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Full Length Research Paper

Expression of *VP60* gene from rabbit haemorrhagic disease virus (RHDV) YL strain under control of the *ats1A* promoter in tobacco

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The *VP60* gene from rabbit haemorrhagic disease virus (RHDV) YL strain in Northeast of China, under control of the *ats1A* promoter from Rubisco small subunit genes of *Arabidopsis thaliana,* was introduced into the transfer deoxyribonucleic acid (T-DNA) region of plant transfer vector pCAMBIA1300 and transferred to tobacco (*Nicotiana tabacum* cv. Petit Havanna SR1) with *Agrobacterium tumefaciens*-mediated method. Polymerase chain reaction (PCR) reverse transcription(RT) and -PCR analysis of the transformed tobacco plants confirmed the integration of the *VP60* gene copy into the plant DNA and *VP60* gene transcription produced. Western blot analysis revealed that the VP60 protein was expressed in tobacco under control of *ats1A* promoter.

Key words: *Agrobacterium tumefaciens,* rabbit haemorrhagic disease virus (RHDV), VP60 protein, *ats1A* promoter, plant-derived vaccines

INTRODUCTION

Rabbit haemorrhagic disease (RHD) is a highly fatal disease affecting rabbits. It is demonstrated that the etiological agent is the rabbit haemorrhagic disease virus (RHDV), a non-enveloped ribonucleic acid (RNA) virus belonging to the genus Lagovirus within the *Caliciviridae* (Parra and Prieto, 1990). The virus capsid is constructed by a unique structural protein VP60 (Ohlinger et al., 1990), which is the main component of commercially used vaccines isolated from liver extracts of infected rabbit. In two decades, the RHDV capsid protein VP60, which is responsible for the induction of protective antibodies, has been successfully expressed in several heterologous systems like *Escherichia coli* (Boga et al., 1994) and *Saccaromyces cerevisiae* (Boga et al., 1997). The development of a subunit RHD-vaccine is necessary for the replacement of the animal vaccine production.

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Abbreviations: RHDV, Rabbit haemorrhagic disease virus; **T-DNA,** transfer deoxyribonucleic acid.

However, these strategies are still inconvenient and expensive for their effective use in animal health. In recent years, transgenic plants have demonstrated considerable potential for the production of vaccines (Ma et al., 2005). Plants are a promising platform for the production of vaccines. The important reason is that it is an economical system as compared to other systems. In this system, desired protein can be produced at 2 to 10% of the cost of microbial fermentation systems and at 0.1% of the cost of mammalian cell cultures, although this depends on the protein of interest, product yield and crop used (Twyman et al., 2003). On the other hand, plants can perform most of the post-translational modifications required for protein stability, bioactivity and favorable pharmacokinetics (Gomord and Faye, 2004).

So far, several plant species suitable for the expression of recombinant proteins with technical and pharmaceutical value have been described. Obvious advantages of plant-derived vaccines are the convenient storage of the material, the lack of human or animal pathogens and the reduction of downstream processes. Storage tissues like cereal and legume seeds with high protein content and excellent storage qualities are favored for the development of edible vaccines. So antigen expression in plant

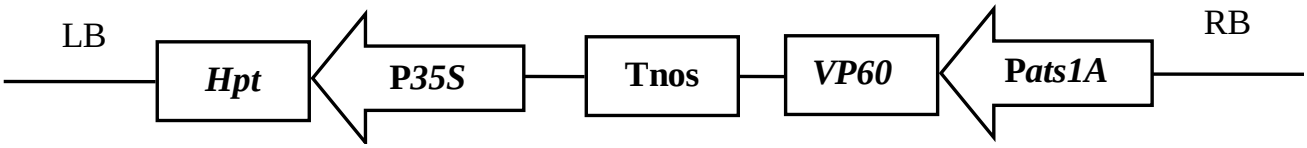

Figure 1. The schematic diagram of T-DNA structure of plant transfer vector pCAMBIA1300-*ats1A*-*vp60*. *Hpt*, Hygromycin phosphotransferase; P*35S,* cauliflower mosaic virus 35S promoter; Tnos, nopaline synthase terminator; P*ats1A*, *ats1A* promoter.

opens an important door to meet the global demand for cheaper, safer and quality vaccines. However, low expression levels combined with the low immunogenicity of plant-derived subunit vaccines have often obstructed the development and commercialization of edible plant vaccines. Optimizations of plant expression systems for vaccine production are essential.

To achieve high level of transcription, the promoter, which drives the transcription, plays an important role in the expression proceeding target gene. The choice of promoters affects transgenic transcription, resulting in changes not only in concentration, but also in the stage, tissue and cell specificity of its expression. The cauliflower mosaic virus 35S (*CaMV35S*) promoter has been used widely because of its strong and constitutive expression, but the *CaMV35S* promoter is lack of the signal peptide and doesn't guide the foreign protein to translocate into the suitable place in cell. In this study, we constructed the plant transfer vector including the *VP60* gene driven by *ats1A* promoter, which is from Rubisco small subunit genes of *Arabidopsis thaliana* and contains the chloroplast transit peptide, transformed tobacco (because the transformation is easier and faster in tobacco) with *Agrobacterium tumefaciens*-mediated method and explored whether the VP60 protein is expressed in tobacco.

MATERIALS AND METHODS

Binary plant transfer vector construction

The *VP60* gene, *ats1A* promoter and Tnos fragment were cloned into the plant transfer vector pCAMBIA1300 and the recombinant plasmid was named as pCAMBIA1300-*ats1A*-*vp60* (Figure 1). The cloning procedures were performed using standard methods (Sambrook and Russell, 2001).

Plant transformation and tissue culture

Sterile tobacco plants *Nicotiana tabacum* cv. Petit Havanna SR1 were cultivated in growth chambers at 24°C with a 16 h/8 h light/dark cycle in Murashige and Skoog (MS) medium containing 30 g/L of sucrose. The binary plant transfer vector was introduced into *A. tumefaciens* EHA105 by electroporation. *N. tabacum* cv. Petit Havanna SR1 was transformed using *A. tumefaciens*-mediated gene transfer, according to the methods of Horsch et al. (1985). Transformants were screened on MS medium, including 0.6 mg/L 6-benzylaminopurine (6-BA), 20 mg/L hygromycin, 300 mg/L carbenicillin and 30 g/L of sucrose.

Nucleic acid isolation and analysis

Plant genomic DNA was extracted from the leaf tissues of transgenic and non-transgenic tobacco according to procedures by Tang et al. (2007). The presence of the different transgenes, *VP60* gene, was confirmed by PCR using the following specific primer pairs: *VP60*fw (5′-TGCTCTAGAATGGAGGGCAAAGCCCGCAC-3′) and *VP60*rv (5′-TCCGAGCTCTTATTATCAGACATAAGAAAA-GCCATTGG-3′), which amplified about 1740 bp full-length fragment *VP60*. PCR condition were 94°C for 4 min, followed by 32 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 2 min and 72°C for 7 min. Total RNA was isolated from leaf tissues of the putative transformants (RNAiso Plus kit, Takara), and used as templates for the complementary deoxyribonucleic acid (cDNA) synthesis (Primescript RT reagent kit, Takara). RT-PCR was carried out by the primers as described previously. PCR condition was the same as aforementioned.

Western blot analysis

Leaf material from plants transformed was ground in liquid nitrogen and the resulting powder was extracted three times with phosphate-buffered saline (PBS) by mixing. Sodium dodecyl sulfate (SDS)-containing sample buffer was added and, after heat denaturation, protein sample was separated on 12% SDS polyacrylamide gels. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham), blocked for 2 h in 10% skim milk-Tris buffered saline (TBS; 0.1 M Tris–HCl, 0.9 NaCl, pH 7.5), and incubated with rabbit antiserum against VP60 protein (the antiserum was prepared by injecting VP60 protein expressed in *E. coli* into rabbit) diluted 1:200 in 2% skim milk-TTBS (0.1 M Tris–HCl, 0.9% NaCl, 0.1% Tween-20, pH 7.5) and anti-rabbit IgG conjugated with horseradish peroxidase (HRP) (CoWin Bioscience Co.) diluted 1:2000 in 2% skim milk- Tris-Tween buffered saline (TTBS). Conjugation antibody was detected using the HRP-DAB system.

RESULTS

To assess the expression of *VP60* gene in tobacco (*N. tabacum* cv. Petit Havanna SR1) plants, a plant transfer binary plasmids was constructed (Figure 1). In the recombinant plasmid pCAMBIA1300-*ats1A*-*vp60*, the expression of *VP60* gene was driven by the *ats1A* promoter. Sequencing of the recombinant plasmid confirmed that no mutations had been introduced and open reading frame (ORF) of *VP60* gene was correct. Following *A. tumefaciens*-mediated transformation, several independent putative transgenic plants rooted on hygromycin–selective medium were assayed by PCR for the detection of *VP60* gene (Figure 2). These plants were positive approximately 90% for *VP60* gene. Transcription

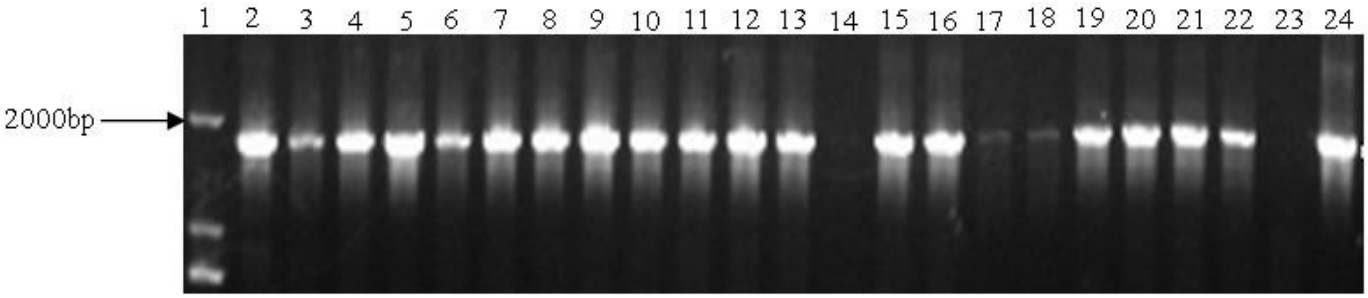

Figure 2. Electrophoresis of genomic DNA PCR. Genomic DNA PCR (about 1740 bp). Lane 1, marker; lanes 2 to 22, transformed tobacco plants; lane 23, non-transformed tobacco plant; lane 24, plant transfer vector pCAMBIA1300-*ats1A*-*vp60* positive control.

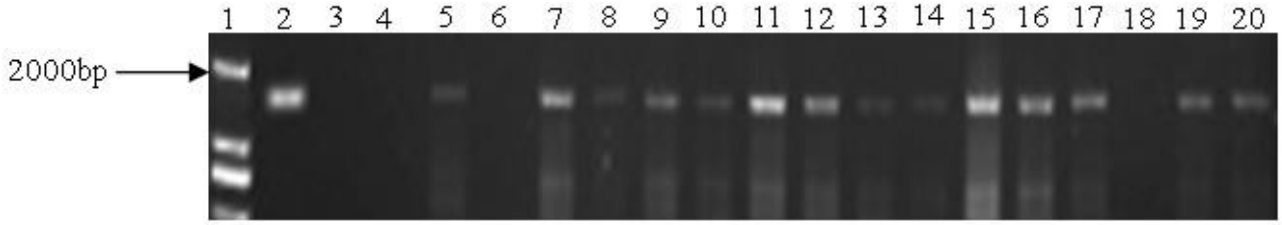

Figure 3. Electrophoresis of RT-PCR. RT-PCR (about 1740 bp). Lane 1, marker; lane 2, plant transfer vector pCAMBIA1300-*ats1A*-*vp60* positive control; lanes 3 and 4, non-transformed tobacco plant; lanes 5 to 20, transformed tobacco plants. RT-PCR, reverse transcripton-polymerase chain reaction.

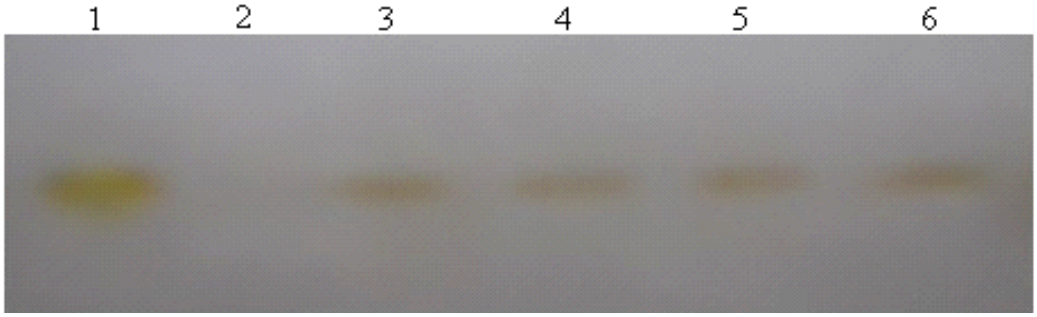

Figure 4. Detection of VP60 protein by Western blot analysis in transformed tobacco. Lane 1, VP60 protein expressed in *E. coli*; lane 2, non-transformed tobacco plant; lanes 3 to 6, different transformed tobacco plants.

of *VP60* gene after its introduction into the plant, RT-PCR was carried out using total RNA extracted from leaves from transformed plants, which were positive for *VP60* gene integration by genome PCR analysis. Some individual transformed plants showed a 1740 bp specific band corresponding to *VP60* gene. Control plant non-transformed, did not show any detectable signal (Figure 3). The presence of VP60 recombinant protein in plants integrating the *VP60* gene was investigated by western blot using leaves from plants (Figure 4). As depicted in Figure 4, there were hybrid responses in transformed plants and there was not in non-transformed plant.

DISCUSSION

In recent years, transgenic plants are one of the most promising substrates to produce pharmaceutical recombinant proteins at a reasonable cost and with high biological security standards, especially in vaccine production. Using plant transformed with *VP60* gene to produce the vaccine against RHD is a typical example. Some achievements have been made in many plants such as tobacco (Mikschofsky et al., 2009a), potato (Castanon et al., 1999; Martín-Alonso et al., 2003) and pea (Mikschofsky et al., 2009b). Most of VP60 expressions in plants are analyzed using the *CaMV35S*

promoter as regulation fragment. In this study, transcription of *VP60* mRNA was driven by the *ats1A* promoter, which is cloned from Rubisco small subunits genes of *A. thaliana*. There is a chloroplast transit peptide included in the promoter.

Wong et al. (1992) reported that the *ats1A* promoter directed approximately 10-fold to 20-fold higher levels of expression than the *CaMV35S* promoter when *ats1A* transit peptide coding sequences was included. Also the target protein fused to the transit peptide was translocated to the chloroplast. There are no reports about expression of the *VP60* gene under regulation of the *ats1A* promoter. Thus, the study was designed to ascertain expression of the *VP60* gene driven by *ats1A* promoter. In summary, our results demonstrate that VP60 protein can be expressed in tobacco under control of the *ats1A* promoter. This study facilitates further development of plant-derived vaccines against RHDV. Work is in progress to examine whether the VP60 protein expressed in transformed tobacco is translocated to the chloroplast.

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