Identification and Characterization of Apple Anthracnose Resistance Genes

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Abstract: Apple is the most widely planted fruit tree in China. Apple anthracnose which caused by the fungus Colletotrichum gloeosporioides is one of the major pathogens causing apple fruit rot and yield loss. In this study, we identified and cloned the Pectinesterase Inhibitor 6-like gene from populations of 'Fuhan' and 'Micui'apples. The Pectinesterase Inhibitor 6-like protein fused to a green fluorescent protein was localized in the cell membrane. The fluorescence quantitative results showed that the expression of Pectinesterase Inhibitor 6-like gene was the highest in sepals and the lowest in styles. The resistant strain was obtained by Agrobacterium-mediated stabilized genetic transformation, and the DNA level identification further proved that the resistant strain was a positive strain successfully transformed.

1 Introduction

Apple belongs to the Rosaceae Malus and is the most widely planted fruit tree in China, mainly distributed in the north temperate zone, including Asia, North America and Europe [1-2]. Rosaceae Malus is one of the most important fruit trees widely planted in the world, and its fruits are very popular among consumers for their unique flavor and rich nutrition. Meanwhile, with the expansion of cultivated area and the development of large-scale cultivation, drought, pests and diseases, and other biotic and abiotic stresses have become an urgent problem to be solved for the upgrading of the apple industry [3]. Among them, apple anthracnose which caused by the fungus Colletotrichum gloeosporioides is one of the major pathogens causing apple fruit rot and yield loss.

Anthracnose is widely distributed worldwide, and the hot and humid climatic conditions provide favorable conditions for its prevalence [4]. After infection, the pathogenic bacteria cause spot formation by invading apple fruits, resulting in reduced fruit quality and even fruit rot, bringing serious economic losses to the apple industry [5-6]. In order to effectively control the occurrence and spread of anthracnose, researchers have been exploring the genetic basis of apple anthracnose resistance, with a view to breeding apple varieties with high disease resistance, high yield and high quality[7].

2 Materials and methods

2.1 Materials

The experimental materials were populations of 'Fuhan' and 'Micui' apples planted in the Liaoning Provincial Institute of Fruit Tree Science (122.15E, 40.17N) which were free from diseases, pests and mechanical damage. The leaves were taken during the growth period and brought back to the laboratory by liquid nitrogen flash freezing and stored in an ultra-low temperature refrigerator at -80 degrees Celsius.

2.2 Method

2.2.1 RNA extraction and cDNA synthesis

Grind apple leaves and extract RNA from apple leaves using the RNAprep Pure Plant Plus Kit. 5×TURE Reaction Mix was used to extract RNA, and 10 μL of the system was configured to synthesize from cDNA according to the instructions. Primers were designed using primer premier 6 software based on transcriptome sequencing data on apple anthracnose resistance (Table 1). cDNA amplification reaction system is shown in Figure 1.

Oligo	5μL
Mix	5μL
positive primer	0.5μL
negative primer	0.5μL
RNA	0.5μL
ddH_2O	Up to 10μL
Total	10μL

Figure 1. cDNA amplification reaction system

The PCR amplification reaction program was performed according to the RNAprep Pure Plant Plus Kit instructions. Agarose gels were prepared for electrophoretic characterization of the PCR products, which were recovered and purified after verifying band size, and then their concentrations were determined. The DNA solution was stored in a refrigerator at -20 ℃.

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2.2.2 Connecting T-vector and transformation into E. coli receptor cells

Gel recycling was performed and T-vector were ligated and transformed into E. coli receptor cells. The resuspended bacterial liquid was spread on solid LB medium containing appropriate antibiotics, incubated at 37 ℃ overnight, and the positive clones were screened by RT-PCR.

2.2.3 Enzymatic digestion of plasmid DNA

RNase/DNase-Free ddH2O	10μL
10×Buffer	2μL
Plasmid DNA	6μL
Restriction endonuclease I	1μL
Restriction endonuclease II	1μL
Total	20μL

Figure 2. The reaction system of Enzymatic digestion of plasmid DNA

The reaction system is shown in Figure 2. The reaction mixture was placed in a 37 ℃ thermostatic water bath, and the digestion reaction was carried out for 2 hours. Use agarose for electrophoresis detection, and select the appropriate size of the target fragment to cut the gel for recovery.

2.2.4 Construction of vector

The DNA sequence of the Apple Anthracnose Resistance gene was screened based on the apple reference genome data and NCBI-BLAST comparison, and was named CDS. Cloning primers were designed using Primer Premier 6 software (Table 1). The successfully sequenced recombinant plasmid DNA and the corresponding vector were subjected to a double digestion reaction and the appropriate size of the bands were cut and recovered, and the enzymatically excised fragments and the corresponding linear vectors were ligated using T4 ligase, mixed and ligated at 4°C overnight. The connection system is shown in Figure 3.

Mix	5μL
positive primer	0.5μL
negative primer	0.5μL
Plasmid DNA	0.5μL
ddH_2O	Up to 10μL
Total	10μL

Figure 3. The connection system

The ligation product was subjected to transformation of E. coli receptor cells, positive clones were selected and plasmid DNA was extracted.

2.2.5 Transformation of agrobacterium sensory cells

- Add 50μL of the sensory state on ice
- Ice bath for 30min, take LB and heat on metal bath for 2min
- Heat-excited carrier for 45sec, temperature 42°C
- Ice bath carrier 2-3min
- Add 200μL of LB
- Shake at 37°C, 165rmp for 40-60min
- Collect the bacterial body by centrifugation at 10000 rpm for 1 min, leave 100μL of supernatant and discard the excess supernatant, resuspend the bacterial body, and then spread the resuspended bacterial solution on the solid LB medium containing the corresponding antibiotics, and incubate inverted at 28 ℃ for 2-3 days
- Screening of colony PCR positive clones

2.2.6 Real-time fluorescence quantitative PCR analysis

Using 238 as the internal reference gene, the primer sequences are listed in Table 1, following the instructions of TIANGEN's SuperReal PerMix Plus (SYBR Green) kit. The reaction system is shown in Figure 4.

2×SuperReal PreMix Plus	5μL
positive primer	0.3μL
negative primer	0.3μL
ROX	3μL
Plasmid DNA	0.4μL
RNase-free H_2O	Up to 10μL
Total	10μL

Figure 4. The reaction system

Amplification procedure: 95℃ 15min 1 cycle, then 95℃ 10sec, 55℃ 30sec and 72℃ 32sec total 40 cycles. Melting curve analysis was performed after this. Two biological experiments were performed for each sample. Finally, quantitative data analysis was performed using the $2^{-\Delta\Delta CT}$ method [8].

Table1. CDS clone primers, Construction of vector and fluorescent quantitative primers

Primer type	Gene	Primer sequences(5'-3')
Cloning primer	CDS	F:ATGAAAATGGCATCTTGTAG R:CTACAAATTAGGAAGGCTTCCCAAGC
Construction of vector	Prompt Conversion	F:GACTCTAGAAAGCTTCTGCAGATGAAAATGGCATCTTGTAGCAGTT R:CATGGTACCGGATCCACTAGTCAAATTAGGAAGGCTTCCCAAGC
	Stable Transformation	F:TTGATACATATGCCCGTCGACATGAAAATGGCATCTTGTAGCAGTT R:gctcaccatGGATCCGGTACCCAAATTAGGAAGGCTTCCCAAGC
Fluorescent Quantitative Primers	Actin	F: TGACCGAATGAGCAAGGAAATTACT R: TACTCAGCTTTGGCAATCCACATC
	CDS	F:GATGTTCTGAGGACGCTGACTAAG R:AAGCCATCCAAGCAAGTGTCTC

3 Results and analysis

3.1 Apple CDS gene cloning

The cDNA of apple leaves was used as a template for PCR amplification, and a clear band with a size of 636 bp was obtained (Figure 5), indicating that the cloning of the apple CDS gene was successful.

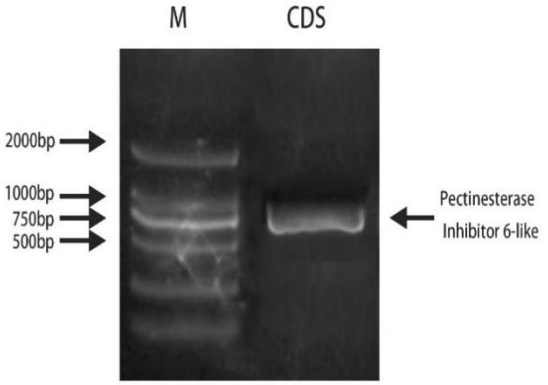

Figure 5. PCR products of CDS gene

3.2 Pectinesterase Inhibitor 6-like gene recombinantly ligated into an overexpression vector

The pectinase inhibitor 6-like vector was double digested with PstIand SpeI. The product obtained by gel recovery was ligated with the purified product of Pectinesterase Inhibitor 6-like gene under the action of recombinant enzyme, and then transformed into E. coli, and then identified by colony PCR (Figure 6). Three monoclonal colonies with positive results were randomly selected and cultured in LB liquid medium containing YEP overnight, and the plasmids were extracted and sent to Taihe Biotechnology Co., Ltd. for sequencing.

According to the sequencing results, the sequenced sequences were compared with those provided in the database by DNAMAN, and the comparison results showed that the similarity between the two was 99.53% (Figure 7). Therefore, the Pectinesterase Inhibitor 6-like gene was cloned in this study.

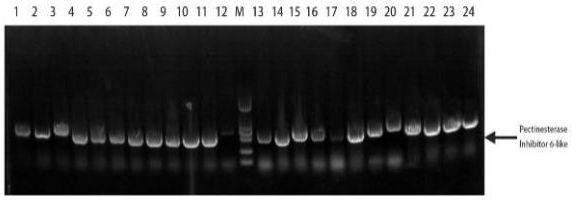

Figure 6. E. coli colony PCR electropherogram

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aim_gene.seq   ATGAAAATGGCATCTTGTAGCAGTTATATTTTTGTGTCAC   40
238.seq        ATGAAAATGGCATCTTGTAGCAGTTATATTTTTGTGTCAC   40
Consensus      atgaaaatggcatcttgtagcagttatatttttgtgtcac

aim_gene.seq   TCATTTTTCATGCAATATGGCTGATTTGTTCAACCCAATT   80
238.seq        TCATTTTTCATGCAATATGGCTGATTTGTTCAACCCAATT   80
Consensus      tcatttttcatgcaatatggctgatttgttcaacccaatt

aim_gene.seq   ATTGGTATCTGGAAAGGGGGACAAAACTTATGTCCAAGAG   120
238.seq        ATTGGTATCTGGAAAGGGGGACAAAACTTATGTCCAAGAG   120
Consensus      attggtatctggaaaggggacaaaacttatgtccaagag

aim_gene.seq   GCCTGCAGTAATTATGTCCAAGAGGCCTGCAGTGTGACCA   160
238.seq        GCCTGCAGTAATTATGTCCAAGAGGCCTGCAGTGTGACCA   160
Consensus      gcctgcagtaattatgtccaagaggcctgcagtgtgacca

aim_gene.seq   GGTATCGGGATGTTTGCATCCACTCGCTCTCACCGTTTTC   200
238.seq        GGTATCGGGATGTTTGCATCCACTCGCTCTCACCGTTTTC   200
Consensus      ggtatcgggatgtttgcatccactcgctctcaccgttttc

aim_gene.seq   GAGCTCAGCCAAGAACAGCCCGAGCAGGTGGGGACGAGCC   240
238.seq        GAGCTCAGCCAAGAACAGCCCGAGCAGGTGGGGCCGAGCC   240
Consensus      gagctcagccaagaacagcccgagcaggtgggc cgagcc

aim_gene.seq   GGGGTTTCAGTTTCATTGAGCGAGGCCAAGAGTGCCACTC   280
238.seq        GGGGTTTCAGTTTCATTGAGCGAGGCCAAGAGTGCCACTC   280
Consensus      ggggtttcagtttcattgagcgaggccaagagtgccactc

aim_gene.seq   GGTACTTGGTAACTCTGAAAACGCACGGGGGTGGTTTGAA   320
238.seq        GGTACTTGGTAACTCTGAAAACGCACGGGGGTGGTTTGAA   320
Consensus      ggtacttggtaactctgaaaacgcacgggggtggtttgaa

aim_gene.seq   GGGGAGAAACGAAGTTGCTTTGTCCGACTGCATTGAGTGC   360
238.seq        GGGGAGAAACGAAGTTGCTTTGTCCGACTGCATTGAGTGC   360
Consensus      ggggagaaacgaagttgctttgtccgactgcattgagtgc

aim_gene.seq   TTTCAGAACGCGATCGACGAGCTTCACAAATCGCTCGATG   400
238.seq        TTTCAGAACGCGATCGACGAGCTTCACAAATCGCTCGATG   400
Consensus      tttcagaacgcgatcgacgagcttcacaaatcgctcgatg

aim_gene.seq   TTCTGAGGACGCTGACTAAGATGACATTCGACACGAAAAT   440
238.seq        TTCTGAGGACGCTGACTAAGATGACATTCGACACGAAAAT   440
Consensus      ttctgaggacgctgactaagatgacattcgacacgaaaat

aim_gene.seq   GGCGGACCTGAATACCTGGCTCAGTGCTGCCCTAACCAAT   480
238.seq        GGCGGACCTGAATACCTGGCTCAGTGCTGCCCTAACCAAT   480
Consensus      ggcggacctgaatacctggctcagtgctgccctaaccaat

aim_gene.seq   GGAGACAGTTGCTTGGATGGCTTTGAGGGCCAGAATGGAA   520
238.seq        GGAGACAGGTGCTTGGATGGCTTTGAGGGCCAGAATGGAA   520
Consensus      ggagacac tgcttggatggctttgagggccagaatggaa

aim_gene.seq   AACCAGTCAAATTGCTACAAGATAGGGTTTTGAAAGTTAC   560
238.seq        AACCAGTCAAATTGCTACAAGACAGGGTTTTGAAAGTTAC   560
Consensus      aaccagtcaaattgctacaaga agggttttgaaagttac

aim_gene.seq   CTATATCACCAGTAATGCACTGGCTCTTGTTAACAAACTT   600
238.seq        CTATATCACCAGTAATGCACTGGCTCTTGTTAACAAACTT   600
Consensus      ctatatcaccagtaatgcactggctcttgttaacaaactt

aim_gene.seq   GCGACTACTGGCTTGGGAAGCCTTCCTAATTTGTA        635
238.seq        GCGACTACTGGCTTGGGAAGCCTTCCTAATTTGTA       635
Consensus      gcgactactggcttgggaagccttcctaatttgta
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Figure 7. The alignment result of Pectinesterase Inhibitor 6-like sequence

3.3 Homology comparison of apple Pectinesterase Inhibitor 6-like protein sequences

We used DNAMAN software to perform multiple sequence comparison of the protein sequences of this gene, and the results are shown in Figure 8. The results show that SNP did not change the amino acid sequence.

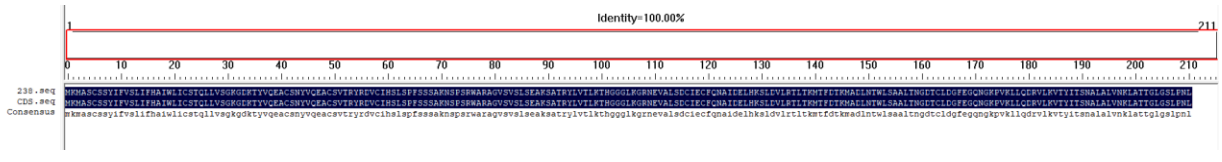


Figure 8. Pectinesterase Inhibitor 6-like is compared by protein homology sequence

3.4 Pectinesterase Inhibitor 6-like subcellular localization

Pectinase inhibitor 6-like protein is one of them and is predicted to be located in the cell membrane. To further determine the localization of pectinase inhibitor 6-like proteins in cells, we cloned full-length pectinase inhibitor 6-like and constructed Pectinesterase Inhibitor 6-like-MT and Pectinesterase Inhibitor 6-like-ER expression vectors (Figure 9). We injected the bacterial fluids containing Pectinesterase Inhibitor 6-like-MT strain and Pectinesterase Inhibitor 6-like-ER into the epidermis of tobacco leaves for transient transformation. The co-injected tobacco was exposed to long sunlight at 25 ℃ for 2-3 days, and after the fusion fragments were fully expressed, the lower epidermis of the injected tobacco leaves was torn open and the fluorescence signals were detected under a photon laser confocal microscope. It was observed that green fluorescence could be observed on the endoplasmic reticulum in tobacco leaves injected with Pectinesterase Inhibitor 6-like-ER broth, proving that it was highly expressed in the cell wall (Figure 10). The results showed that the protein encoded by this gene co-localized with the cell membrane dye FM4-64, indicating that the protein is located in the cell membrane. In addition, there is also a GFP signal in the inner part of the chloroplasts, and the punctate structure is likely to be the organelles such as mitochondria, lysosomes, Golgi, vesicles and other organs, so the protein is also likely to function in the cytoplasm of the cell organelles. But the specific location of the function needs to be confirmed using the marker for the organelles of the mitochondria, lysosomes, Golgi, vesicles and other organelles.

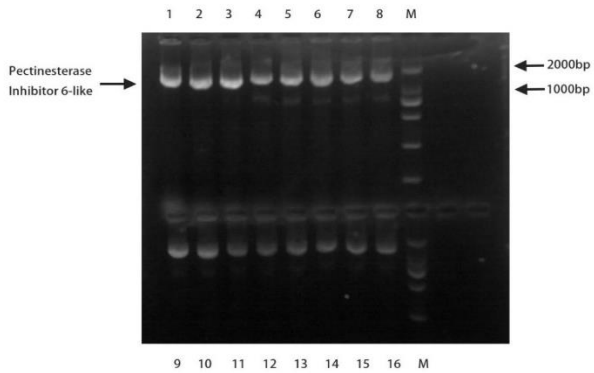


Figure 9. Agrobacterium tumefaciens colony PCR electropherogram

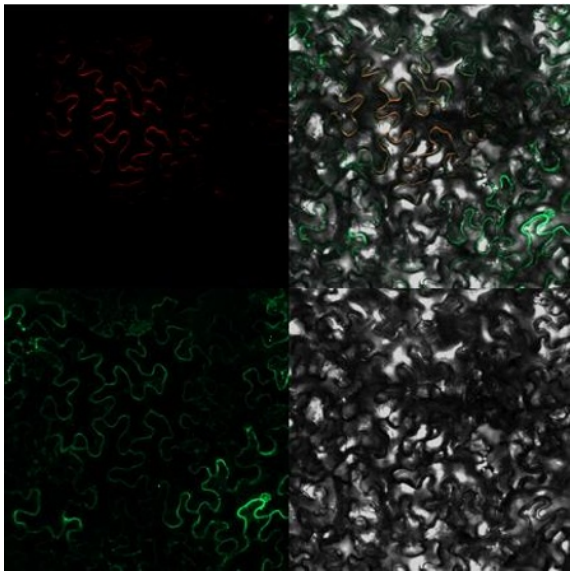
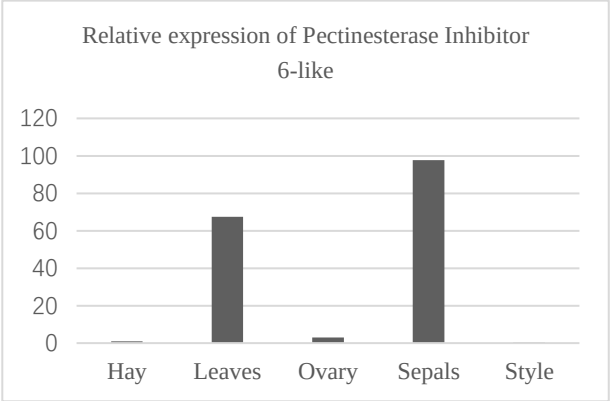


Figure 10. Subcellular location of Pectinesterase Inhibitor 6-like

3.5 Fluorescence quantitative PCR analysis

The results of fluorescence quantification are shown in the table 2 below, and it can be concluded that the highest expression was found in sepals, The lowest expression was found in the styles.

Table 2. Pectinesterase Inhibitor 6-like relative expression of Pectinesterase Inhibitor 6-like overexpression transgenic plants



3.6 Obtaining transgenic apple plants

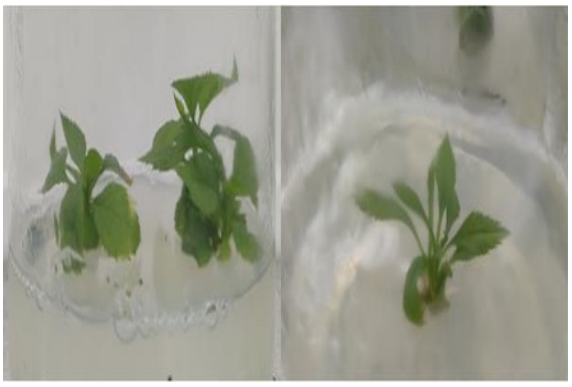


Figure 11. Transgenic resistant seedlings and DNA water bottle test results

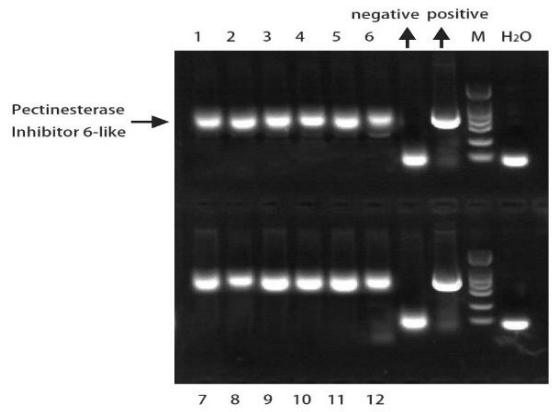


Figure 12. The validation of agrobacterium by PCR

The resistant strain was obtained by Agrobacterium-mediated stabilized genetic transformation, as shown in Figure 11. And the DNA level identification further proved that the resistant strain was a positive strain successfully transformed (Figure 12). In order to further clarify the gene function, phenotypic observation was carried out at a later stage to determine the changes in the content and nature of cell wall pectin in the transgenic strain and to clarify the biological function of the Pectinesterase Inhibitor 6-like gene.

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