Cloning and Expression of Cucumber HPL Gene
استنساخ واستخراج جين HPL في نبات الخيار

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المستخلص:

الخيار من النباتات المزروعة وينتمى الى الفصيلة القرعية التي تضم القرع و الكوسا الي اخره يرجع أصل الخيار الي الهند وغرب القارة الاسيوية، وتوجد بها نكهات مميزة ، والنكهة تجعل منه مذاق قوي ورائع اضافة محايدة للأطعمة التي تحتوي تركيب (nonadienal). واجريت التجارب للبحث الجين (Hydroperoxide) لمعرفة خصائص المواد العطرية التي يرتبط تركيب جزء من هذه الاصناف المعروفة باسم المواد العطرية التي تكون النكهات مميزة واكتر قوة. وقد تم اختيار دراسة الاختلافات في المحتوى الرئيسي للمواد العطرية nonadienal وبأستخدام اثنين من الانزيمات الرئيسية وهي انزيم hydroperoxide و hydrogenperoxide التي تشكف نوع صنف الجينات الموجود في نبات الخيار. في حين ان جزء من الجينات تم استنساخه في المختبر من الخيار البري الذي كانت فيه المادة العطرية أعلى من الخيار المزروع. والجينات التي تم استنساخها ثلاث جينات هي (جين 013551 csa، 003558csa) و جين csa003557(وتم حقن النبات المزروع بها فوجد تغير في طعم ثمار النبات بفضل الجينات المستنسخة.

الكلمات الدالة:– المواد العطرية ،الخيار، جين، استخراج، Escherichia Coli.

Abstract

Experiments were established when the HPL gene existence in cucumber with characteristics of aromatic substances nonadienal synthesis is closely related. the Cultivars of varied size and color are now grown in warm areas worldwide, were selected to study the differences in the main content of aromatic substances and apparently utilizing the data for future studies of the genetic differences of the different flavor varieties of cucumbers. Moreover, analysis of control of azelaic diene Aldehyde synthesis in two key enzymes namely hydro peroxide enzyme and

hydrogen peroxide cleaving the expression of the enzymatic synthesis of each member of the gene family in cucumber),The experimental results obtained were clearly approved that the level of differences existed between cucumbers varieties in aromatic content was mainly due to their genetic differences. The wild cucumber aromatic substance content is much higher than the cultivated ones. Genetically, the HPL family genes expression in various organs of cucumber was studied and the amount of csa013551 expression in various organs of cucumber is very low, not even the expression in the roots and fruits. Two other genes csa003557 and csa003558 in each organ. We succeed in Cloning full-length coding sequence (cds) of HPL gene sequence into csa003558 and successfully proteins induced the expression of each gene were explored .

Keywords: The cucumber,aromatic substances, HPL gene expression, Escherichia Coli

Introduction

Lipid hydroperoxidelyase (HPL) is widely distributed in plants. Plant lipoxygenase (LOX) pathway downstream enzymes, catalyzed the LOX reaction product - lipid hydro peroxide cleavage generating the short-chain Aldehyde and the oxygen-containing acid [1]. HPL catalytic products (six-carbon Aldehyde and aldol) are an important component of the characteristic flavor of fruits, vegetables, and green leafy food additives, to restore the aromatic flavor of the fruit and vegetable accordingly. These flavor compounds are also widely used in the perfume and cosmetics that the manufacturing industries considered as a high economic value component [2]. Matsui et al in 1996 [3] reported that, since the HPL was cloned using cDNA, the research on this enzyme was gradually got attentions, especially the HPL gene transferred research. People start one

after another to conduct research in this gene from different plant namely, green pepper, cucumber, melon, Arabidopsis, tomato, guava, citrus

The HPL facilitated production of volatile Aldehyde from fatty acids. The long term objective of this project is to change the taste in the cucumbers specifically. The enzymes Lipoxygenase and Hydroperoxide Lyase (HPL) play key roles in the production of these aldehydes. Lipoxygenases add molecular oxygen to linoleic acid or linolenic acid to produce hydroperoxides HPO

Studying of molecular genetics system has constrains since many plants produce volatile Aldehyde such as (Z)-3-hexenal and (E)-2-hexenal as a defense mechanism (Matsui, 2006; Tiget, 2001). The cucumber plant, for example, produces Aldehyde among other products, in response to mechanical wounding (Matsui, 2006). These Products include hexane, 12-oxo-(Z)-9-dodecenoic acid, (Z)-3-nonenal, and 9-oxo-nonanoic acid. The C-9 Aldehyde specifically is important flavor compounds in cucumbers and melons (Matsui, 2006). These Aldehydes also have been shown to repel insects, lead to the production of plant antibiotics such as phytoalexins and in some cases act as antibacterial and antifungal Agents against plant pathogens (Matsui, 2008). Studies in some plants have shown that absence of the HPL enzyme in plant leaves can lead to as high as a two-fold increase in aphid fecundity in the leaves (Vancanneyt, 2001). Enzymes Lipoxygenase and Hydroperoxide Lyase (HPL) play key roles in the production of these Aldehyde's. Lipoxygenases , add molecular oxygen to linoleic acid or linolenic acid to produce hydroperoxides (HPO), while HPL subsequently cleaves the HPO into Aldehydes then plants produce volatile Aldehyde such as (Z)-3-hexenal and (E)-2-hexenal as a Defense mechanism (Matsui, 2006; Tiget, 2001

Materials and Methods

1Plant material:

In our study, 22 different cucumber plant materials covering , cucumber resources cucumber characteristics determination of aromatic substances, experimental material from the Chinese Academy of Agricultural Sciences Institute of Vegetables and Flowers, functional genomics biotechnology cucumber core germplasm bank, were planted at the Institute of vegetables and Flowers's glass greenhouse.

2Chemicals:

75μm Carboxen/PDMS manual SPME, Supelco manufactured in the United States; purchased from Suning a Finnigan Trace MS gas chromatography - mass spectrometer Finnigan manufacturing Matsuhashi mixer. For standard nonadienal purchased from SIGMA.

.3Experimental Methods:

1Samples before processing:

13-10) days after pollination) fresh cucumber, peeled, made into a paste with a small universal grinder, weighed samples in duplicate the 5g quickly loaded vials, then 50μl 100ppm nonadienal as a standard goods was added , so that the concentration of the standard sample and ultimately achieve 1ppm. Another plus, as a control.

1,4Experimental conditions-:

(1) Solid phase micro extraction:

Extraction head temperature of 250 ° C, time 45min; extraction conditions of 40 ℃ extraction 10min, needle distance liquid level height 2cm; adsorptions: 220 ° C, 1min.

(2)Mass spectrometry conditions:

The socer temperature of 200° C, ionization mode EI electronic energy 70ev. The filament current 0.2mA, test voltage 350V, mass range 32.6 - 449.4m / Z.

(3)The chromatography conditions:

The capillary column Supelcowax10, carrier gas He, flow rate 0.8mL/min, after desorption shunt, split ratio 50:1, shunt speed 10mL/min, constant pressure 35kpa, injector temperature 220 ° C, interface temperature 200 ° C, the starting temperature of 36 ℃, maintained 2min, 25 ° C / min rose to 70 ° C, and then rose 7 ℃ / min to 150 ° C, final rose to 30 ℃ / min to 190 ° C, maintaining 7min.

.4Calculation Method: C = S0 / (S-S0) * C0 Where

C: the concentration of the analyst;

S: Not plus standard before analyst peak area;

S0: the peak area in the standard material was added;

C0: the added nonadienal standard concentration

Results and Analysis-:

The Experimental reference Adoption of cucumber aroma substances measured by solid phase micro-extraction, GC-MS and Standard Addition the Cucumber aroma components nonadienal quantitative analysis. Because this experiment only concerned with the trans, cis -2,6 - nonadienal the relative content of this component, it is based on the solid phase micro extraction imbalance theory, the experimental method of Adoption of such made some improvements, including the extract shorten the time from 35min to 10min, split ratio from 12:1 to 50:1, and the use of anti-Shun of -2,6 - nonadienal standard as a frame of reference,

using the internal standard method and external standard the relative quantification method combined with the standard addition method, to

avoid the difficulties as well as the internal standard internal standard selection instability problems in the basement, but also due to a combination of the external standard method, so do not consider the correction factor was the problem. Shorten the experimental time, to improve the efficiency of the experiment, and also to make more accurate relative quantification. The measurement results are shown in Table 2-1.

Measurement showed that (1) wild cucumber (87), the content of aromatic substances is much higher than the cultivated varieties, is about 13 times the average content of cultivated varieties of aromatic substances, lay the foundation for future cross-breeding methods to improve existing varieties scent . (2) Cultivar No. 56 the K2148 nonadienal the content is about 5-8 times that of the other varieties, can be used as a good material for research cucumber aromatic substances related gene.

2.2HPL gene expression analysis and cloning:

2.1Materials and methods:

2.2 Materials:

.1Plant material {Plant treatment{

According to aromatic substances measured, the relatively high content No, 56 cucumber K2148 experimental material was selected. This material which was planted at the Chinese Academy of Agricultural Sciences, the Institute of Vegetables and Flowers's glass greenhouse, was subjected to root, stem, leaf, male, young material of fruit sampling for gene expression analysis. Moreover, about 12 days after pollination melon for cloning material. Frozen for sampling after and stored in the refrigerator at -80 .°C

.2Enzymes, reagents and kits:

The nucleic acid molecular weight standards Marker, Oligo (dT) 18, dNTP, SYBR Premix Ex Taq, L share from Takara. RNA rapid extraction and purification kit, the plasmid small mention kit, the rapid recovery of DNA

purification kit pEASY-T1 carrier, E.coli DH5α competent cells were purchased from a wholly-gold biological Limited. Taq PCR mix, Platinum Taq PCR mix purchased from the days of root biomass Limited. Reverse transcriptase MMLV purchased from the Promega. Ampicillin and other biochemical reagents

were purchased from the Ameresco company. The remaining chemical reagents were analytical grade.

.3preparation of the medium and antibiotics:

-(1) LB medium to prepare (1 L): 5g yeast extract, 10g tryptone, 10g NaCl, plus 15g agar preparation of a solid medium؛

1000 -(2)x ampicillin (Ampicillin): water formulated as 100mg/ml liquor, and filter-sterilized؛

2.3Experimental Methods:

.1Plant RNA extractions:

The cucumber of leaves were collected as in liquid nitrogen and stored at -80°C until use. Immediately before extraction, the frozen material was ground to a fine powder in liquid nitrogen with mortar and pestle. Unless otherwise specified, a whole cucumber was used in the extraction،

.2Total RNA Extraction:

.3Material and Methods :

Total RNA extraction of each was done using a Each sample was ground to a fine powder in liquid nitrogen, and 200 mg of each sample was collected in a 1.5 ml micro centrifuge tube, and immediately mixed with 300 ~ 500ul l of the buffer Into the Clean Bench 0.45ml RL

(confirm Added β-mercaptoethanol), mixing shock operation, added to the sample; RLT. for 3 min and then cooled. The solution was transferred to a spin column, placed in a 1.5 ml collection tube and centrifuged for 2 min

at 12000 rpm. The flow-through was carefully transferred to a new micro centrifuge tube. Care was taken not to disturb the pellet that had formed in each of the flow-through tubes.230ul of ethanol was added to each of the tubes, and the tubes were mixed. The mixture in each tube was transferred into its own RNeasy spin column placed in a 2.0 ml collection tube, and the spin column was spun for 30 s at 12000rpm. The flow through was discarded. 350 the protein solution RW1 was added to each spin column and the column was centrifuged for 30 s at 12000 rpm (flow through was discarded). Prepare DNase working solution, take 10ul DNase 70ul of the buffer RDDmix, containing DNase was added onto the membrane of each column, and the column was allowed to sit for 15 min at room temperature .

30l of buffer RW1 was added to each spin column and the columns were spun for 15 s at 12000rpm. Then 500 l of buffer RPE was added to each spin column and the columns were spun at 12000 rpm for 2 min. The columns were spun once more for 1 min to dry any remaining ethanol. New RNase free collection tubes replaced the old ones, 50ul of RNase free water was added to each tube and the columns were spun at 12000rpm1min to elute the RNA. RNA was stored at -80°.

.4cDNA template synthesis:

(1Add in 0.3ml RNase Free PCR tube: RNA 2 ~ 4μg, Oligo (dT) 18)TAKARA 14nM) 1μl, to complement with RNase Free H2O 13.5μl؛

(2)Mix set PCR instrument, 70 ° C 10min quickly removed, placed on ice Cooling 5min؛

(3) And then successively added: 5X MMLV Buffer 5μl dNTP (Takara 2.5mM(

5μl RNase Inhibitor (Takara 40U/μl), 0.5 μL of MMLV (Promega 200U/μl) 1μl؛

(4)Mix, 42 ℃ for 60min, 72 ℃ 5min to terminate the reaction, −20.℃

.5PCR reaction− ؛

.6Real time PCR reaction system (repeat 3 times

Fresh	MQ water	11.7μl	11.7 μl
RT−PC	RT− PCR Buffer (2 ×)	32.5	32.5μl
Forwa	Forward primer (3 μM	6.5μ	6.5 μl
Revers	Reverse primer (3 μM)	6.5μ	6.5 μl
cDNA	cDNA template	6.5	6.5μl
50x dy	50x dye II	1.3	1.3μl

.7 Clone the PCR reaction−؛

(1) Reaction system: 2x PCR mix 10μl

Primer	primer L (20μM)	1μl	1 μl
Primer	primer R (20μM)	1μl	1 μl
Plate	Plate 3 ~ 1	~ 1μl	
ddH2	ddH2O	20μl	20μl

(2) Light mix

3.3 PCR products were purified (full−gold DNA purification recycling kit:(

(1) Cut the strip from the agarose gel, placed in a 1.5ml centrifuge tube

(2)adding an appropriate amount of sol solution, 55ºC water bath to glue block completely dissolved

(3)The above solution was added to the adsorption column, 4 °C or −20 ºC placed 3min a 12000rpm centrifugation 30s Discard the waste liquid collection tube

(4)Add 700 μl rinse liquid, 12000rpm centrifugation 30s, drained waste liquid collection tube, repeat

12000 (5)rpm centrifuged 2min, removing the rinse liquid

(6)Remove the adsorption column, placed in a new tube, add the appropriate preheated elution buffer, at room temperature

Place 2min, 12000rpm centrifugal 1min

(7)Takes 2 ~ 4μl on a 1% agarose gel electrophoresis

4.4Objective fragment and the vector:

(1) Connection system (5 μl): DNA 4μl

PEASY-T1 1μl

(2)Mixing, at room temperature for 15 ~ 20 min

4.4Plasmid extraction (full gold kit(

(1) Picked colony PCR analysis of monoclonal adding 2ml LB (with appropriate antibiotics) 8 ~ 12h, shake bacteria

(2)To preserve the bacteria, the same time learn about 1ml broth cells were collected by, 12000rpm centrifugation 30s

(3)The supernatant was added 250μl solution containing RNase I, mixing shock, then add 250μl solution II

Mix by inverting 10 times or so, the solution became clear by adding 350μl solution III, mix by inversion

12000 (4)rpm centrifugal 10min, the supernatant was added to the adsorption column

12000 (5)rpm centrifugal 30s, drained collection tube waste liquid

(6)Add 700 μl rinse liquid, 12000rpm centrifugation 30s, and drained waste liquid collection tube, repeat

12000 (7)rpm centrifuged 2min, drained collection tube waste liquid

(8)Remove the adsorption column, placed in a new 1.5 ml tube, add the appropriate preheated elution buffer, place 2min at room temperature, 12000rpm centrifugation for 1min

2 ~ 1 (9)μl on a 1% agarose gel electrophoresis

4.6Transformed into E. coli T:

5 (1μl legation products added 50μl E.coli DH5α competent cells, mix, ice bath 30min

(2)Water bath 42 ºC for 60 ~ 90s, quickly centrifuge tube inserted into the ice for 5min

(3)Add 500μl LB without antibiotics shake with slow roll 160rpm at 37 ºC for 45min

(4)Centrifugation 10s about 12000rpm, the cells were collected, applied to the LB plate (containing the corresponding antibiotic(

(5)Tablet inverted culture 8 ~ 16h at 37℃

4.7Colonies PCR identification :

(1) Plate number, generally opt for 6 to 10 spots colony PCR identification, if the connection is not on, you can continue to pick 10 plaques were identified

(2)Preparation of PCR samples according to the plaque number, packing, and then enter the Clean Bench Operation dip plaque with a small pipette tip, and then put the PCR tubes hanging rub ensure bacilli enter the PCR tube

(3)PCR reaction was performed, and do not consider the annealing temperature, according to the fragment length can set the appropriate extension time, days root general Taq enzymatic extension efficiency is 2KB/Min

(4)Run the agarose gel detection and identification, singled out to amplify the correct bacteria further shake bacteria, selected 3-5 strains sequenced .

4.8Primer design for gene expression analysis-؛

Table2. 1 HPL gene RT-PCR primers sequences

Primer name Sequence (5' to 3)

Csa003557-F CCGTCTTCCACGCCAACA

Csa003557-RTGGGTCCAAATAAGCACAG

Csa003558-F CATCATCCATACTTTCCGTTTA

Csa003558-RCCTGTTTCTCGGCTTCG

Csa013551-F TCGTTCCCACCATCCAC

Csa013551-RAGCCACCATAGGCGTTG

Table 2.3 RT-PCR primers sequences

QRT-Cs-actin F	ATTCTTGCATCTCTAAGTACCTTCC
QRT-Cs-actin R	CCAACTAAAGGGAAATAACTCACC
QRT-Csa003558 F	CATCATCCATACTTTCCGTTTA
QRT-Csa003558 R	CCTGTTTCTCGGCTTCG

Table 2.4- primers designed for cloning of Csa-033558

Csa003558 F TCTTCTTCAGAACACCCAC

Csa003558 R TTAGGCTTTAGTCAACGATT

Table 2.5 HPL gene of primers for vector construction and restriction enzyme digestion

Primer name	Sequence (5' to 3('
pET48 F	GGAATTCTATGACTTCATCTTCTTCAGAACACCCAC
pET48 R	AAGGAAAAAAGCGGCCGCTTAGGCTTTAGTCAACGATT
pET-28 F	GGAATTCATGACTTCATCTTCTTCAGAACACCCAC
pET-28 R	AAGGAAAAAAGCGGCCGCTTAGGCTTTAGTCAACGATT

5.2 Results and Analysis-:

.1 Total RNA extraction integrity testing-:

Cucumber roots, stems, leaves, male flowers, young fruit were used for total RNA extraction. the extraction process

affected by RNA degradation reaction when looking for

better RT-PCR and Real-Time PCR operation.

.2 Analysis of gene expression results-:

Different organs

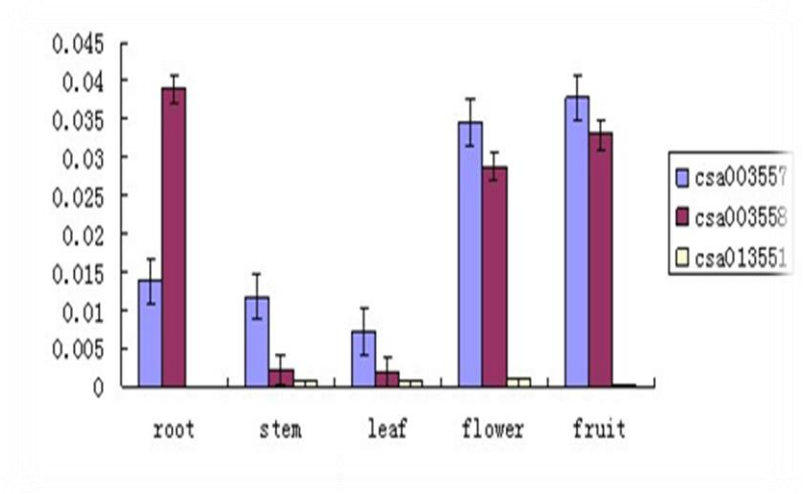

Figure 4- HPL gene expression in the different organs of cucumber

.3 HPL gene expression results-:

The HPL family genes fluorescence quantitative PCR results showed that the amount of csa013551 expression in various organs of cucumber was very low, not even the expression in the roots and fruits. Two other genes csa003557 and csa003558 each organ, there is a certain amount of expression, and the former expression in various organs uproot them outside the rest were higher than the latter.

.4 Gene Cloning

Gene expression in various organs of cucumber was varied. Amount of higher HPL gene expression in the fruit csa003557, cloning csa003558. Successfully, the full length cds sequence of cas003558 a HPL gene was cloned . Partial cds sequence following PCR electrophoresis which sequenced by the Beijing Genomics Institute, Appendix sequencing results, specific analysis is as follows. Csa0033558 Sequence was {TAA TGA

TAG} 1434bp, 478 amino acids, the molecular weight of the protein is 54, 31 KD.

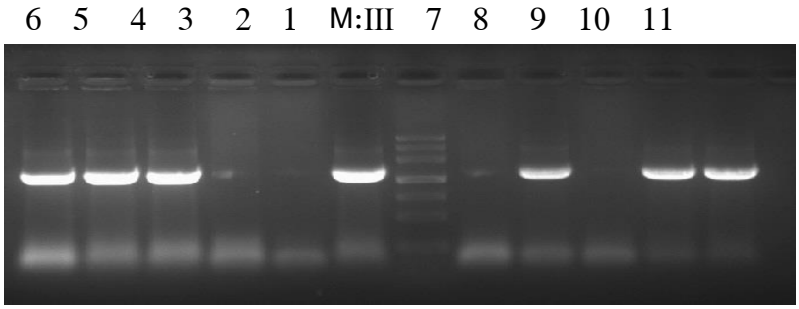

Figure 5- Electrophoresis of genes amplified with PCR

Conclusion

The experiment studied the main differences of the content of aromatic substances in the different varieties of cucumbers. Analysis the LOX pathway control with cucumber features aromatic substances synthesis is closely related to the two key enzymes - lipoxygenase and hydro peroxide lyase synthesis members of the

gene family expression in the cucumber, and clone a part of the gene and its prokaryotic expression in- vitro. The main conclusions were as follows:

.1Cucumber aromatic substances content was the level of existence of differences between the varieties, not necessarily linked to such differences and varieties of regional, mainly due to their genetic differences result;

the wild cucumber aromatic substance content is much higher than the cultivars trying to cultivated species and the wild species hybridization method to improve the existing varieties fragrance the tasteless flavor quality and poor.

.2Study the HPL family genes expression in various organs of cucumber. : Amount of csa013551 expression in various organs of cucumber is very low, not even the expression in the roots and fruits. Two other genes

csa003557 csa003558 each organ, there is a certain amount of expression, and the former is the

rest of the various organs other than to uproot them. in expression were higher than the latter.

.3HPL gene csa003558 with a cds sequence length1434bp, encoding 478 amino acids, with molecular weight of 72KDa.

.4csa033558 gene and prokaryotic expression of the carrier the PET48b pairs of the DNA fragments using T4 DNA ligase, we succeed in construction of the prokaryotic expression vector, and transformed into E. coli. Extraction of the plasmid transformed into BL21.

.5Protein induces expression by SDS-PAGE analysisBL21 at 22 degrees, 30 degrees and 37

degrees of IPTG induced by 22h, 15h and 8h, the total protein extract of E. coli by SDS-PAGE analysis.Results under the conditions of 22 degrees

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