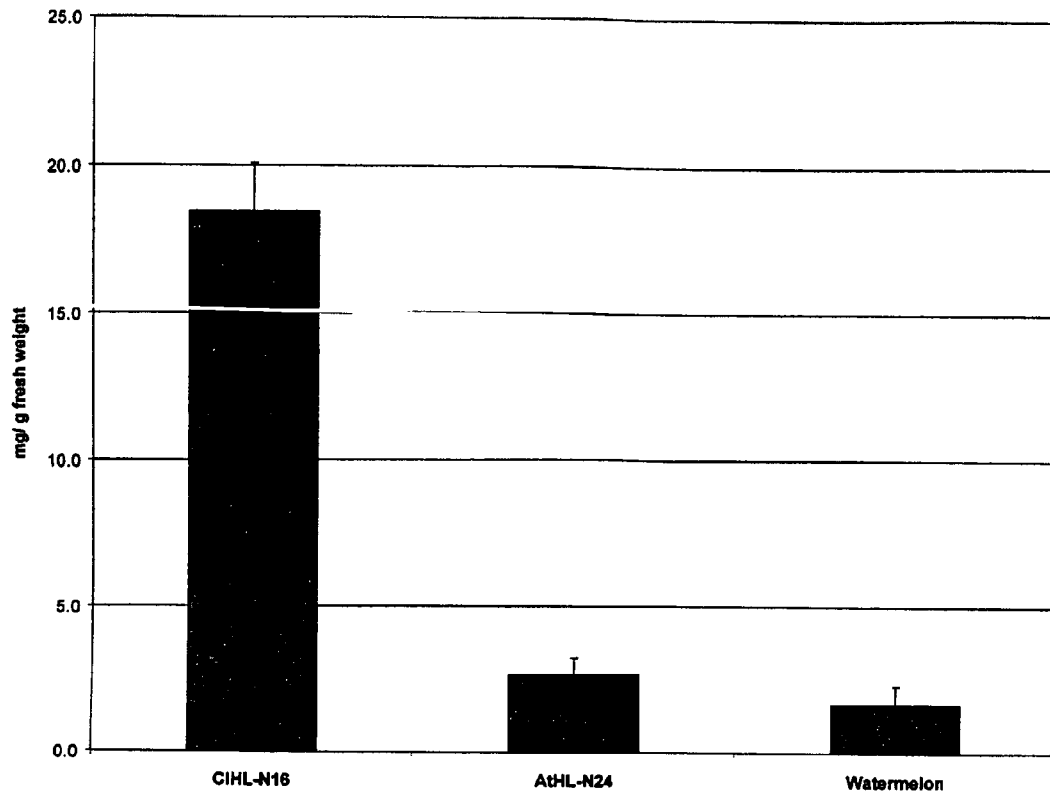
1	Met	Lys	Val	Thr	Met	Thr	Ser	Gly	Gly	Met	Pro	Ser	Ile	Pro	Ser	15
16	Ser	Ile	Ser	Pro	Pro	Pro	Val	Thr	Leu	Pro	Leu	Arg	Asn	Ile	Pro	30
31	Gly	Ser	Tyr	Gly	Leu	Pro	Leu	Phe	Gly	Ser	Ile	Gly	Asp	Arg	Leu	45
46	Asp	Tyr	Phe	Trp	Phe	Gln	Gly	Pro	Glu	Lys	Phe	Phe	Arg	Ser	Arg	60
61	Met	Glu	Lys	Asn	Gln	Ser	Thr	Val	Phe	Arg	Thr	Asn	Val	Pro	Pro	75
76	Ser	Phe	Pro	Phe	Phe	Phe	Thr	Asp	Pro	Arg	Val	Ile	Ala	Val	Leu	90
91	Asp	Cys	Lys	Ser	Phe	Ala	His	Leu	Phe	Asp	Met	Glu	Ile	Val	Glu	105
106	Lys	Lys	Asn	Val	Leu	Val	Gly	Asp	Phe	Met	Pro	Ser	Thr	Ser	Phe	120
121	Thr	Gly	Asn	Met	Arg	Val	Cys	Ala	Tyr	Leu	Asp	Thr	Ser	Glu	Ser	135
136	Gln	His	Ser	Lys	Ile	Lys	Asn	Phe	Val	Met	Asp	Val	Leu	Arg	Arg	150
151	Ser	Ser	Arg	Ile	Trp	Ile	Gln	Glu	Leu	Glu	Ser	Asn	Leu	Ser	Thr	165
166	Met	Trp	Asp	Ser	Ile	Glu	Ser	Glu	Ile	Ala	Lys	Asp	Thr	Lys	Ser	180
181	Ser	Phe	Arg	Asn	His	Leu	Gln	Pro	Thr	Leu	Phe	Asn	Phe	Phe	Ser	195
196	Lys	Thr	Leu	Ala	Gly	Ala	Asp	Thr	Ala	Lys	Ser	Pro	Glu	Val	Ala	210
211	Lys	Ser	Gly	Tyr	Ile	Asp	Val	Ile	Ile	Trp	Leu	Gly	Leu	Gln	Leu	225
226	Val	Pro	Thr	Ile	His	Ile	Gly	Ile	Leu	Gln	Pro	Leu	Glu	Glu	Ile	240
241	Phe	Leu	His	Ser	Phe	Arg	Leu	Pro	Phe	Phe	Pro	Ile	Ala	Ser	Arg	255
256	Tyr	Gln	Arg	Leu	Tyr	Asp	Phe	Ile	Gln	Lys	Glu	Gly	Glu	Glu	Val	270
271	Val	Glu	Arg	Gly	Val	Ser	Glu	Phe	Gly	Leu	Thr	Lys	Asp	Glu	Ala	285
286	Ile	His	Asn	Leu	Ile	Phe	Thr	Met	Gly	Phe	Asn	Ala	Tyr	Gly	Gly	300
301	Phe	Ser	Leu	Phe	Phe	Pro	Val	Leu	Leu	Asp	Arg	Ile	Leu	Asn	Asp	315
316	Lys	Thr	Gly	Leu	Gln	Gln	Arg	Ile	Leu	Glu	Glu	Val	Lys	Ala	Lys	330
331	Thr	Gly	Ser	Gly	Leu	Thr	Phe	Glu	Ser	Val	Lys	Glu	Met	Asp	Leu	345
346	Ile	Tyr	Ser	Val	Val	Tyr	Glu	Thr	Leu	Arg	Leu	Asp	Pro	Pro	Val	360
361	Pro	Thr	Gln	Tyr	Ala	Arg	Ala	Arg	Lys	Asp	Phe	Lys	Leu	Ser	Ser	375
376	Tyr	Asp	Ser	Ala	Tyr	Ser	Ile	Lys	Lys	Gly	Glu	Leu	Leu	Cys	Gly	390
391	Tyr	Gln	Pro	Leu	Val	Met	Arg	Asp	Pro	Lys	Val	Phe	Asn	Lys	Pro	405
406	Lys	Thr	Phe	Asn	Pro	Gly	Arg	Phe	Arg	Gly	Glu	Lys	Gly	Ala	Ala	420
421	Leu	Leu	Asp	Tyr	Leu	Phe	Trp	Ser	Asn	Gly	Pro	Gln	Thr	Gly	Leu	435
436	Pro	Ser	Glu	His	Asn	Lys	Gln	Cys	Ala	Gly	Lys	Asp	Leu	Val	Val	450
451	Leu	Thr	Ala	Val	Val	Phe	Val	Ala	Tyr	Ile	Phe	Arg	Arg	Tyr	Asp	465
466	Trp	Ile	Ala	Gly	Glu	Gly	Gly	Ser	Ile	Thr	Ala	Phe	Gln	Arg	Thr	480
481	Asn															481

FIGURE 2

Citrullus lanatus Hydroperoxide Lyase Nucleotide Sequence

1	ATGAAGGTCACCATGACCTCCGGCGGAATGCCTTCCATACCTTCATCGATTTCCGCCACCG	60
61	CCGGTCACTTTACCGCTCAGAAATATCCCGGCAGCTACGGTTTGCCGCTGTTCCGGATCC	120
121	ATCGGTGACCGGCTGGATTACTTCTGGTTTCAAGGACCCGAGAAGTTCCTCAGGTCTCGG	180
181	ATGGAGAAGAATCAAAGTACGGTTTTTCAGAACGAATGTTCCCTCCGTCGTTCCCTTCTTC	240
241	TTCACCGATCCGAGAGTGATTGCGGTCTGGATTGCAAGTCGTTTGCATCTATTCGAC	300
301	ATGGAAATCGTGGAGAAGAAGAATGTTCTGGTCGGTGATTTCATGCCGAGCACAAAGTTTC	360
361	ACCGGAAATATGAGAGTCTGTGCGTATTTGGATACGTCGGAATCTCAACACTCGAAGATA	420
421	AAAAACTTCGTCATGGACGTTCTGCGGCGGAGCTCGAGGATTGGATACAGGAGTTGGAA	480
481	TCGAACTATCGACCTCTGCGGACAGCATAGAATCCGAAATCGCAAAGGACACAAAATCC	540
541	AGCTTCAGAAACCATCTCCAACCACTCTTTTCAATTTCTTCTCCAAAACCCCTGGCCGGC	600
601	GCCGACACTGCAAAATCACCGGAAGTGGCTAAATCCGGCTACATCGACGTCATAATTTGG	660
661	CTGGGGCTCCAGCTGGTCCCCACCATCCACATCGGCATTTCCAAACCCCTGGAAGAAATA	720
721	TTCTCCACTCTTTCCGATTACCCCTCTTCCCATCGCCTCTCGCTACCAAAGACTCTAC	780
781	GATTTTCATCCAAAAGAAGGGGAAGAAGTGGTTGAGCGAGGCGTTTCGGAGTTCGGGTTG	840
841	AGGAAGGATGAAGCAATTCACAATCTCATCTCACCATGGGATTCACGCCTACGGTGGT	900
901	TTCAGTCTCTTCTTCCCGGTTCTACTCGATCGGATACTCAACGACAAAACCGGTTTACAA	960
961	CAGAGAATCCTCGAGGAAGTCAAGGCAAAAACCGGCTCCGGTCTGACATTCGAGTCGGTC	1020
1021	AAGGAGATGGATCTCATCTACTCCGTCGTTTACGAGACTCCGGCTTGACCCGCCGGTT	1080
1081	CCAACCCAGTACGCGAGAGCCAGAAAGGATTTCAAGCTAAGTTCCTACGATTCAGCGTAT	1140
1141	AGCATCAAGAAAGGGGAGCTGCTTTGTGGTATCAGCCGCTGGTGATGAGAGACCCGAAG	1200
1201	GTGTTCAATAAACCGAAGCGTTTAAATCCGGGCCGGTCCGGGGAGAGAAGGGGGCGGCG	1260
1261	CTGCTGGATTATTTGTTCTGGTCGAACGGGCCGACACGGGACTACCGAGCGAGCATAAC	1320
1321	AAGCAGTGCGCCGGAAGGATTTGGTGGTGTGACGGCAGTGGTGTTCGTGGCTTACATA	1380
1381	TTTCGAAGGTATGATTGGATTGCAGGGGAAGGAGGTTTCGATTACAGCTTTTCAAAGGACC	1440
1441	AACTGAAGTGAATATATATATATATGTAGATTGAGAACTGCAGCTTTTTTGTTCATGG	1500
1501	CTTCTTTTTTATGTATGATGTGGAGCCCAATGAAAAAATGGAAAAATTAATCAATA	1561
1561	AAATTAAGATTCCATTTAAAAAAGCAAAAAAAGCAAAAAAAGCAAAAAAAGCAAAAAA	1620
1621	AAAAAAGCAAAAAAAGCAAAAAAAGCAAAAAAAGCAAAAAAAGCAAAAAAAGCAAAAAA	1632

Figure 3



CIHL-N16: Watermelon HL transgenic tobacco

AtHL-N24: Arabidopsis HL transgenic tobacco

Figure 4

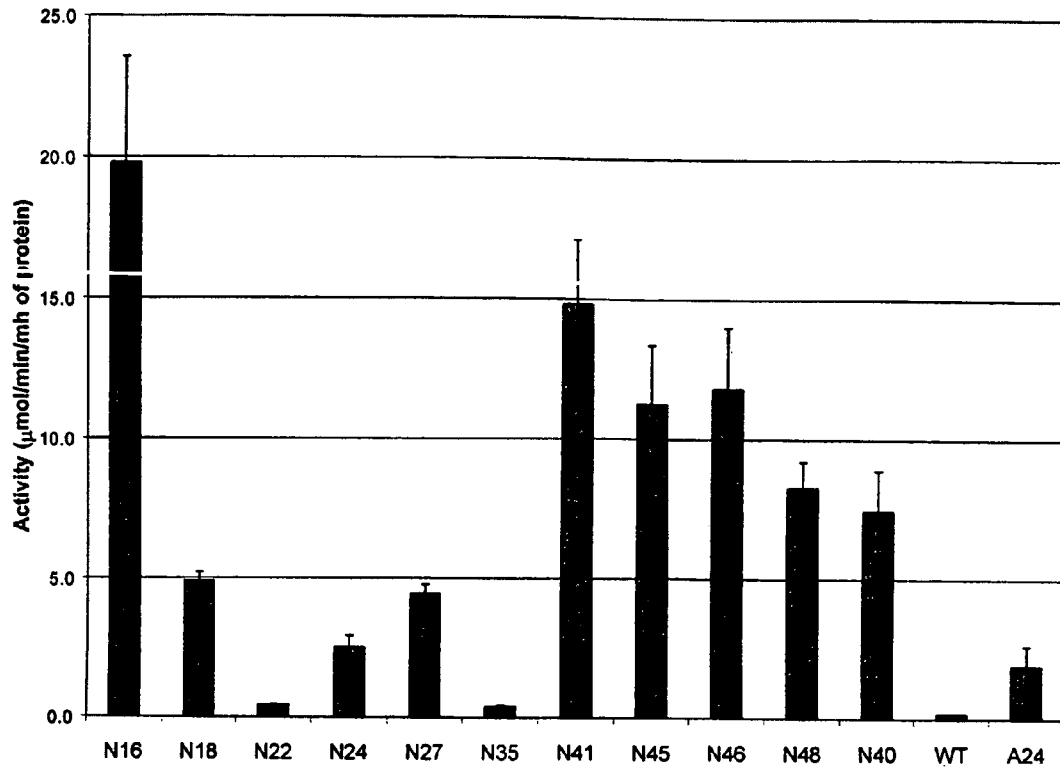


Figure 5

1

**RECOMBINANT WATERMELON
(CITRULLUS LANATUS) HYDROPEROXIDE
LYASE AND USES THEREOF**

FIELD OF THE INVENTION

This invention relates to the production of a hydroperoxide lyase (HL) protein in host cells via recombinant expression of the protein. Recombinant HL protein, DNA sequences encoding the protein, vectors containing these DNA sequences and hosts containing the vectors are provided, together with methods for recombinantly producing such protein, DNA sequences, vectors and hosts.

BACKGROUND OF THE INVENTION

Fatty acid hydroperoxide lyase (HL) is an enzyme of the octadecanoid pathway that cleaves a C—C bond in the hydroperoxides of polyunsaturated fatty acids to generate aldehydes and ω -oxo-acids. This enzyme is widely distributed in plants and is involved in the biosynthesis of volatile aldehydes and alcohols. Its presence in plants can be recognized by the fact that crushed leaves of nearly every plant generate a grassy smell that is characteristic of C₆-aldehydes. The short-chain volatile aldehydes and their reduced derivatives, alcohols, are important constituents of the characteristic flavors of fruits, vegetables and green leaves.

The six-carbon volatile compounds, leaf aldehyde [(2E)-hexanal] and leaf alcohol [(3Z)-hexanol], are important components of the aroma and flavor of fruits and vegetables and are associated with 'green notes' of leaves. These compounds are widely used as flavors in foods and beverages and some of the C₆-aldehydes are also reported to have anti-microbial properties. These volatile compounds are produced by fatty acid hydroperoxide lyase (HL) by cleaving 13-hydroperoxy fatty acids, mainly from linolenic and linoleic acids.

Commercial production of natural 'green note' compounds is generally achieved by fractional distillation of essential oils such as mint oil or by the combined action of lipoxygenase and hydroperoxide lyase on unsaturated fatty acids using plant material from different sources. These processes provide low yields and are dependant on specific plant materials. Therefore, there is a need for improved methods of generating 'green note' compounds.

SUMMARY OF THE INVENTION

It has now been found that high reproducible yields of "green note" compounds (e.g., [(2E)-hexanal] can be obtained independent of plant materials and in the absence of unwanted side reaction (e.g. isomerase activity) by transfer of the gene coding for HL from plant into host cells, subsequent expression of the gene, addition of linolenic acid as substrate and lipoxygenase (or soybean as the enzyme source) to produce the leaf aldehyde [(2E)-hexanal].

Thus, in a first aspect of this invention, there are provided isolated DNA sequences encoding a protein having the amino acid sequence of SEQ ID NO: 1. In a preferred embodiment the isolated nucleic acid encodes a polypeptide having at least about 90% amino acid sequence similarity to SEQ ID NO: 1 and having HL activity.

In another aspect of the invention, there is provided an expression vector comprising a nucleic acid molecule encoding a polypeptide having at least about 90% sequence similarity to SEQ ID NO: 1 and having HL activity.

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In yet another aspect of the invention there is provided a host cell transformed with an expression vector comprising a nucleic acid molecule encoding a polypeptide having at least about 90% sequence similarity to SEQ ID NO: 1 and having HL activity. In a preferred embodiment the host cell is *E. coli*.

In another aspect of the invention there is provided a method of making hydroperoxide lyase comprising culturing a host cell transformed with an expression vector comprising a nucleic acid molecule encoding a polypeptide having at least about 90% sequence similarity to SEQ ID NO: 1 and having HL activity under conditions that enable expression of the nucleic acid, and isolating the polypeptide from the host cells.

The invention also provides transgenic plants comprising a recombinant nucleic acid molecule comprising a nucleic acid molecule encoding a polypeptide having at least about 90% sequence similarity to SEQ ID NO: 1 and having HL activity.

In another aspect of the invention there is provided a method for producing green note compounds comprising reacting fatty acid hydroperoxide in the presence of isolated recombinant protein having the amino acid sequence of SEQ ID NO: 1 or a polypeptide having at least about 90% sequence similarity to SEQ ID NO: 1 and having HL activity; and reacting the resulting aliphatic aldehydes in the presence of isomerase and/or alcohol dehydrogenase.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic drawing of the hydroperoxide lyase pathway.

FIG. 2 is the amino acid sequence of the watermelon (*Citrullus lanatus*) hydroperoxide lyase (SEQ ID NO: 1).

FIG. 3 is the nucleotide sequence of the watermelon (*Citrullus lanatus*) hydroperoxide lyase gene (SEQ ID NO: 2).

FIG. 4 is a bar graph showing the activity of HL over-expressed in tobacco plants. N lines contain watermelon HL; WT is a non-transformed tobacco plant line; and A24 is the highest expresser of *Arabidopsis* HL.

FIG. 5 is a bar graph showing C₆-aldehyde formation from HL over-expressed in tobacco leaves in comparison to normal (non-transformed) watermelon leaves. C1HL-Ni6: watermelon HL transgenic tobacco; A1HL-N24: *Arabidopsis* HL transgenic tobacco.

DETAILED DESCRIPTION OF THE
INVENTION

Fatty acid hydroperoxide lyase (HL) has been cloned from a variety of sources. The gene was first cloned from the bell pepper fruit. (Matsui et al., 1996). Moxon et al. reported in 1997 that the product of the cDNA associated with an *Arabidopsis* EST sequence 94J16 has HL activity when expressed in *E. coli*. Both Bate et al. (1998) and Matsui et al., (1999) have since confirmed that this *Arabidopsis* cDNA encodes an HL. Many additional HLs have been identified and cloned from several plant species, including tomatoes and potatoes. However, until now, the gene has not been isolated from watermelon, whose leaves are reported to be among the highest C₆-aldehyde producers among plant tissues.

A watermelon (*Citrullus lanatus*) HL cDNA was cloned via RT-PCR. For cloning the gene, degenerate primers were designed based on conserved sequences of published sequences of several HL and AOS genes. The full sequence

of the HL cDNA obtained using the primers was determined using RACE. The nucleotide sequence of the *Citrus lanatus* HL gene is shown in FIG. 3, while the derived amino acid sequence is shown in FIG. 2. The watermelon polypeptide sequence has highest sequence similarity to cucumber 13-HL and 63, 59, 59, 58, 57 and 47% sequence identity with HL from guava, tomato, *Nicotiana attenuate*, potato, alfalfa, green pepper, *Arabidopsis* and barley, respectively.

As used herein the term "substantially homologous," means that a particular sequence, for example, a mutant sequence, varies from a reference sequence by one or more substitutions, deletions, or additions, the net effect of which does not result in an adverse functional dissimilarity between the reference and subject sequences. For purposes of the present invention, DNA sequences having at least 90 percent homology (or sequence similarity), encoding equivalent biological properties, and showing equivalent expression characteristics are considered substantially homologous. Sequences having lesser degrees of homology, encoding comparable HL activity, and showing equivalent expression characteristics, e.g., fragments of the nucleotide sequence SEQ ID NO: 2 are considered substantial equivalents. Generally, homologous DNA sequences can be identified by cross-hybridization under standard hybridization conditions of moderate stringency.

The present invention also provides vectors and expression vectors containing the DNA sequences of the present invention, hosts containing such vectors for the production of proteins with HL activity, and processes for the production of such DNA sequences, recombinant vectors and host cells.

The invention also provides recombinant proteins with HL activity. Specifically a protein with HL activity is defined to include a polypeptide having the amino acid sequence SEQ ID NO: 2 or any protein or polypeptide having an amino acid sequence which is substantially homologous to the amino acid sequence SEQ ID NO: 2 and further having the following biological activity: When the protein or polypeptide is incubated under suitable conditions and a suitable amount of substrate such as 13-(S)-linolenic acid hydroperoxide is added, the formation of (3Z)-hexenal and (2E)-hexenal is observed.

As used herein the term recombinant proteins having at least about 90% sequence similarity to SEQ ID NO: 1 and having HL activity includes proteins modified deliberately, as for example, by addition of specific sequences that preferably bind to an affinity carrier material. Examples of such sequences are sequences containing at least two adjacent histidine residues (see in this respect European Patent No. 282 042). Such sequences bind selectively to nitrilotriacetic acid nickel chelate resins (Hochuli and Dobeli, Biol. Chem. Hoope-Seyler 368, 748 (1987); European Patent No. 253 303). Proteins with HL activity which contain such a specific sequence can, therefore, be separated selectively from the remaining polypeptides. The specific sequence can be linked either to the C-terminus or the N-terminus of the proteins with HL activity.

Methods for the expression, isolation and purification of watermelon HL proteins and proteins having at least about 90% sequence similarity to SEQ ID NO: 1 and having HL activity are also provided. For example, recombinant HL protein can be prepared by cloning of DNA sequences encoding proteins with HL activity. DNA sequences encoding proteins with HL activity can be cloned using a variety of techniques. Using the methods described in this application cDNAs encoding proteins with HL activity or fragments thereof can be produced. These cDNAs can be isolated and

amplified by PCR technique using oligodeoxynucleotide DNA primers by conventional techniques.

The cDNA (SEQ ID NO: 2) encoding the amino acid sequence SEQ ID NO: 1 was obtained using the DNA primers described in the examples. By using conventional techniques, this cDNA has been isolated from RT-PCR products using RNA derived from watermelon (*Citrus lanatus*) leaves. A RT-PCR band of an expected size was obtained from watermelon leaf total RNA using a degenerate primer pair. The full length watermelon HL cDNA was isolated and cloned into a CMV ³⁵S-driven binary vector.

The cDNA may be obtained not only from RT-products, but by other conventional techniques, e.g., by cloning genomic DNA, or fragments thereof, purified from the desired cells or plant tissues. These procedures are described by Sambrook et al., in "DNA Cloning: A Practical Approach", Vol. I and II, D. N. Glover, ed., 1985, MRL Press, Ltd., Oxford, U. K; Benton and Davis, Science 196, 180-182 (1977); and Grunstein and Hogness, Proc. Nat. Acad. Sci. 72, 3961-3965 (1975).

To obtain cDNA encoding watermelon proteins with HL activity cDNA libraries obtained using RNA isolated from watermelon leaf may be screened by conventional DNA hybridization techniques by the methods of Benton and Davis, supra, or Grunstein and Hogness, supra, using labeled HL gene fragments, for example. Clones which hybridize to the labeled gene fragments are analyzed, e.g., by restriction endonuclease cleavage or agarose gel electrophoresis. After isolating several positive clones the positive insert of one clone is subcloned, e.g., into phagemids, and sequenced by conventional techniques.

Clones derived from genomic DNA may contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will not contain intron sequences. In the molecular cloning of the gene from genomic DNA, DNA fragments are generated, some of which will encode the desired gene. The DNA may be cleaved at specific sites using various restriction enzymes. Alternatively, one may use DNase in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication. The linear DNA fragments can then be separated according to size by standard techniques, including but not limited to, agarose and polyacrylamide gel electrophoresis and column chromatography.

Whatever the source, the DNA sequence encoding proteins with HL activity may be cloned into a suitable vector for propagation of the DNA by methods known in the art. Any commercially available vector may be used. For example, the DNA may be inserted into a CMV-S35 promoter-driven binary vector. Appropriate vectors for use with bacterial hosts are described by Pouwels et al., in "Cloning Vectors: A Laboratory Manual", 1985, Elsevier, N.Y. As a representative but nonlimiting example, useful cloning vectors for bacterial use can comprise a selectable marker and a bacterial origin of replication derived from commercially available plasmids which are in turn derived from the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega Biotec, Madison, Wis., USA).

The DNA sequences encoding proteins with HL activity inserted in these commercially available vectors can be verified by methods known in the art, e.g., by standard nucleotide sequencing techniques.

DNA sequences that code for proteins with HL activity from plants other than watermelon may be used herein.

Accordingly, while specific DNA has been cloned and sequenced in relation to the DNA sequence in watermelon leaves, any plant cell or tissue potentially can be used as the nucleic acid source of the protein with HL activity. DNA sequences having at least about 90% sequence similarity to SEQ ID NO: 2 are then selected for use in the present invention.

Cloned DNA sequences that code for watermelon proteins having HL activity or DNA sequences that encode a polypeptide having at least about 90% sequence similarity to SEQ ID NO: 1 and having HL activity can be expressed in hosts to enable the production of these proteins with greater efficiency. Techniques for these genetic manipulations are specific for the different available hosts and are known in the art.

For expression of proteins with HL activity in hosts, in principle, all vectors which replicate and express DNA sequences encoding the proteins with HL activity in the chosen host are suitable. Expression vectors suitable for use in prokaryotic host cells are mentioned, for example, in the textbook "Molecular Cloning—A Laboratory Manual", Cold Spring Harbor Laboratory (1982), of Maniatis et al. Examples of other vectors are plasmids of the pDS family (Bujard et al., Methods in Enzymology, eds. Wu and Grossmann, Academic Press, Inc., Vol. 155, 416–433 (1987)).

Such prokaryotic expression vectors which contain the DNA sequences coding for proteins with HL activity operatively linked with an expression control sequence can be incorporated using conventional methods into any suitable prokaryotic host cell. The selection of a suitable prokaryotic host cell is determined by different factors which are well-known in the art.

Suitable prokaryotic host organisms include gram-negative and gram-positive bacteria, for example *E. coli* and *B. subtilis* strains. Examples of prokaryotic host organisms are *E. coli* strain M15 (described as strain OZ 291 by Villarejo et al. in J. Bacteriol. 120, 466–474 [1974] and *E. coli* W3110 [ATCC No. 27325]). In addition to the aforementioned *E. coli* strains, however, other generally accessible *E. coli* strains such as *E. coli* 294 (ATCC No. 31446) and *E. coli* RR1 (ATCC No. 31343) can also be used.

Plants can also be used as hosts for the recombinant production of proteins with HL activity. Transfer of the gene coding for the protein with HL activity may be achieved by a variety of methods (for review see Potrykus and Spangenberg, eds., Gene transfer to plants. A laboratory manual, Springer Verlag, Heidelberg, Germany (1995)), whereby the HL gene is integrated into the chromosome of the host plant. Homologous expression—overexpression—of the protein with HL activity can be achieved, for example, by transforming a watermelon plant with the nucleic acid of SEQ ID NO: 2 or a nucleic acid encoding a polypeptide having at least about 90% sequence similarity to SEQ ID NO: 1 and having HL activity. Other examples for plant hosts for the production of recombinant HL protein include, but are not limited to maize (*Zea mays*, Ishida et al., Nature Biotechnology 14, 745–750 (1996)), flax (*Linum usitatissimum*, Dong and Mchughen, Plant Sci. 88 (1), 61–71 (1993)) and soybean (*Glycine max*, Christou et al., Tibtech 8, 145–151 (1990)).

Transgenic plants of the invention can be used as a source or green note compounds, for example, by homogenizing plant tissues or biomass with linolenic acid and lipoxygenase or soybean as an enzyme source. Also, the nucleic acids of the invention can be expressed in microorganisms, such as bacteria and yeast, and their biomass can be used in the same way to produce green note compounds. The green note compounds can be isolated from the transformed organisms by any process known to those of skill in the art.

For the isolation of small amounts of proteins with HL activity expressed in prokaryotic host cells for analytical purposes, e.g., for polyacrylamide gel electrophoresis, the host cells can be disrupted by treatment with a detergent, e.g., sodium dodecyl sulphate (SDS). Larger quantities of the HPO lyase protein can be obtained by mechanical [Charm et al., Meth. Enzymol. 22, 476–556 (1971)], enzymatic (lysozyme treatment) or chemical (detergent treatment, urea or guanidinium hydrochloride treatment, etc.) treatments followed by use of known methods, e.g., by centrifugation at different gravities, precipitation with ammonium sulphate, dialysis (at normal pressure or at reduced pressure), preparative isoelectric focusing, preparative gel electrophoresis or by various chromatographic methods such as gel filtration, high performance liquid chromatography (HPLC), ion exchange chromatography, reverse phase chromatography and affinity chromatography (e.g., on Sepharose.RTM. Blue CL-6B).

The proteins of the invention that have HL activity can be used in the production of natural "green note" compounds by catalyzing the formation of aldehydes from fatty acid hydroperoxide. The term "green note" compounds relates to leaf aliphatic aldehydes and leaf aliphatic alcohols, e.g., (3Z)-hexenol and (2E)-hexenal. Hence the present invention also provides a process for the production of natural "green note" compounds, which process comprises the steps of:

a) reacting fatty acid hydroperoxide in the presence of a protein having the amino acid sequence of SEQ ID NO: 1 or a polypeptide having at least about 90% sequence identity to SEQ ID NO: 1 and having HL activity; and

b) reacting the resulting aliphatic aldehydes in the presence of isomerase and/or alcohol dehydrogenase.

In the process for the production of "green note" compounds the watermelon HL protein can be used in isolated form, or alternatively, in form of cell-free extracts obtained from host cells containing vectors for the production of protein with HL activity. In a preferred embodiment of the invention, the process for the production of natural "green note" compounds is employed to produce (3Z)-hexenol. A preferred process for producing (3Z)-hexenol comprises the steps of:

a) reacting 13-(S)-hydroperoxide linolenic acid and/or hydrolyzed linseed oil in the presence of recombinant proteins of the invention having HL activity; and

b) reducing the resulting (3Z)-hexenal with alcohol dehydrogenase.

The green note compounds, e.g. (2E)-hexenal and (3Z)-hexenol, prepared by the process of the present invention can be used as odorant and/or flavorant and worked into odorant and/or flavorant compositions in a manner known in the art.

Having now generally described this invention, the same will become better understood by reference to the specific examples, which are included herein for purpose of illustration only and are not intended to be limiting unless otherwise specified.

EXAMPLE 1

Cloning of Full Length cDNA of Watermelon Hydroperoxide Lyase

A pair of degenerate primers was designed from published HL sequences of several plants and synthesized. Total RNA was isolated from watermelon leaf tissues using the Trizol® reagent (Life Technologies) and the protocol suggested by the manufacturer. RT-PCR was conducted using the total RNA as template and a RT-PCR band of ~500 base pairs, which is the expected size for a HL protein was obtained. The RT-PCR product was partially sequenced, demonstrating that the RT-PCR product contained a portion of the coding region of watermelon HL.

Sequencing of the full length cDNA was accomplished using Clontech's SMART RACE kit. Two primers were designed from the partial sequence obtained:

Forward primer: (SEQ ID NO:3)
CCG GCT CCG GTC TGA CAT TCG AGT CCG

Reverse primer: (SEQ ID NO:4)
GCT CGC TCG GTA GTC CCG TCT GCG GCC CG

With these gene-specific primers and primers supplied by the kit, the sequence of the coding region of the watermelon HL was obtained.

From the sequence obtained, the following primer pair was designed for cloning the full length cDNA of watermelon HL:

5'-end primer: (SEQ ID NO:5)
CGC ACT AGT ATG AAG GTC ACC ATG ACC TC

3'-end primer: (SEQ ID NO:6)
GGT AAG CTT CAG TTG GTC CTT TGA AAA GC

The 5'-end primer was designed to contain the SpeI-recognition site just before the start codon, while the 3'-end primer contained the HindIII recognition site just after the stop codon. The PCR product obtained using this primer pair was ligated into cloning vector pGEM-T Easy (Promega) by TA ligation.

EXAMPLE 2

Expression of Watermelon Hydroperoxide Lyase cDNA in Plants

The isolated cDNA was then digested with SpeI and ligated into a CMV ³⁵S promoter-driven binary vector pKYLX71:35S2 digested with XbaI, which produces a compatible 5'-extension. The binary vector was transferred into *Agrobacterium tumefaciens* strain GV3850 using freeze-thaw method and its integrity was confirmed by PCR. Using *Agrobacterium* containing the watermelon HL, tobacco plants were transformed by a leaf disc method, and the transgenic lines were selected for antibiotic resistance. The HL activity of the tobacco leaf tissues was analyzed and confirmed that transformation and expression of the watermelon HL cDNA had occurred. The enzyme extracts

obtained from leaf tissues were reacted with 13-hydroperoxy linolenic acid in the presence of yeast alcohol dehydrogenase (ADH) and NADH, where the production of aldehydes was measured by the reduction of NAD consumed by ADH converting aldehyde to alcohol spectrophotometrically. The products formed from 13-hydroperoxy linolenic acid in the leaf tissues of the transformed tobacco leaf were confirmed to be (3Z)-hexenal and (2E)-hexenal by GC-MS.

EXAMPLE 3

Comparison of Hydroperoxide Lyase Activity in Transgenic Plants

To compare the watermelon HL activity with another HL gene, HL cDNA was isolated from *Arabidopsis* and transgenic tobacco plants were generated following the same procedure described in example 2. HL activity was measured spectrophotometrically as described in example 2. The results shown in FIG. 4 demonstrated the much higher activity of watermelon HL than *Arabidopsis* HL.

EXAMPLE 4

Comparison of C₆ Aldehyde Production from Transgenic Plants

Twenty mg of leaf tissues of transgenic tobacco plants over-expressing watermelon HL or *Arabidopsis* HL or watermelon were homogenized with 5 mg of linolenic acid (Sigma) and 25 mg of soybean acetone powder as a lipoxygenase source in 2.0 mL of water at room temperature for 3 minutes. 0.224 mg (2.0 micromole) of (2E)-heptenal was added as an internal standard. The reaction products were extracted with 1 mL of pentane and analyzed with GC-MS. The results shown in FIG. 5 demonstrated the higher production rate of (3Z)-hexenal by watermelon HL expressed in tobacco plants than *Arabidopsis* HL as well as watermelon leaves.

EXAMPLE 5

Comparison of hydroperoxide lyases *C. lanatus*, *Arabidopsis thaliana* and *N. tabacum* expressed in *E. coli*

To compare the relative activities HLs from *C. lanatus* (C1HL), *Arabidopsis* (AtHL) and *N. tabacum* (NtHL), we produced these proteins in *E. coli* and measured HL enzyme activity in crude extracts of the *E. coli* cultures. Table I demonstrates that these clones all possess HL enzyme activity when compared to the vector control. Activity for the full-length C1HL protein was considerably higher (approximately 250-fold or more) compared to NtHL and AtHL.

TABLE I

HL activity of crude <i>E. coli</i> extracts containing (His) ₆ -tagged protein or (His) ₆ -tagged fusion protein	
<i>E. coli</i> lysates expressing the following constructs	HL Activity (nmol/mg protein/min)
vector control	9.02 ± 2
C1HL-3-3 lysate	2,379 ± 516
NtHL-F6-3 lysate	28 ± 2.4
AtHL-F5-14 lysate	57

Values represent means±SE of replicates (n=3-6) except the AtHL for which we only have one replicate so far.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 6

<210> SEQ ID NO 1

<211> LENGTH: 481

<212> TYPE: PRT

<213> ORGANISM: Citrullus lanatus

<400> SEQUENCE: 1

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Met Lys Val Thr Met Thr Ser Gly Gly Met Pro Ser Ile Pro Ser Ser
 1          5          10          15
Ile Ser Pro Pro Pro Val Thr Leu Pro Leu Arg Asn Ile Pro Gly Ser
          20          25          30
Tyr Gly Leu Pro Leu Phe Gly Ser Ile Gly Asp Arg Leu Asp Tyr Phe
          35          40          45
Trp Phe Gln Gly Pro Glu Lys Phe Phe Arg Ser Arg Met Glu Lys Asn
          50          55          60
Gln Ser Thr Val Phe Arg Thr Asn Val Pro Pro Ser Phe Pro Phe Phe
 65          70          75          80
Phe Thr Asp Pro Arg Val Ile Ala Val Leu Asp Cys Lys Ser Phe Ala
          85          90          95
His Leu Phe Asp Met Glu Ile Val Glu Lys Lys Asn Val Leu Val Gly
          100          105          110
Asp Phe Met Pro Ser Thr Ser Phe Thr Gly Asn Met Arg Val Cys Ala
          115          120          125
Tyr Leu Asp Thr Ser Glu Ser Gln His Ser Lys Ile Lys Asn Phe Val
          130          135          140
Met Asp Val Leu Arg Arg Ser Ser Arg Ile Trp Ile Gln Glu Leu Glu
          145          150          155          160
Ser Asn Leu Ser Thr Met Trp Asp Ser Ile Glu Ser Glu Ile Ala Lys
          165          170          175
Asp Thr Lys Ser Ser Phe Arg Asn His Leu Gln Pro Thr Leu Phe Asn
          180          185          190
Phe Phe Ser Lys Thr Leu Ala Gly Ala Asp Thr Ala Lys Ser Pro Glu
          195          200          205
Val Ala Lys Ser Gly Tyr Ile Asp Val Ile Ile Trp Leu Gly Leu Gln
          210          215          220
Leu Val Pro Thr Ile His Ile Gly Ile Leu Gln Pro Leu Glu Glu Ile
          225          230          235          240
Phe Leu His Ser Phe Arg Leu Pro Phe Phe Pro Ile Ala Ser Arg Tyr
          245          250          255
Gln Arg Leu Tyr Asp Phe Ile Gln Lys Glu Gly Glu Glu Val Val Glu
          260          265          270
Arg Gly Val Ser Glu Phe Gly Leu Thr Lys Asp Glu Ala Ile His Asn
          275          280          285
Leu Ile Phe Thr Met Gly Phe Asn Ala Tyr Gly Gly Phe Ser Leu Phe
          290          295          300
Phe Pro Val Leu Leu Asp Arg Ile Leu Asn Asp Lys Thr Gly Leu Gln
          305          310          315          320
Gln Arg Ile Leu Glu Glu Val Lys Ala Lys Thr Gly Ser Gly Leu Thr
          325          330          335
Phe Glu Ser Val Lys Glu Met Asp Leu Ile Tyr Ser Val Val Tyr Glu
          340          345          350
Thr Leu Arg Leu Asp Pro Pro Val Pro Thr Gln Tyr Ala Arg Ala Arg

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-continued

355	360	365	
Lys Asp Phe Lys Leu Ser Ser Tyr Asp Ser Ala Tyr Ser Ile Lys Lys			
370	375	380	
Gly Glu Leu Leu Cys Gly Tyr Gln Pro Leu Val Met Arg Asp Pro Lys			
385	390	395	400
Val Phe Asn Lys Pro Lys Thr Phe Asn Pro Gly Arg Phe Arg Gly Glu			
405	410	415	
Lys Gly Ala Ala Leu Leu Asp Tyr Leu Phe Trp Ser Asn Gly Pro Gln			
420	425	430	
Thr Gly Leu Pro Ser Glu His Asn Lys Gln Cys Ala Gly Lys Asp Leu			
435	440	445	
Val Val Leu Thr Ala Val Val Phe Val Ala Tyr Ile Phe Arg Arg Tyr			
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465	470	475	480

Asn

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 <211> LENGTH: 1632
 <212> TYPE: DNA
 <213> ORGANISM: Citrullus lanatus

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atcggtgacc ggctggatta cttctggttt caaggaccg agaagttctt caggtctcgg     180
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ttcaccgatc cgagagtgat tgcggttctg gattgcaagt cgtttgcgca tctattcgac     300
atggaaatcg tggagaagaa gaatgttctg gtcggtgatt tcatgccgag cacaagtttc     360
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ttcagtctct tcttccgggt tctactcgat cggatactca acgacaaaac cggtttacia     960
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agcatcaaga aaggggagct gctttgtggg tatcagccgc tggatgatgag agaccggaag    1200
gtgttcaata aaccgagac gtttaatccg ggccggttcc ggggagagaa gggggcggcg    1260
ctgctggatt atttgttctg gtcgaacggg ccgacagcgg gactaccgag cgagcataac    1320
aagcagtgcg ccgggaagga tttggtggtg ctgacggcag tgggttctgt ggcttacata    1380
    
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tttcgaaggt atgattggat tgcaggggaa ggagggtcga ttacagcttt tcaaaggacc 1440
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cttctttttt atgtatgagt gtggagccca aatgaaaaaa attggaaaaa ttaatcaata 1560
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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 3
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<220> FEATURE:
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<400> SEQUENCE: 4
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<210> SEQ ID NO 5
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Artificial sequence designed from watermelon
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<223> OTHER INFORMATION: Artificial sequence designed from watermelon
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<400> SEQUENCE: 6
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```

What is claimed is:

1. An isolated nucleic acid sequence encoding the polypeptide of SEQ ID NO:1.
2. The isolated nucleic acid of claim 1 wherein the nucleic acid has the sequence of SEQ ID NO: 2.
3. A vector comprising a nucleic acid sequence encoding the polypeptide of SEQ ID NO: 1.

- 55 4. An isolated host cell transformed with a vector comprising a nucleic acid sequence encoding the polypeptide of SEQ ID NO: 1.
5. The host cell of claim 4 wherein the host cell is *E. coli*.

* * * * *