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Full Length Article

Agroinfiltration Transient Assay to Evaluate the Potential of ihpRNA Constructs for Lily Mottle Virus Resistance

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Abstract

In order to obtain a resistant strain of Lily mottle virus (LMoV), three RNA interference (RNAi) vectors with intron hairpin RNA structure (ihpRNA) were constructed and transformed into *Agrobacterium* GV 3101 strain. The gene hotspots of LMoV coated protein (CP) and cylindrical inclusion (CI) were selected, and the positive and negative fragments of the target gene were inserted into both sides of the Pdk intron fragment of plant expression vector pRNAi-LIC by one-step, zero-background ligation-independent cloning (OZ-LIC) method. Antiviral analysis was performed using *Agrobacterium*-mediated transient-expression assay. RT-PCR and indirect ELISA results showed that the hairpin RNA from the reverse repeats of CI-517 bp and CI-295 bp could interfere the virus effectively, while the CP-400 bp hairpin's impact was not significant. The work illustrates that the genetically modified genome could LMoV. © 2019 Friends Science Publishers

Keywords: Lily mottle virus; ihpRNA interference; Host-pathogen interactions; Resistance detection

Introduction

Plant antiviral genetic engineering provides a new strategy for synthesizing plant resistance viruses, among which RNA-mediated resistance virus is favored by increasing number of researchers (Kusaba, 2004). Studies have shown that ihpRNA with intron as a spacer sequence shows high silencing efficiency (Wesley *et al*., 2001) with a maximum ihpRNA silencing efficiency of 300-600 bp length stem (Helliwell and Waterhouse, 2003). Therefore, the synthesis of an efficient ihpRNA interference vector is the key technology to apply the antiviral genetic engineering. The OZ-LIC method developed by Xu *et al*. (2010) enabled to build ihpRNA interference vector in one single step. Compared with the traditional Cleavage method and Gateway technique, this method has zero-background and low-cost advantages (Yan *et al.*, 2013). Chen *et al*. (2011) used this method to obtain an efficient ihpRNA.

LMoV is one of Potyvirus. Once LMoV infects lily, lily shows vein clearing, leaf mottle, twisted bifurca-tion, flower breaking and reduced bulb yields (Asjes, 2000). Conventional detoxification is difficult to remove LMoV completely within a short time. With the development of molecular biology, antiviral genetic engineering has been increasingly applied to anti-LMoV (Zhang *et al*., 2017). Xu *et al*. (2011) constructed virus resistant RNAi vector to Lily symptomless virus (LSV) and LMoV binary virus by Gateway technology. However, synthesizing virus that shows resistance to LMoV alone has not been reported.

Potyvirus protein function studies show that CI protein is responsible for cell-to-cell movement and ATPase/RNA helicase and CP protein is involved in genome replication, aphid transmission, cell-to-cell and systemic movement and virus assembly (Urcuqui-Inchima *et al*., 2001). In this study, the genes of these two hot-spot sequence proteins were selected, and the RNAi vector which generates RNA hairpin structure was synthesized using OZ-LIC method.

Materials and Methods

Plants, Bacterial Strains, Plasmids and other Reagents

LMoV, tobacco plants, pMDTM18-CI plasmid, pMDTM18-CP plasmid, *Escherichia coli* (*E. coli*) strain DH5a and *Agrobacterium* strain GV3101 were preserved in the lab. T4 DNA polymerase, dATP, dTTP, Gel purification kit and restriction enzymes were purchased from TAKARA (DaLian, China). The pRNAi-LIC vector was obtained from Professor Yule Liu's group of Tsinghua University.

Cloning of the Target Gene

According to the whole genome sequence of LMoV-DL (GeneBank accession number: HM222521), 3681-4197 and 4305-4599 of CI gene sequence (CI517, CI295) were selected and 1-400 of CP gene sequence (CP400) was used as the target fragments.

CI517-LIC1/CI517-LIC2 were used as primers to amplify CI517 positive fragment and CI517-LIC3/CI517-LIC4 were used to amplify CI517 reverse fragment with pMDTM18-CI plasmid as template. Similarly, CI295 and CP400 positive and negative fragments were chosen for amplification. (Table 1) The PCR products were purified using Gel purification kit.

Making ihpRNA Interference Vector

Synthesis of RNAi vector using OZ-LIC method was described in Xu *et al*. (2010). Purified forward and reverse fragments were treated with T4 DNA polymerase and dATP. The pRNAi-LIC vector was digested with Sma I and treated with T4 DNA polymerase and dNTP. Prepared forward/reverse fragments and pRNAi-LIC vector were mixed and incubated. The mixture was then transformed into *E. coli* DH5a competent cells. The plasmid was extracted and verified by restriction enzyme digestion according to the restriction sites of the vector and primer.

Agroinfiltration

RNAi vectors were transformed into *Agrobacterium* strain GV3101 competent cells by freeze-thaw method and plated in YEB liquid medium. The plasmids were extracted from A. tumefaciens for digestion. Leaves of tobacco plants were infiltrated by *Agrobacterium* engineering bacteria. For comparison, plants were agroinfiltrated with pRNAi-LIC vector. After 4 days, the agroinfiltrated leaves were challenged by LMoV.

RT-PCR and Indirect ELISA Analysis

Total RNA was extracted after 13 day post inoculation (dpi), and then RT-PCR was performed to detect LMoV. For RT-PCR, LMoV-CP1 and LMoV-CP2 were forward and reverse primers. Indirect ELISA was performed using a method described in Xu *et al*. (2017).

Results

Amplification of the Target Fragment

The sequencing PCR product fragments were shown in Fig. 1, which were ready for the following experiments.

Restriction Enzyme Analysis of RNAi Vector

BamH I/Sac I Digestion: the pRNAi-LIC vector has three restriction sites. Three bands occurred after digestion if the target fragment is not the cleavage site. Four bands occurred if the target fragment has one cleavage site (Fig. 2).

Pst I/Kpn I or Sal I/Kpn I Digestion: 2663 bp, 2219 bp and 2429 bp fragments were produced, respectively (Fig. 2). The plasmids were identified as RNAi-CI517, RNAi-CI295 and RNAi-CP400, respectively.

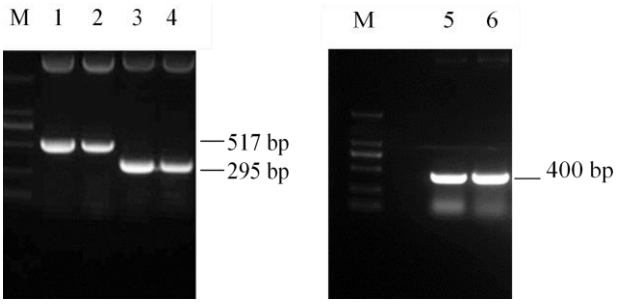

Fig. 1: Amplification of target genes
M: DL2 000 DNA marker; 1,2; 3,4; 5,6: sense and anti-sense fragments of CI_{517}, CI_{295} and CP_{400}

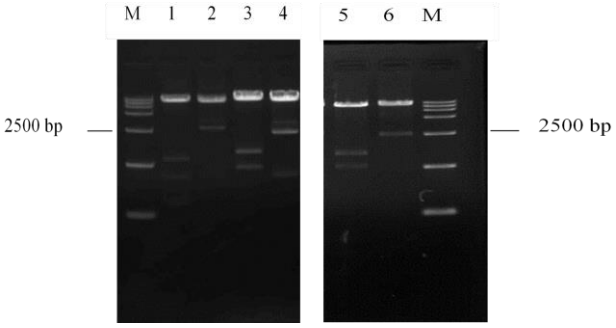

Fig. 2: Restriction enzyme analysis of RNAi-CI_{517}, RNAi-CI_{295} and RNAi-CP_{400}
M: DL15000 DNA marker; 1, 3, 5: RNAi-CI_{517}, RNAi-CI_{295} and RNAi-CP_{400} digested by *Bam*H I/*Sma* I;
2,4: RNAi-CI_{517}, RNAi-CI_{295} digested by *Pst* I/*Kpn* I; 6:RNAi-CP_{400} digested by *Sal* I/*Kpn* I

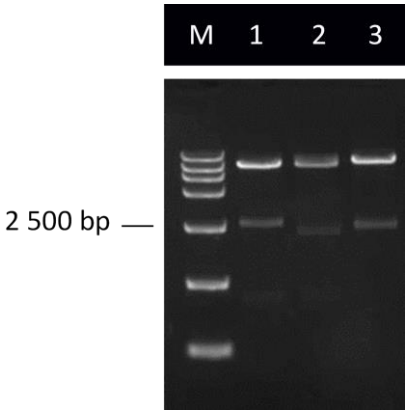

Fig. 3: Identification of *Agrobacterium* engineering bacteria
M: DL15000 DNA marker; 1,2: RNAi-CI_{517}, RNAi-CI_{295} digested by *Pst* I/*Kpn* I;
3: RNAi-CP_{400} digested by *Sal* I/*Kpn* I

The Effect of Transient Interference

As shown in Fig. 3, the plasmids extracted from GV3101 produced 2663 bp, 2219 bp and 2429 bp bands, respectively. The interference vector was successfully transferred into *A. tumefaciens.*

Table 1: Primers used in this work

Name	Sequence
CI_{517}-LIC1	5'-CGACGACAAGACCCTCTGCAGTTGAAGAGCGTGAAATGGT-3'(*Pst* I)
CI_{517}-LIC2	5'-GAGGAGAAGAGCCCTCAAAGTCGCGTAGCTGGTT-3'
CI_{517}-LIC3	5'-CCAGCACGGAACCCTCAAAGTCGCGTAGCTGGTT-3'
CI_{517}-LIC4	5'-AGAGCACACGACCCTGGTACCTTGAAGAGCGTGAAATGGT-3'(*Kpn* I)
CI_{295}-LIC1	5'-CGACGACAAGACCCTCTGCAGGAGTTCACCACGCAGCATC-3'(*Pst* I)
CI_{295}-LIC2	5'-GAGGAGAAGAGCCCTATGATATTCGTGGCCACAACAA-3'
CI_{295}-LIC3	5'-CCAGCACGGAACCCTATGATATTCGTGGCCACAACAA-3'
CI_{295}-LIC4	5'-AGAGCACACGACCCTGGTACCGAGTTCACCACGCAGCATC-3'(*Kpn* I)
CP_{400}-LIC1	5'-CGACGACAAGACCCTGTCGACTGGCGTGTGGCTCATGATG-3'(*Sal* I)
CP_{400}-LIC2	5'-GAGGAGAAGAGCCCTCCAAGTAAGGAGTGCATGT-3'
CP_{400}-LIC3	5'-CCAGCACGGAACCCTCCAAGTAAGGAGTGCATGT-3'
CP_{400}-LIC4	5'-AGAGCACACGACCCTGGTACCTGGCGTGTGGCTCATGATG-3'(*Kpn* I)
LMoV-CP1	5'-TGGGCACCTTGTGAATAACA-3'
LMoV-CP2	5'-TGCTGTATGCCTCTCCGTGT-3'

The RNAi-CI517 and RNAi-CI295 treatment groups were detected by RT-PCR without specific band amplification (Fig. 4), and compared with the empty vector control, the RNAi-CP400 treatment group, and leaf without chlorisis and shrinkage (Fig. 5). Meanwhile, indirect ELISA results (Table 2) showed similar conclusion. Although the symptoms were lighter than the control group in the RNAi-CP400 treatment group, the virus test results were positive. Therefore, we concluded that RNAi-CI517 and RNAi-CI295 showed good antiviral effect, while RNAi-CP400 demonstrated no obvious antiviral effect.

Discussion

Gene sequences derived from plant virus have been introduced into a wide variety of plant species to produce transgenic plants for protecting against virus infection (Sherman *et al*., 2015). In this study, the RNAi vector containing ihpRNA was constructed using OZ-LIC method, and the feasibility and the effectiveness were verified. This method only requires four LIC linkers and a two-step polymerase treatment. With incubation overnight at 4℃, the connection efficiency was greatly enhanced. In order to investigate the effect of hairpin RNA derived from the reverse repeats of the pathogen, the invading virus by the transient expression method was mediated by *A. Tumefaciens* (Ding *et al*., 2011). The results showed that RNAi-CI517 and RNAi-CI295 showed significant interference effects, and the RNAi-CP400 had no obvious resistance to infection. The RNAi vectors' different antiviral effects depends on the length and the base sequence of stem and loop of ihpRNA, which can change the free energy of the secondary structure of the ihpRNA and as a consequence, affect its stability and silencing efficiency (Hirai *et al*., 2007).

This work is novel in the field of LMoV disease resistance research. RNAi-CI517 and RNAi-CI295 show high silence efficiency, which can be transformed into the lily plant as the resistance strains. Meanwhile, dsRNA *in vitro* through synthesizing prokaryotic expression vector with ihpRNA are obtained, and plants were imparted with anti-viral ability once the dsRNA enters into plants *via* the absorption of leaf epidermal cells (Tenllado *et al*., 2004).

Table 2: Resistance effects of tobacco leaves with instant interfered of LMoV by indirect ELISA

Treatment	RNAi-CI_{517}	RNAi-CI_{295}	RNAi-CP_{400}	pRNAi-LIC	Negative control	Blank
OD_{450}	0.328	0.404	0.743	1.035	0.261	0.081
P/N	1.37	1.79	3.68	5.30	/	/
-/+	—	—	+	+	/	/

Note: P/N < 2 is negative; P/N > 2 is positive

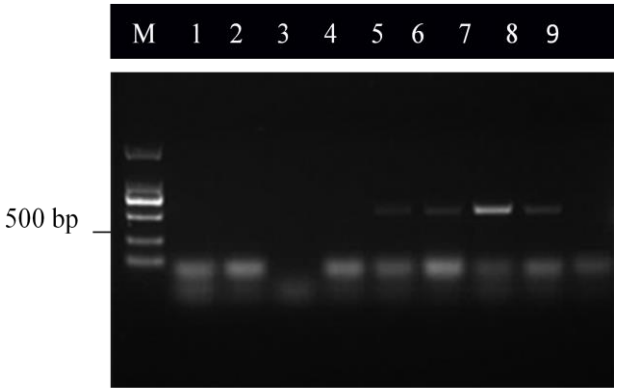

Fig. 4: PCR detection of LMoV
M: DL2000 DNA marker; 1,2; 3,4; 5,6: Inoculate LMoV after agroinfiltration with RNAi-CI_{517}; RNAi-CI_{295}; RNAi-CP_{400}; 7,8: Vector alone; 9: Healthy plant

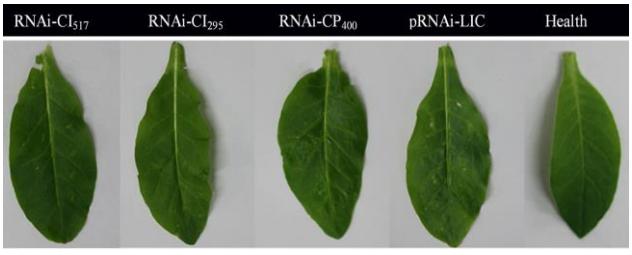

Fig. 5: Symptoms of tobacco leaves with instant interfered of LMoV

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