# Cloning and expression analysis of hydroperoxide lyase gene in *Nicotiana tabacum*

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**Abstract:** Tobacco is an important economic crop in the world. In order to explore the role of hydroperoxide lyase (HPL) in the formation of tobacco quality, HPL was cloned from tobacco leaves. The bioinformatics analysis of the gene and its promoter was carried out. The relationship of HPL between *Nicotiana tabacum* and *Capsicum annuum* is closer. Tobacco HPL gene contains two exons and one intron, and its expression was regulated by a variety of factors in tobacco. HPL could be expressed in roots, stems and leaves, but its expression was the highest in leaves. These results will lay a foundation for studying the function of HPL in improving tobacco quality.
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**Keywords:** *Nicotiana tabacum*; *HPL*; tobacco quality; expression analysis

## 1. Introduction

Hydroperoxide lyase (HPL) is a type of membrane binding heme-ferrothionein, belonging to the cytochrome P450 family (Zhao, 2004). HPL is involved in the lipoxygenase pathway in plants and is a key enzyme in the downstream of the pathway, which can cleave fatty acid hydroperoxides catalyzed by lipoxygenase to form oxoacids and short-chain volatile aldehydes (Chen et al. 2012). Its products are specific aroma components, which can be used as additives for foods and perfumes, and can also protect plants from pathogens and pests (Xie, 2014; Wu, 2011). Depending on the position of the peroxy group of the catalyzed substrate, HPL can be classified into two types of isoenzymes. The first type isozyme (also named 13-HPL) can catalyze the fracture of 13 peroxy groups, and the second type isozyme (also named 9-HPL) catalyze the fracture of 9 peroxy groups (Liu et al. 2008).

Since the first HPL gene was cloned from sweet pepper (Matsui et al. 2006), the cDNA sequences of HPL was obtained from various plants such as Arabidopsis thaliana and melon, tomato, green bell pepper, olive, cucumber and so on. And the research on HPL gene was deepened gradually. HPL is involved in many stages of plant growth and development, such as plant specific aroma, resistance to pests and diseases, response to stress and signal transduction (Zhao et al. 2004). It was found that HPL can regulate the defense response of tomato to Prodenia litura and Alternaria by regulating the release of green leaf volatiles and the expression of jasmonic acid gene (Xin et al. 2014). In order to improve the flavor and quality of lettuce, HPL gene was cloned from Trifoliate orange with strong smell and it was over-expressed in lettuce, laying a foundation for the improvement of lettuce varieties (Xie et al. 2016). Through the study on catalytic products (C6 and C9 volatiles) of HPL, it was pointed out that C6 and C9 volatiles are very important for the odor of fruits and vegetables (Chen et al. 2012). The different ratios of C6 and C9 aldehyde lead to different odors. C6 aldehyde imparts a grassy scent to plants while C9 aldehyde increases strong fragrance (Chen et al. 2012). It was prospected that HPL may have a good application in improving plant resistance to disease and plant flavor (Zhao, 2004).

Tobacco is an important economic crop in the world, and its yield and quality was focused on. HPL is involved in the metabolism of plant fatty acid (Li, 2005), and its catalysate (volatile short-chain aldehydes and oxyacids) has special odors which are the important indicator of tobacco quality. It is unclear whether the quality and resistance of tobacco can be improved by regulating the expression of HPL. Here, we cloned the HPL gene from tobacco, and its expression characteristic was analyzed, hoping to improve tobacco quality and resistance.

## 2. Material and Methods

### 2.1 Plant material and treatment

Tobacco (K326) was planted in greenhouse. The leaves with good growth conditions were selected, washed with distilled water, dried, and wrapped with tin foil. The liquid nitrogen was used to freeze leaves quickly and then stored at -80 ℃ for RNA extraction.

### 2.2 Total RNA extraction of tobacco leaves and reverse transcription

Tobacco leaves (about 0.1 g) stored at -80 ℃ were quickly ground in liquid nitrogen. Total RNA was extracted according to the Trizol method. Then electrophoresis was used to analyze the quality of

RNA. The cDNA was obtained by following the instructions of the reverse transcription kit.

**2.3 Cloning of tobacco HPL gene**

The cDNA sequence of tobacco HPL gene was searched on NCBI. The primers were designed based on the sequence, and the upstream and downstream primers were used the Xba I and Sma I restriction sites (underlined portions), respectively. The primer sequences were as follows: 5'-GCTCTAGAATGTCCACAATAATGGCGAAAAT GAT-3', 5'-TCCCCCGGGTCAACTGGCTTTTTTCACAGATG TGAT-3'. The cDNA obtained by reverse transcription was used as a template for cloning. The PCR cycle conditions were as follows: pre-denaturation at 94 ℃ for 5 min, denaturation at 94 ℃ for 30 s, annealing at 55 ℃ for 30 s, extension at 72 ℃ for 92 s for a total of 30 cycles; 72 ℃ for a final extension of 10 min.

**2.4 Bioinformatics analysis of tobacco HPL gene**

The open reading frame of tobacco HPL gene sequence was obtained with the online tool ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/). The homology alignment of nucleic acid sequences was performed with NCBI Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The promoter sequence was analyzed with the online tool PlantCARE. (http://bioinformatics.psb.ugent.be/webtools/plantcare /html/). Phylogenetic tree was constructed with software DNAMAN.

**2.5 Specificity analysis of tobacco HPL gene expression**

The roots, stems and leaves of tobacco plants (K326) in seedling stage, blossom stage and mature stage were sampled separately. These tissues were quickly frozen with liquid nitrogen and then stored at -80 degrees for RNA extraction. Total RNA was extracted with TRIzol method, and cDNA obtained by reverse transcription was used as template. The tobacco actin gene (GenBank: AF126810) was used as a reference gene for real-time quantitative RT-PCR. The primers for actin were CTGAGGTCCTTTTCCAACCA and TACCCGGG AACATGGTAGAG. The primers for HPL were GTTTTGAGTTGTGGCAGCTA and GAGTATCACACAAGGGGGAG. Three replicates were performed for each sample. The qPCR reaction system was performed with the described procedure (Li et al, 2016).

## 3. Results

**3.1 Cloning of HPL gene**

Total RNA was extracted from tobacco leaves, and cDNA obtained by reverse transcription was used as a template. A 1494bp gene fragment was cloned by PCR (Figure 1), which was consistent with HPL in tobacco, indicating that HPL gene has been successfully cloned from tobacco.

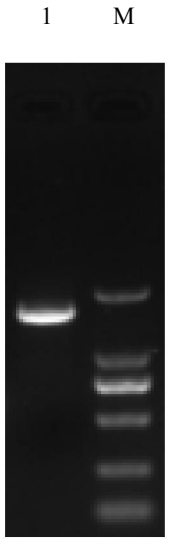


**Figure 1. PCR result of HPL gene from tobacco.**

Lane 1: PCR product, lane M: DL2000 DNA Maker.

**3.2 Bioinformatics analysis of HPL from tobacco**

The HPL cDNA sequence from tobacco consists of 1494 bp and contains a complete open reading frame encoding a protein consisting of 497 amino acids (Figure 2).

After searching for the hydroperoxide lyase gene sequences of other plants on NCBI, including Arabidopsis thaliana, Maize, Cotton, Grape, Pepper, Potato, Cucumber, etc., multiple sequence alignment was performed using DNA Man. It was found that the similarity of gene sequences reached 60.93%, indicating that the cloned fragment was indeed the HPL gene of tobacco. The analysis of phylogenetic tree showed that the relationship of HPL between *Nicotiana tabacum* and *Capsicum annuum* is closer (Figure 3).

Nucleic acid alignment was performed by using NCBI to obtain the corresponding DNA sequence, and it is found that tobacco HPL gene contains two exons and one intron. The promoter sequence of tobacco HPL is obtained, and its cis-acting elements are analyzed by using the online tool PlantCARE. There are many cis-acting elements responding to environmental conditions, such as ABRE elements in response to abscisic acid, CGTCA-motif elements in response to methyl jasmonate and WUN-motif elements in response to damage stress. TCCC-motif elements are light responsive elements. These

elements indicate that HPL gene is regulated through various ways.

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1    ATGTCCACAATAATGGCGAAAATGATGAGCGGATCTACTCCTATTAATCCTGGTAGTACTGGCCGTACATCCTCACCGTCGCTAACTCCG
1     M  S  T  I  M  A  K  M  M  S  G  S  T  P  I  N  P  G  S  T  G  R  T  S  S  P  S  L  T  P
91   CCGCCAGCTTCTCTTCCCGTCCGTACAATTCCCGGCGGCTACGGTTGGCCGTTGTTAGGACCAATATCTGATAGATTGAATTATAATTGG
31    P  P  A  S  L  P  V  R  T  I  P  G  G  Y  G  W  P  L  L  G  P  I  S  D  R  L  N  Y  N  W
181  TTCCAGGTACCTAACACTTTTTTTACCAAGAGAATAGAAAAGCACAAGAGCACAGTTTTTAGAACTAATGTGCCTCCTTGTTTTCCGTTT
61    F  Q  V  P  N  T  F  F  T  K  R  I  E  K  H  K  S  T  V  F  R  T  N  V  P  P  C  F  P  F
271  TTTCTTGGTGTTAATCCGAATGTGGTGGCGGTTCTTGATGTCAAGTCGTTTTCGCATCTGTTTGATATGGAGATTGTAGAGAAAGCTAAT
91    F  L  G  V  N  P  N  V  V  A  V  L  D  V  K  S  F  S  H  L  F  D  M  E  I  V  E  K  A  N
361  GTCCTTGTTGGGGATTTCATGCCTAGTGTTCAGTATACTGGAGATATGCGTGTTTGTGCTTATCTTGATACTTCTGAACCTAAACATACT
121   V  L  V  G  D  F  M  P  S  V  Q  Y  T  G  D  M  R  V  C  A  Y  L  D  T  S  E  P  K  H  T
451  CAGATTAAGAACTTTTCATTGGACATTCTAAAAAGAAGCTCAAAAACATGGGTGCCAACACTTGTCAATGAACTCAACACCATGTTTGAA
151   Q  I  K  N  F  S  L  D  I  L  K  R  S  S  K  T  W  V  P  T  L  V  N  E  L  N  T  M  F  E
541  ACTTTCGAATCAGATATCTCAAAATCAAACTCAGCTTCTCTTCTCCCTACTATGCAAAAATTCCTCTTCAACTTTTTTTCCCTCACTCTC
181   T  F  E  S  D  I  S  K  S  N  S  A  S  L  L  P  T  M  Q  K  F  L  F  N  F  F  S  L  T  L
631  TTAGGAGCCAATCCATCAGCTTCACCAGAAATAGCCAATTCTGGCTACGTAATGCTTGACCCGTGGCTAGCTATTCACCTTGCACCTACT
211   L  G  A  N  P  S  A  S  P  E  I  A  N  S  G  Y  V  M  L  D  P  W  L  A  I  H  L  A  P  T
721  GTTAGTATTGGCGTACTTCAACCCCTTGAAGAAATATTTGTCCACTCTTTTAGTTACCCTTTTTTCCTTGTCAAAGGTGGTTATGAAAAA
241   V  S  I  G  V  L  Q  P  L  E  E  I  F  V  H  S  F  S  Y  P  F  F  L  V  K  G  G  Y  E  K
811  CTCATACAATTTGTCAAAAATGAAGCTAAGGAAGTTTTAAATAGAGGTAAATCAGAGTTTGGACTTACTGAACAAGAAGCTATACATAAC
271   L  I  Q  F  V  K  N  E  A  K  E  V  L  N  R  G  K  S  E  F  G  L  T  E  Q  E  A  I  H  N
901  CTTTTGTTCATTCTTGGGTTCAATGCTTTTGGTGGTTTCTCTATTTTTTTGCCAACCCTTTTGGGAAATCTTGGAGATGAGAAAAATGCA
301   L  L  F  I  L  G  F  N  A  F  G  G  F  S  I  F  L  P  T  L  L  G  N  L  G  D  E  K  N  A
991  GAGTTACAAGAGAAATTGAGAAATGAAGTTAGAGAGAAAGTTGGATTAAAGCCAGAAAATTTGAGTTTTGAGAGTGTTAAAGAAATGGAA
331   E  L  Q  E  K  L  R  N  E  V  R  E  K  V  G  L  K  P  E  N  L  S  F  E  S  V  K  E  M  E
1081 CTTGTTCAGTCTTTTGTATATGAAACACTTAGACTTAGTCCACCTGTGCCAACTCAATATGCAAGAGCAAGAAAAGATTTTAAGCTAAGT
361   L  V  Q  S  F  V  Y  E  T  L  R  L  S  P  P  V  P  T  Q  Y  A  R  A  R  K  D  F  K  L  S
1171 TCACATGATTCAGTTTATGAAATCAAGAAAGGTGAACTTCTTTGTGGATATCAGCCATTGGTTATGAGAGATCCAAAGGTGTTTGATAAT
391   S  H  D  S  V  Y  E  I  K  K  G  E  L  L  C  G  Y  Q  P  L  V  M  R  D  P  K  V  F  D  N
1261 CCTGAAAAGTTTGTGTTGGAAAGGTTTACAAAGGAAAAAGGGAAAGAATTGCTGAATTATTTGTTTTGGTCAAATGGACCACAGACTGGG
421   P  E  K  F  V  L  E  R  F  T  K  E  K  G  K  E  L  L  N  Y  L  F  W  S  N  G  P  Q  T  G
1351 AGACCAACTGAATCAAACAAACAATGTGCTGCTAAGGATATTGTTACTCTTACTGCTTCTTTGATTGTGGCTTATGTTTTTCAAAGGTAT
451   R  P  T  E  S  N  K  Q  C  A  A  K  D  I  V  T  L  T  A  S  L  I  V  A  Y  V  F  Q  R  Y
1441 GATTCAGTGAGTTTCTCTTCTGGTTCAATCACATCTGTGAAAAAAGCCAGTTGA
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**Figure 2. Full-length cDNA sequence and deduced amino acid sequence of NtHPL**

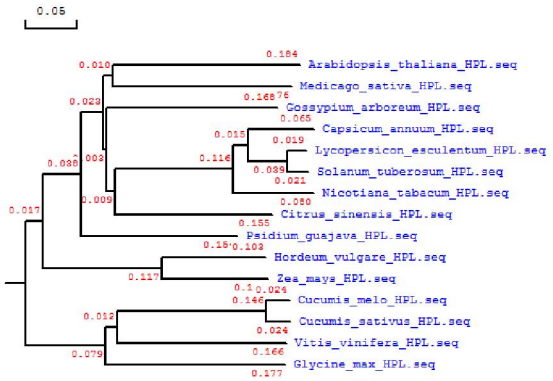


**Figure 3. Phylogenetic tree of HPL genes**

### 3.3 Specificity analysis of tobacco HPL expression

It can be seen that the expression levels of tobacco HPL are different at different growth stages (Figure 4). At mature stage, the expression level of HPL in leaves is 15.56 times and 15.2 times compared to roots and stems, respectively. At blossom stage, the expression level of HPL in leaves is 37.35 times and 25.46 times compared to roots and stems respectively. At seedling stage, the expression level of HPL in leaves is 6.52 times and 23.16 times compared to roots and stems, respectively. In general, tobacco HPL is mainly expressed in leaves at seedling stage. The result may be consistent with the function of HPL, which can catalyze the cleavage of hydroperoxide to produce volatile substances such as short-chain

aldehydes and oxoacids, and the leaves are the main sites for synthesis of plant fatty acid.

## 4. Discussion

Fatty acid metabolism is an important metabolic process during plant growth and development. Hydroperoxide lyase is an enzyme at the downstream of the lipoxygenation pathway, and its catalytic product is the main component of volatiles in leaves. It has been found that HPLs are involved in variously physiological processes in plants, such as seed dormancy and wound stress response. Its catalytic product can be used as an odorant of plants and as a signal molecule to participate in the plant defense responses. Tobacco is an important economic crop and aroma is an important indicator of tobacco quality. The catalytic product of HPL is volatile short-chain aldehyde, which make the plant fragrant. HPL promoter in tobacco has many components responding to environmental stress, such as ABRE and WUN-motif components, indicating that HPL may be regulated by many factors. The specificity expression analysis shows that it is mainly expressed in tobacco leaves and less expressed in stem and root, which may be consistent with the function of HPL. In order to improve the quality of tobacco, we cloned the HPL gene from tobacco, constructed the overexpression vector of HPL gene and transferred it into Agrobacterium successfully, which lay a foundation for further study on the function of tobacco HPL.

**Table 1. Analysis of cis-acting elements in tobacco HPL promoter**

| Site name | Core sequence | Function |
|---|---|---|
| ABRE | ACGTG | cis-acting element in the abscisic acid responsiveness |
| ARE | AAACCA | cis-acting regulatory element essential for the anaerobic induction |
| AT-rich element | ATAGAAATCAA | binding site of AT-rich DNA binding protein (ATBP-1) |
| Box 4 | ATTAAT | part of a conserved DNA module involved in light responsiveness |
| CAAT-box | CAAAT | common cis-acting element in promoter and enhancer regions |
| CAT-box | GCCACT | cis-acting regulatory element related to meristem expression |
| CGTCA-motif | CGTCA | cis-acting regulatory element involved in the MeJA-responsiveness |
| G-Box | CACGTT | cis-acting regulatory element involved in light responsiveness |
| MBS | CAACTG | MYB binding site involved in drought-inducibility |
| O2-site | GTTGACGTGA | cis-acting regulatory element in zein metabolism regulation |
| TATA-box | TATA | core promoter element around -30 of transcription start |
| TC-rich repeats | GTTTTCTTAC | cis-acting element involved in defense and stress responsiveness |
| TCCC-motif | TCTCCCT | part of a light responsive element |
| TCT-motif | TCTTAC | part of a light responsive element |
| TGACG-motif | TGACG | cis-acting regulatory element involved in the MeJA-responsiveness |
| WUN-motif | TAATTACTC | wound-responsive element |

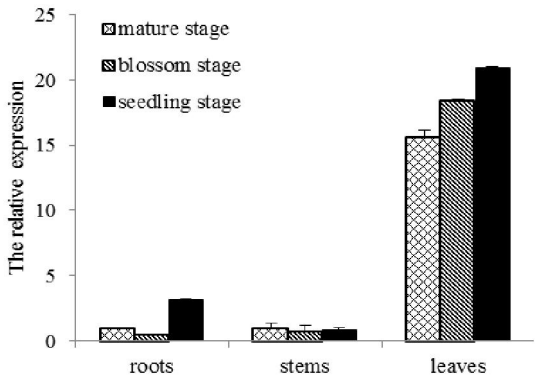


**Figure 4. The expression analysis of tobacco HPL**

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