Agrobacterium-mediated Transient Expression of Foreign Gene in Arabidopsis thaliana

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Abstract  Viral vector-mediated inoculation is a normal method of transient expression of foreign gene in the plants. In this study, we transiently expressed the foreign GUS gene in Arabidopsis thaliana by using Agrobacterium-mediated leaf-infection and detected the expression of GUS gene after ago-inoculation by GUS staining method and semi-quantitative RT-PCR. The results showed that GUS was expressed on 7-15 day after inoculation in Arabidopsis plants. Moreover, we successfully obtained fast expression of the viral suppressor HCPro in Arabidopsis plants, providing a new system for the fast expression of foreign gene and the study of gene function in plants.

Keywords  Arabidopsis thaliana; Leaf-infection; Foreign gene, Agrobacterium

The expression methods of foreign gene include stable expression and transient expression in the plants (Walmsley et al., 2000), the transient expression system has the advantages of simplicity, rapidity and high efficiency (Shivprasad et al., 2000). Viral vector-mediated leaf-infection is a common method for transient expression of foreign gene in the plants. People have transiently expressed a variety of foreign proteins in vitro tobacco leaves by using this inoculation (Kathuria et al., 2002; Wang et al., 2002). Some researchers used viral vector-mediated leaf-infection to expressed high levels green fluorescent protein (GFP) in tobacco (N. tabacum) (Jia et al., 2003). Liu et al successfully expressed human acidic fibroblast growth factor (ha FGF) with viral vector-mediated leaf-infection in tobacco (N. tabacum) (Liu et al., 2007). In 2008, Yang et al transiently expressed GFP in tobacco (N. tabacum) by using viral vector 30B that is derived from Tobacco mosaic virus (TMV) (Yang et al., 2008). In 2013, we used viral vector-mediated method to transiently express haFGF in pea (Pisum sativum) plants (Yang Liping et al., 2013). In 2016, we established and optimized the viral vector-mediated root vacuum inoculation in tobacco (N. tabacum), and successfully expressed the foreign gene efficiently with this method (Yang Liping et al., 2016). The studies have shown that these plant transient expression systems and the study of inoculation methods provide a research basis for plant bioreactors application, and also provide an effective platform for the rapid production of medicinal proteins and vaccines.

In this study, we explored an Agrobacterium-mediated inoculation for rapid expression of foreign gene and gene function study in the plants. Agrobacterium-mediated leaf-infection was used to transiently express the reporter gene GUS in Arabidopsis thaliana plants. The results of gene expression detection analysis and GUS staining showed that we successfully expressed the GUS gene in Arabidopsis by using Agrobacterium-mediated leaf-infection. Further research results had shown that the viral suppressor HC-Pro was expressed by this method in the plant, which provides a new expression system for the study of its function. Agrobacterium-mediated leaf-infection has the advantages of simplicity, rapidity and high expression efficiency, which provides a research basis for the fast expression and the functional identification of foreign gene in the plants.
1 Results and Analysis

1.1 The expression of GUS gene by Agrobacterium-mediated method

First, we transformed the expression vector pCAMBIA2301-GUS into Agrobacterium EHA105, then we used the Agrobacterium-mediated leaf-infection to transiently express the reporter gene GUS in Arabidopsis thaliana, and also observed and photographed for 14 days continuously. GUS staining was used to detect the expression of GUS gene in Arabidopsis. The results showed that 7 days after agro-inoculation, GUS began to expressed in the wound site of infected leaves (Figure 1A); 10 days after agro-inoculation, Agrobacterium was passed around through vascular bundles, and it can be observed that GUS expressed spread out from the wound site of infected leaves (Figure 1B; Figure 1C); 14 days after leaf-infection, GUS expressed heavily in uninfected stems (Figure 1D) and new leaves (Figure 1E).

1.2 The rapid expression of GUS gene in Arabidopsis thaliana

The plant materials from 7-10 days after inoculation were extracted for gene expression detection. The results showed that the reporter gene GUS was successfully expressed in Arabidopsis thaliana, indicating the effectiveness of Agrobacterium-mediated leaf-infection for fast expression of foreign gene (Figure 2A). In order to further determine whether the foreign gene transiently expressed in the new leaves and new stems, we detected the reporter gene GUS by RT-semi-quantitative PCR. The total RNA was extracted form the infected leaves (15 days after inoculation) and the new leaves to reverse transcription, and obtained cDNA was used as template to detect the expression of reporter gene. The result showed that GUS was expressed in both leaves on 15 days after Agrobacterium inoculation and new leaves (Figure 2B), that indicated the Agrobacterium-mediated leaf-infection can realize the expression of foreign gene in Arabidopsis.
1.3 The expression of HCPm by Agrobacterium-mediated method in Arabidopsis thaliana

We have successfully constructed the expression vector PBI121-HCPm. In order to further study the function of HCPm, we used Agrobacterium-mediated method to express HCPm in Arabidopsis thaliana. Arabidopsis with stretched leaves were selected as plant materials for leaf-infection inoculation. When Arabidopsis infected with Agrobacterium carrying empty vector was used as control (Figure 3A), the phenotype of Arabidopsis expressing HCPm were obviously changed. 7 days after inoculation, the infected leaves showed mild curled and deformed (Figure 3B); 10 days after inoculation, some infected leaves were obvious curled and deformed, and the new leaves were serrated (Figure 3C), which was very similar to the phenotype of HCPm transgenic Arabidopsis obtained previously (Yang et al., 2016), indicating that HCPm began to express in the new leaves. 14-15 days after inoculation, the leaves were more serrated, some leaves were severely curled and deformed, and appeared yellow and wilting symptoms (Figure 3D). The phenotype change of Arabidopsis after inoculation suggested that HCPm expression changed the expression of host endogenous gene and the regulation mechanism of gene expression. The total RNA were extracted form infected plants to semi-quantitative RT-PCR detection, and the result confirmed that HCPm expressed in Arabidopsis (Figure 3E).

2 Discussion

Viral-derived expression vectors are often used for the transient expression of foreign genes in plants. The main inoculation methods of the plant transient expression system mainly include viral vector-mediated leaf-infection and vacuum infiltration (Kapila et al., 1997). The researchers used viral-mediated method to transiently express acidic fibroblast growth factor (aFGF) in tobacco (Liu et al., 2007). This method has the advantages of fast and high expression efficiency and is widely used by people. In this study, we transformed plant expression vectors pCAMBIA2301-GUS and pBI121-HCPm into Agrobacterium EHA105, and studied the transient expression of foreign genes in Arabidopsis plants by Agrobacterium-mediated leaf infection. In this method, the expression vectors and foreign genes carried by Agrobacterium are rapidly expressed mainly through the transmission and spread of Agrobacterium (Figure 1). Our results indicated that the reporter gene GUS (Figure 2) and functional protein HCPm (Figure 3) were successfully expressed with this inoculation method.

In this Agrobacterium-mediated leaf-infection, the expression vector carried by Agrobacterium may be a plant binary expression vector, Arabidopsis or other crops can be selected as the infection material. However, the host is limited by viral-derived expression vectors in viral-mediated transient expression systems. For example, expression vectors derived from Tobacco mosaic virus (TMV) are limited to tobacco and other host materials (Liu et al., 2007; Yang et al., 2008). Therefore, this Agrobacterium-mediated inoculation method further expands the host range and is suitable for the rapid expression of genes and the study of gene function in plants, providing a new system for plant transient expression systems.

Figure 3 The phenotype of Arabidopsis thaliana expressed HCPm and the detection of gene expression
Note: A: mock-infected Arabidopsis thaliana served as controls; B: The leaves appeared curled and deformed after agro-inoculation; C-D: The infected Arabidopsis thaliana exhibited obvious deformed leaves, new leaves exhibited obvious serration; E: The detection of HCPm gene expression in infected plants, Actin served as controls
3 Materials and Methods
3.1 Materials and sources
The *Arabidopsis thaliana* seeds and strains are kept by our laboratory; plant expression vector pCambia-GUS is kept by our laboratory.

3.2 Preparation of Agrobacterium tumefaciens resuspension
The EHA105-pBI121-HC-Pro Agrobacterium liquid was activated and cultured in 50 mL LB liquid medium, and 20 \( \mu \text{mol} \) AS and 100 \( \mu \text{mol} \) MES were added. Centrifuge at 4 000 r/min for 10 min to collect *Agrobacterium*. Formulated solution (100 mL of ddH\(_2\)O was added with 1 mmol of MgCl\(_2\), 1 mmol of MES and 100 \( \mu \text{mol} \) of AS), *Agrobacterium* was resuspended at a concentration of about \( OD_{600}=1.0 \), and the *Agrobacterium tumefaciens* resuspension was placed at room temperature for 3 hours for leaf infection.

3.3 Agrobacterium-mediated Arabidopsis inoculation
The stretched leaves of *Arabidopsis thaliana* were selected, and the syringe with the needle removed was used to infect the *Agrobacterium tumefaciens* resuspension into the leaf tissue, and each plant was infected with 2-3 leaves. The plants were cultivated under dark conditions for 24 hours and then transferred to normal conditions. The cultivation temperature of *Arabidopsis thaliana* was 22°C~25°C.

3.4 Semi-quantitative RT-PCR to detect gene expression
We used TRIzol method to extract the total RNA of the plant, semi-quantitative RT-PCR detection, pre-denaturation: take 2.5 \( \mu \text{g} \) of RNA, adjusted to 10 \( \mu \text{L} \) system with ddH\(_2\)O, pre-denatured the RNA first, the pre-denaturation conditions: treatment at 70°C for 5 min; 4°C, 5 min. The pre-denatured RNA was reverse-transcribed, and the post-transcriptional cDNA was used as the template for PCR detection.

3.5 GUS staining analysis
The GUS staining solution and the GUS staining buffer were configured as the GUS staining working solution according to the volume ratio of 1:50, and placed in darkness at -80°C for standby use. Put the *Arabidopsis* plant material in a 50mL beaker, added the appropriate amount of GUS staining working solution to completely cover the plant material. Wrapped the beaker with foil paper and used the vacuum infiltration for staining. The vacuum pressure was 0.08 MPa and the time was 5 minutes. We observed that the plant material was immersed in the bottom of the GUS staining solution, and the experiment was repeated twice. The whole beaker was incubated at 37°C for 24 hours. With the extension of incubation time, GUS expression parts or sites showed blue or blue spots (The whole process is protected from light). Plant materials were fixed with 30 mL Carnoy's fluid at room temperature for 1-3 hours. Then the decolorization process was carried out. The volume fractions of ethanol were set as 25%, 50%, 70%, 85% and anhydrous ethanol, and the decolorization process was conducted at each concentration for 1-3 hours. We observed and took pictures under the microscope. Samples can be stored in 75% ethanol at 4°C for 1-3 months.

Authors' contributions
Taicheng Jin was the executor of this experimental study, completed the data analysis and paper writing. Liping Yang directed the experimental design and paper modification; Chenjing Lang, Yue Wang, Dawei Meng and Yanju Wu participated in the completion of relevant experiments. All authors read and approved the final manuscript.

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