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Full Length Article

Regeneration of Transgenic Lily after Transformation of LSV and LMoV Gene by using Agrobacterium-Mediated System

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Abstract

The pH7GWIWG2 (II) expression vectors of capsid protein gene sequences constructed in our lab contain Lily symptomless virus (LSV) and Lily mottle virus (LMoV). *Lilium orientalus* 'Sorbonne' and *Lilium longiflorum*,'White Heavenan' are the receptor, using Agrobacterium-mediated transformation to lily. The results show that: 'Sorbonne' and 'White Heaven' were infected with Agrobacterium bacteria (OD_{600} = 0.8) 15 min, fostered dark for 4 d, respectively adapter red off bacteria with cefotaxime (300 mg/L) train the filter to the selection medium containing 23.0 mg/L and 25.0 mg/L hygromycin to obtain transgenic lily. RT-PCR assay prove that the two encoding viral capsid protein gene have expressed in the lily genome, ELISA and Western blot show that the transformation of lily significantly enhance the resistance to LSV and LMoV. © 2018 Friends Science Publishers

Keywords: Transgenic lily; Agrobacterium-mediated system; Dual anti-virus; RNA interference

Introduction

Lily virus disease has been one of the unrooted diseases that plague the production of lily bulb and fresh cut flowering in China. Since the first lily necrotic spot disease was found in Oregon by American scholars Brierley and Smith (1994), 14 lily viruses have been reported at home and abroad. Among them, Lily symptomless virus (LSV) and Lily mosaic virus (LMoV) are the most harmful to lily (Kaminska, 1996; Asjes and Blom-Barnhoorn, 2002; Sharma *et al.*, 2005). The main symptoms are plant degeneration and dwarfism, degreening stripes in leaves, little and small bud formation, and some petals are deformed or even unable to bloom (Hagita and Sasaki, 1994; Cohen *et al.*, 1996).

In recent years, plant antiviral genetic engineering based on RNA interference technology (RNAi) has shown lots of potential applications in the prevention and treatment of plant virus diseases (Xu *et al.*, 2017). Many studies have shown that double strains RNA is an efficient inducer of gene silencing, and expression or introduction of dsRNA can induce gene silencing of specific Gene (Grishok *et al.*, 2001; Sijen *et al.*, 2001). It was found that resistant plants, which were obtained by transformation of potato Y virus (PVY) gene into tobacco could be resistant to such pathema. Using the replicase gene fragment containing Barley Yellow Dwarf virus (BYDV) PAV strain, a hair-clip RNA vector was constructed, and infected into barley to obtain strong resistant plants and stable filial generation (Wang *et al.*, 2000; Kalantidis *et al.*, 2002) detected a fully resistant strain in regenerated tobacco plants with reverse repeat cDNA sequence of Cucumber Mosaic virus (CMV), and confirmed that tobacco virus resistance is directly related to the production of siRNA. But so far, there are no reports on the cultivation of disease-resistant lily using RNAi technique. In this study, two kinds of virus capsid protein genes of LSV and LMoV were transformed into lily by RNA interference technique to provide technical support for radical cure of lily virus disease and enhancing the domestic production of lily seed ball in China.

Materials and Methods

Experimental Material

Lilium orientalus (Sorbonne) and *Lilium longiflorum* (*Lilium longiflorum*) White Heavenan (*Lilium longiflorum*) were propagated and maintained by our laboratory on Murashig and skoog medium containing 20 μg/μL hygromycin and conforin by immunoblots. All transgenic plants and wild-type plants were grow in glass house at 23^{o}C, and 16-h-light-8-h-dark cycles.

Medium: MS 30 g/L, sucrose 5 g/L, Agar powder, pH 5.8.

Agrobacterium tumefaciens suspension medium: peptone 5 g/L, yeast extract 1 g/L, beef extract 5 g/L, $MgSO_4$ $7H_2O$ 0.493 g/L, pH 7.0

Strain and Plasmid

Agrobacterium tumefaciens EHA105 was preserved in our laboratory. The hpRNAi expression vector pH7GWIWG2 (II-LSV-LMoV) was constructed by our laboratory (Fig. 1), which showed hygromycin resistance in plants.

Preparation of Agrobacterium Bacteria and Infection of Explants

Root carcinoma agrobacterium single colony was taken in containing 50 mg/L spectinomycin and 50 mg/L rifampicin YEB liquid medium, at 28°C, and 180 RPM oscillation for the night training, centrifugal collecting bacteria, suspended in liquid medium, MS shaking culture to OD_{600} value of 0.8 or so, as a fluid of infection. The culture methods of 3 d lily bulbs leaves in the fluid of the infection for 15 min, taken out the blade after infection, blot, sterile filter vaccination was conducted in the common culture medium dark culture, 4 d after turn to choose medium, 25°C, 16 h. d^{-1}. About 25 d secondary, the removal of the Browning death of explants.

RT-PCR

A small amount of leaves were cut from transgenic plants, the total RNA was extracted by TRIzol method (Bao biologic engineering Co., Ltd.), reverse transcribed to cDNA, PCR amplification. Primers were designed according to the genomic sequences of LSV and LMoV, L-F: 5'- ggggacaagtttgtacaaaaaagcag -3'; L-R: 5'- ggggaccactttgtacaagaaagct- 3'. The reaction system was up to 20 μL, the cycle parameters were at 94°C for 5 min, denatured at 94°C for 30 s, annealed at 57°C for 30 s, and 35 cycles for 72°C, 45 s, then 72°C for 10 min.

Western Blot

The infected LSV and LMoV virus lily in our laboratory were ground with appropriate amount of quartz sand, and the phosphate buffer containing 0.05% -mercaptoethanol (pH 7.0) was added at 1:10. Eight different plants of two transgenic lily varieties at flowering stage were inoculated by friction, and the virus-free non-transgenic lily was used as the positive control. After 5 days of inoculation, the incidence of LSV and LMoV were investigated regularly, and ELISA was detected. The virus-free lily was used as negative control. The results showed that the absorbance value of infected lily was more than twice as high as that of seedling lily, and less than twice that of disease-resistant plant (Sutula *et al.*, 1986). Western blot was used for the detection of transgenic lily and non-transgenic lily. The purified LSV particles were used as positive control and virus-free non-transgenic lily as negative control.

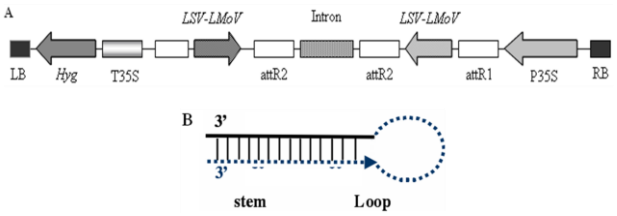

Fig. 1: A: The scheme of recombinant expression vector pH7GWIWG2 (II)-LSV-LMoV; B: hpRNA structure
P35S: promoter; T35S: terminator; Hyg: Hygromycin resistance marker

Results

RT-PCR Detection of Transgenic Plants

In order to testify that we have got the stable transgenic plants, we randomly selected 21 strains and 8 strains of hygromycin from "Sorbonne" and "White Paradise" for PCR detection (Fig. 2 and 3). 17 strains of Sorbonne have amplified a relatively clear 873 BP fragment, and 6 strains of "White Paradise" have amplified the target fragment, indicating that the exogenous gene has been integrated into the genome of lily.

Inoculation of Transgenic Plants with Disease Resistance

Infecting LSV and LMoV viruses into transgenic and non-transgenic plants. After 5 days, there was no disease in transgenic or non-transgenic plants. After 15 days, the leaves of non-transgenic lily were twisted and dark spots appeared, while there were 2 strains of transgenic lily "Sorbonne" and 1 strain of "White Paradise". After one month of infecting, the non-transgenic plants were obviously short, the stems were slender, the leaves were severely distorted, and a large number of withered spots appeared, while the transgenic plants were resistant to LSV and LMoV viruses, showing strong capability of virus resistance and growing well (Fig. 4). This indicated that LSV and LMoV gene sequences had been expressed in the transformed plants, but whether the disease resistance could be permanently inherited still needed to be further identified.

ELISA and Western Blot

After 10 days of inoculation, the transgenic lily "Sorbonne" and "White Paradise" were tested by ELISA. The results indicated that 38% Sorbonne had resistance to LSV virus, and 75% Baiduan had ability resistance to LSV. Sorbonne and White Paradise were 38% and 75% against LSV and LMoV, respectively. Sorbonne and White Paradise were 38% and 75% against LSV and LMoV, respectively (Table 1). But no transgenic lily was resistant to disease (Fig. 5).

Table 1: Resistance assay of transgenic lily by ELISA test to LSV and LMoV

Transgenic Lines	Number of tested plants	Resistant LSV (%)	Resistant LMoV (%)	Resistant LSV- LMoV (%)
'Sorbonne'	8	4(50)	3(38)	3(38)
'White Heaven'	8	7(88)	6(75)	6(75)
CK (Sorbonne)	10	0(0)	0(0)	0(0)
CK (White Heaven)	10	0(0)	0(0)	0(0)

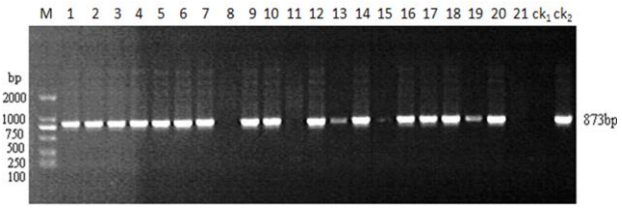

Fig. 2: The RT-PCR identification of transgenic *Lilium oriental*, 'Sorbonne'
M: DL2000 DNA marker; CK_1: negative control; CK_2: positive control; 1~21: resistant lily

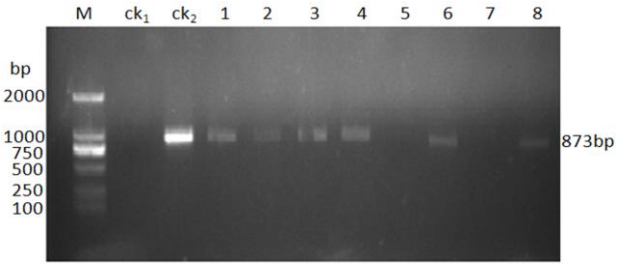

Fig. 3: The RT-PCR identification of transgenic *Lilium longiflorum*, 'White Heaven'
M: DL2000 marker; CK_1: negative control; CK_2: positive control; 1~8: resistant lily

The results of Western blot analysis showed that there were lighter bands in transgenic "Sorbonne" and "White Paradise" than in negative controls, while the same bands were found in non-transgenic "Sorbonne" and "White Paradise" as compared with purified LSV particles. It was proved that transgenic lily was resistant to LSV.

Discussion

At present, the mechanism of interference RNA has been studied more and more clearly. It has been reported that the virus resistance of transgenic plants obtained by using plant virus gene has been reported successfully in many plants (Wang *et al.*, 2000; Yan *et al.*, 2012). Traditional multi-gene recombination usually requires the construction of multiple intermediate vectors. In this study, LSV and LMoV coat protein genes were recombined and ligated *in vitro* using overlapping extension PCR technique, which greatly reduced the number of ligation and transformation. At the same time, the probability of base mutation is reduced, and the restriction of restriction endonuclease of DNA and the

Fig. 4: Resistance comparison between transgenic and non-transgenic lily
A: Leaf symptoms (1: control plant, 2: transformed plant); B: Plant growth situation (1: control plant, 2: transformed plant)

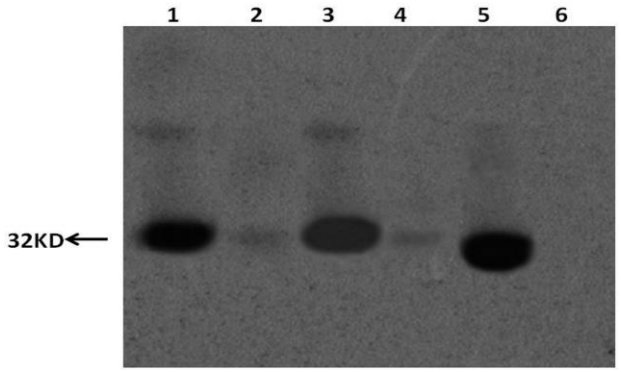

Fig. 5: Western blot analysis for LSV
1: 'Sorbonne' control plant; 2: 'Sorbonne' transformed plant; 3: 'White Heaven' control plant;
4: 'White Heaven' transformed plant; 5: Purified LSV; 6: negative control

restriction of endonuclease site inside gene is eliminated. The hpRNAi expression vector pH7GWIWG2 (II -LSV-LMoV) constructed by the Gateway technique breaks through the traditional plasmid construction model and can be efficiently and quickly constructed into the expression vector only by BP reaction and LR reaction. From the PCR amplification of the target fragment to the acquisition of the expression vector, it takes only a very short time to achieve the expected results, and the correctness of the gene location and reading framework is maintained (Xu *et al.*, 2011). At the same time, the constructed double resistant carrier can effectively express dsRNA, which can be used directly for the transformation of *Agrobacterium tumefaciens*, which provides a guarantee for the high efficiency genetic transformation of *Lilium*.

It is generally believed that monocotyledonous transformation rate is relatively low in plant genetic

transformation, and RNA silencing can be induced by RNAi technique with only small fragments to induce plant virus resistance (Sijen *et al.*, 1996). In this study, *Agrobacterium tumefaciens* mediated the establishment of a good genetic transformation system of Oriental *Lilium* (Xu *et al.*, 2008). The fusion gene of LSV and LMoV was transferred into "Sorbonne" and "White Paradise", which proved that antiviral transgenic lily could be obtained by using RNAi technique (Xu *et al.*, 2008). The fusion gene of LSV and LMoV was transferred into "Sorbonne" of Oriental *Lilium* and "White Heaven" of *Lilium chinensis*. It was proved that antiviral transgenic lily could be obtained by using RNAi technique. The resistant plants obtained by hygromycin resistance screening were lower, and hygromycin sensitivity to different varieties of lily was also different, "Sorbonne" was more sensitive to hygromycin than "Baiduan", and RT-PCR was used to detect hygromycin in antagonistic plants. Among 21 strains of Sorbonne, 17 were positive seedlings, and 6 of 8 were positive seedlings. It was preliminarily proved that two genes encoding the capsid protein of virus had been expressed in the genome of lily. ELISA analysis of PCR positive plants showed that a small number of plants showed positive results, which might be related to the amount of inoculation and the resistance of transgenic plants, and the disease resistance was also different among individuals and varieties in addition to the type of virus. The results of Western blot analysis showed that there were also slight bands in transgenic plants, indicating that a small number of LSV were not completely silenced.

In this study, the plants resistant to LSV and LMoV were obtained by RNAi mediated disease resistance, but the transformation efficiency was still very low (Lindbo *et al.*, 2001) in the study of transgenic plants, it was found by chance that some endogenous genes could not be expressed after introducing full-length or partial genes into plant cells, but the transcription of these genes was not affected by any way. The results of RT-PCR detection and disease resistance inoculation also proved that RT-PCR technique had the same effect on lily virus resistance. Lily is often infected by many viruses, especially by LSV and LMoV. So far, no effective control methods have been found. Using RNAi technique, the varieties of virus-resistant lily were cultivated, which opened up a new way for molecular breeding of resistance to virus in lily.

Conclusion

In this study, the expression vector of LSV-LMoV was constructed by Gateway, and the transgenic lilies were obtained successfully by agrobacterium-mediated.

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