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Expression and detection of the FMDV VP1 transgene and expressed structural protein in *Arabidopsis thaliana*

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Expression and detection of the FMDV VP1 transgene and expressed structural protein in *Arabidopsis thaliana*

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Abstract: To explore the feasibility of developing a new type of plant-derived foot-and-mouth disease virus (FMDV) oral vaccine, the plant seed-specific expression vector p7SBin438/VP1 carrying the VP1 gene of the FMDV strain O/China/99 was constructed and transformed into *Agrobacterium tumefaciens* strain GV3101. This strain was used for transformation of *Arabidopsis thaliana* via the floral-dip method. The kanamycin-resistant transgenic plants were selected, and the VP1 gene and protein expressions were confirmed by PCR and sandwich-ELISA, respectively. The ELISA positive plants were further confirmed by Western blot assay, and the VP1 gene in the transgenic progeny was confirmed by PCR. The results showed that the *Agrobacterium*-mediated floral-dip transformation method could produce transgenic *Arabidopsis* containing the VP1 gene. Therefore, this protocol provides a successful strategy to deliver viral genes to leguminous plants for genetic studies and for production of low-cost oral vaccines.

Key words: Foot-and-mouth disease virus (FMDV), VP1 gene, seed-specific expression vector, transgenic *Arabidopsis thaliana*

Introduction

Foot-and-mouth disease (FMD) is an acute, febrile and a highly contagious disease caused by the foot-and-mouth disease virus (FMDV), and mainly infects cloven-hoofed animals (1). Although it results in a low mortality rate, the disease reduces animal reproductive performance, and its outbreaks affect international trade, often resulting in heavy direct and indirect economic losses. At present, in the prevention and control of FMD, a policy that combines immunization and culling has been

adopted (2). A safe and effective vaccine is the prerequisite for the successful control, prevention, and even eradication of FMD. The FMD attenuated vaccine has a good and long-lasting immunity, and can be injected in a small volume. However, the attenuated vaccine has shortcomings in terms of virulence distribution and genetic reversions. There are many worldwide reports of FMD caused by the use of attenuated vaccines, and many countries have expressly prohibited their use as a result (3). Although the inactivated vaccine overcomes some

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disadvantages of the attenuated vaccine, it requires large vaccine doses and induces only a short duration of immunity and limited spectrum of antiviral immune responses. Additionally, incomplete inactivation of the vaccine virus may also cause a new outbreak of the disease, and all of these limitations will become increasingly prominent (4). Therefore, research institutions around the world are working on the development of safe and effective new FMD vaccines.

The edible vaccines produced in a plant reactor, a new trend in vaccine research, may overcome the disadvantages of previous generation inactivated vaccines by having the additional advantages of the low cost of production, storage and transportation not requiring a special cold-chain system (5,6). In recent years, there have been reports both in China and Argentina on the use of FMDV VP1 protein expressed in transgenic plants as a vaccine antigen. The Borca group used VP1 protein produced by a plant expression system to immunize mice, which successfully produced VP1 specific antibodies and were effectively protected (7-9). Sun et al. (10) transferred the FMDV VP1 gene into tobacco and *Chlamydomonas chloroplasts*, and obtained their expression. Pan et al. (11) transferred the FMDV structural polyprotein P1-2A and protease 3C gene into tomato plants, and foliar extracts from P1-2A3C transgenic tomato plants elicit a protective response in guinea pigs.

Ideal transgenic plants often require high-level expression of the exogenous gene in a specific location and a specified period of time. Therefore, targeting and optimizing gene expression in plant seeds or other specific tissue is one strategy for accumulating the target protein, and research on and applications of tissue-specific promoters are gaining increased attention (12-14). While constitutive promoters are currently widely used in plant expression vectors, we used the seed-specific promoter 7S to replace the cauliflower mosaic virus (CaMV) 35S constitutive promoter in the plant constitutive expression vector pBin438, and constructed the seed-specific vector p7SBin438/VP1 expressing the FMDV VP1 gene. Using this construct, we subsequently produced transgenic *Arabidopsis thaliana* with efficient expression of the VP1 structural protein in

pods by the floral-dip transformation method with *Agrobacterium tumefaciens* GV3101. This study explored the application potential of *A. thaliana* model plant to investigate its feasibility for further research on transformation of FMDV immune genes into leguminous plant seeds.

Materials and methods

Reagents

Taq polymerase, BamH I, Sal I, and Hind III restriction enzymes were purchased from Promega (Madison, WI, USA). FMD positive bovine serum and ELISA kits were kindly provided by Prof. Junwu Ma (Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences). AKP labeled IgG was from Sigma (St. Louis, MO, USA). 6-Benzylamino purine (6-BA) and 2-morpholinoethanesulfonic acid (MES) were from Beijing Jinke Biotech Co. (Beijing, China). Nitrocellulose filters and NBT/BCIP were from AMRESCO (Solon, OH, USA). All reagents were purchased from Sigma (St. Louis, MO, USA), except where otherwise stated.

Plasmids and E. coli strains

pGEM/VP1 plasmid carrying the VP1 gene of FMDV O/China/99 strain was stored in our laboratory. *A. tumefaciens* strain GV3101 and plant constitutive-expression vector pBIN438 were kindly provided by the Crop Institute, Chinese Academy of Agricultural Sciences. The pU8-2 plasmid carrying plant seed-specific promoter 7S was provided by the Institute of Microbiology, Chinese Academy of Sciences.

Construction of seed-specific vector p7SBin438/VP1

For the construction of p7SBin438/VP1, a pair of primers (7S1: 5'-CCCAAGCTTCCTATCTGTCACTTC-3'; 7S2: 5'-CGGGATCCGAGACTGGTGATT-3') containing the Hind III and BamH I restriction sites (underlined), respectively, were designed to amplify the 7S promoter from plasmid pUC18 carrying a seed-specific promoter. The resulting PCR fragments and pBIN438 plasmid were digested with Hind III and BamH I. The fragments of interests were purified from an agarose gel and

ligated with T4 DNA ligase, resulting in the seed-specific plasmid p7SBin438. A pair of primers (VPR1: TGACCACCTCCACAGGCGAGCCGGCTGAC-3'; VPR2: TCAGACAAGAGCTGTTTCACAGGCGCCAC-3') containing the BamH I and Sal I restriction sites (underlined), respectively, were designed to amplify the VP1 gene from pGEM/VP1. The resulting PCR fragments and p7SBin438 plasmid were digested with BamH I and Sal I. The fragments of interests were ligated with T4 DNA ligase, resulting in p7SBin438/VP1 (Figure 1). The recombinant was verified by restriction enzyme analysis, PCR, and sequencing. The vector p7SBin438/VP1 was transferred to *A. tumefaciens* strain GV3101 by triparental mating as described previously (15). The bacteria were cultured at 28 °C in the dark in liquid Yeast Extract and Beef (YEB) medium supplemented with 50 mg/L kanamycin, 25 mg/L streptomycin, and 50 mg/L rifampicin to screen and identify the target gene.

Transformation of *Arabidopsis thaliana*

A. thaliana was transformed by the floral-dip transformation method described previously (16,17). Briefly, *Arabidopsis* plants were grown in a 10 cm diameter pot until they began to bolt. The main bolts were clipped, and transformation was conducted when the lateral branches grew to 2 to 10 cm and there were many unopened flower clusters on the top. Two days before vacuum infiltration, a colony of *Agrobacterium* carrying p7SBin438/VP1 plasmid was selected and inoculated in 5 mL YEB medium with antibiotics and grown at 28 °C and shaken at 250 rpm

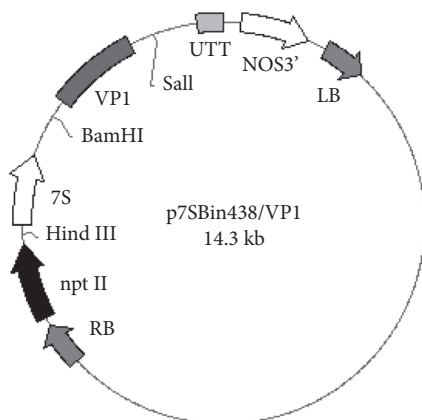


Figure 1. Construction of plant seed-specific expression vector p7SBin438/VP1.

for 2 days. The bacteria were then inoculated into 500 mL of YEB media at a ratio of 1:100 and grown until the OD_{600} reached 0.8. After centrifugation at $3500 \times g$ for 15 min, the *Agrobacterium* was resuspended in dipping solution (1/2 MS salt, $1 \times$ Vit B5, 50 g/L sucrose, 0.5 g/L MES, 0.044 μ M 6-BA, and 200 μ L/L Silwet L-77, adjusted with KOH to pH 5.7) and adjusted to $OD_{600} = 0.8$. The above-ground parts of the plant were immersed in dipping solution for 2 to 3 min with gentle agitation. The dipped plants were placed under a dome for 24 h to maintain high humidity. The plants were grown in a greenhouse until maturity. The dry seeds were harvested as T_0 seed and stocked in a tube.

Resistance screening of transgenic *Arabidopsis thaliana*

T_0 seeds were surface sterilized in 10% sodium hypochlorite for 10 min, followed by washing with sterile water 5 to 6 times, and resuspended in a small amount of sterile water. After vernalization at 4 °C for 2 days, the seeds in the water solution were drawn with a 1 mL pipette, and uniformly sowed on MS medium (50 mg/L kanamycin). They were then transferred to a 20 ± 2 °C climate chamber with continuous illumination and cultivated until the seed pods turned yellow, after which T_1 seeds were harvested.

PCR detection of transgenic *Arabidopsis*

Fifty milligrams of leaves from screened transgenic *Arabidopsis* were harvested and minced in liquid nitrogen. DNA was isolated using the CTAB (hexadecyl trimethyl ammonium bromide) method as described previously (15). The leaves from wild-type *Arabidopsis* were used as negative control. PCR was performed using a commercial kit (Takara, Dalian, China) with the VPR1 and VPR2 primers according to the manufacturer's instructions. Amplification conditions were 94 °C, 3 min; followed by 30 cycles of 94 °C, 50 s; 55 °C, 50 s; 72 °C, 1 min; and a final elongation 72 °C, 10 min.

ELISA detection of transgenic *Arabidopsis*

Fifty milligrams of leaves and legumen were harvested from transgenic or wild-type *Arabidopsis* (negative control) and minced in isolation buffer (PBST, 0.029% NaN_3 , pH 7.4) followed by centrifugation at $8000 \times g$ for 20 min. The supernatant

was collected and the target VP1 protein was detected using a sandwich-ELISA kit (Junwu Ma, Lanzhou, China). Whole inactivated FMDV antigen was used as positive control.

Western blot analysis

Fifty milligrams of leaves and legumen of transgenic *Arabidopsis* were minced in liquid nitrogen followed by mixing with 150 μ L of protein isolation buffer (50 mmol/L Tris-CL, pH 7.5, 10 mmol/L EDTA, 100 mmol/L NaCl, 0.5% Triton X-100, 14 mmol/L mercaptoethanol, and 1 mmol/L PMSF). The mixture was centrifuged at 10,000 rpm for 20 min at 4 °C. The supernatant was harvested and mixed with the same volume of loading buffer followed by boiling for 5 min. Aliquots of 50 mg proteins were separated by 12% SDS-polyacrylamide gel, and transferred to nitrocellulose filters. The filters were blocked with TBST buffer containing 5% skimmed milk, incubated with FMD bovine serum (1:12,000) at 37 °C for 2 h, and followed by the addition of AP-conjugated rabbit anti-bovine IgG (1:15000) at 37 °C for 2 h. Binding was visualized by incubation with NBT/BCIP substrate solution.

Analysis of the transgenic *Arabidopsis thaliana* progeny

After the T₁ transgenic seeds were surface sterilized, they were sown on MS medium containing 50 mg/L kanamycin (at 20 \pm 2 °C, and continuous light) for germination. The T₂ seedlings were then transferred into soil to grow. The genomic DNA of leaves of kanamycin-resistant putative transgenic plants was isolated, and the VP1 gene fragments were amplified as described above.

Results

Construction of seed-specific expression vector p7SBin438/VP1 and sequence analysis

The recombinant p7SBin438/VP1 vector was constructed as described in *Materials and methods*, and the inserted fragments (7S and VP1) were confirmed by digestion with BamH I/Sal I or Hind III/BamH I, which yielded the expected sizes of about 650 bp and 500 bp, respectively. PCR analysis was also performed using VPR1/VPR2 or 7S1/7S2 primer sets to verify the presence of the inserts in the

p7SBin438/VP1 plasmid. The corresponding PCR products of expected sizes were obtained, indicating that the seed-specific expression vector p7SBin438/VP1 carrying VP1 gene was successfully constructed (Figure 2). Finally, the sequencing results showed that the VP1 gene was correctly cloned into the plant expression vector, and the sequence is 100% identical to the corresponding coding sequence of the parent O/China/99 strain.

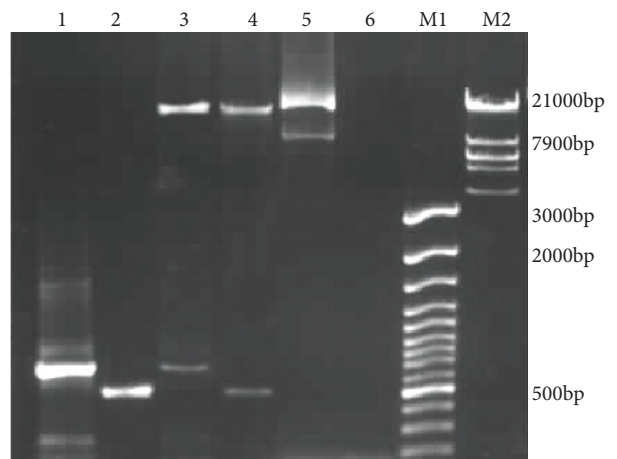


Figure 2. Identification of recombinant plasmid p7SBin438/VP1 by enzyme digestion and PCR.

Lane M1, 100 bp DNA Ladder Plus Marker; Lane M2, Low Range II Marker; Lane 1, PCR product of VP1; Lane 2, PCR product of 7S; Lane 3, p7SBin438/VP1 digested with BamH I/Sal I; Lane 4, p7SBin438/VP1 digested with Hind III/BamH I; Lane 5 Recombinant plasmid p7SBin438/VP1; Lane 6, Negative control.

Arabidopsis transformation and resistant seed selection

A. thaliana inflorescences were transformed by the floral-dip method. After the maturity of *Arabidopsis*, T₀ *Arabidopsis* seeds were harvested and sown on the MS medium containing 50 mg/L kanamycin following sterilization. The majority of seedlings turned yellow 2 weeks after kanamycin resistance selection and then gradually died. The kanamycin-resistant seedlings gradually grew and rooted quickly. The untransformed plants could not root and subsequently withered. The control groups

grew well. After growing 4 to 8 rosette leaves, the kanamycin-resistant seedlings were transplanted into soil soaked with Hoagland nutrient fluid for further cultivation.

Expression of the VP1 gene in transgenic Arabidopsis

Genomic DNA in leaves of kanamycin-resistant Arabidopsis seedlings was extracted, and the target VP1 gene was detected with primers VPR1/VPR2 (Figure 2). The VP1 gene was amplified in 10 out of 16 (62%) kanamycin-resistant strains, indicating that T-DNAs containing the VP1 gene were transferred from *Agrobacterium* into the Arabidopsis genome. The results in Figure 2 are representative positive PCR amplifications of the VP1 gene in 4 of the transgenic plants and 1 non-transgenic plant.

Expression of VP1 proteins in transgenic Arabidopsis

Total proteins were extracted from the pods of PCR positive plants, and the VP1 content was detected by ELISA. Only 3 out of 10 plants had positive OD values at or greater than 2-fold higher than that of negative control samples (Figure 3). The OD values of these 3 plants (named transgenic line 1, 2, and 3) gradually declined proportionally to the increase in dilution of the transgenic plant protein extracts, similar to the results with control whole FMDV antigen. The OD values of control plants that were not transformed were low and changed

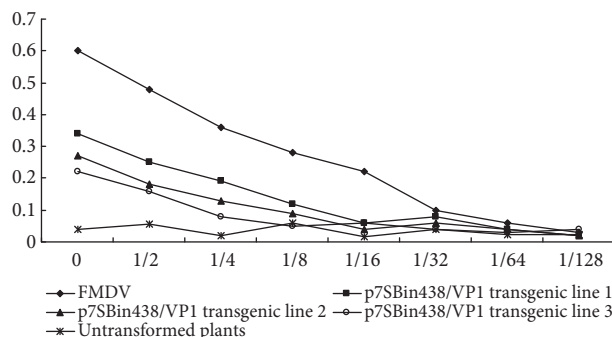


Figure 3. Quantity of target protein in transgenic plants tested with sandwich-ELISA. The data show a specific VP1 protein expression in 3 positive transgenic Arabidopsis. The transgenic lines 1, 2, and 3 have positive OD values at or greater than 2-fold higher than those of negative control samples. The OD values of control plants that were not transformed were low.

irregularly with the increase in dilution of protein extracts. These results indicate that the 3 transgenic Arabidopsis specifically expressed the target VP1 protein.

Tissue specific expression of VP1 in transgenic Arabidopsis

The total protein in the leaves and pods of 3 plants (transgenic lines 1, 2, and 3) confirmed by ELISA to be transgenic were extracted for Western blot assay. As shown in Figure 4, a shallow band of the expected size for VP1 can be seen on the blot of the pod samples for the transgenic lines 2 and 3. For *A. thaliana* pod samples, no specific band can be seen for the protein extract of the pods of transgenic line 1 and non-transformed plant, and of the corresponding leaves of transgenic plants. These results indicate that seed-specific promoter 7S was able to induce the VP1 gene to express the target protein specifically in the pods of transgenic Arabidopsis.

VP1 gene expression in progeny of transgenic Arabidopsis

In order to determine whether the VP1 gene was inherited by the progeny of transgenic plants, T₂ seeds

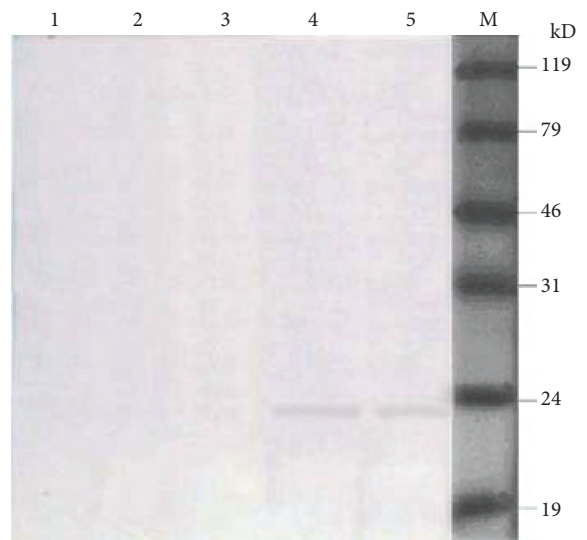


Figure 4. Western blot analysis of the expressed VP1 protein.

Lane M, Protein Molecular Weight Marker; Lane 1, Pod of non-transformed plant; Lane 2, Leaves of transgenic lines 1, 2, 3; Lane 3, Pod of transgenic line 1; Lane 4, Pod of transgenic line 2; Lane 5, Pod of transgenic line 3.

were grown in a greenhouse. The genomic DNA was extracted from the leaves in individual seedlings. The VP1 gene was detected by PCR as described above (data not shown), indicating that the VP1 gene was transmitted to the progeny.

Discussion

Of the 5 identified antigenic sites of O-type FMDV, 3 are located on VP1. In particular, the antigen site 1 comprises the G-H loop on VP1 and the C-terminal residues ~200-213. The amino acids on the loop contain T and B cell epitopes that can stimulate immune responses, and both regions on VP1 play a major role in determining the antigenicity and immunogenicity of the virus (18). Therefore, we selected the FMDV VP1 gene in our studies to insert into a plant seed-specific expression vector. We successfully obtained transgenic plants expressing the VP1 gene in their pods by transforming the p7SBin438/VP1 vector into the model *A. thaliana* plant with the floral-dip method. Altogether, our results indicated that with the guide of the 7S promoter the VP1 gene could be expressed in the pods as well as the seeds of transgenic *A. thaliana*. This study laid a good experimental foundation for our future studies on transfer of FMDV immune genes into seeds of leguminous plants.

Floral-dip of *A. thaliana* primarily uses the flower reaching anthesis as the competent cells for the *Agrobacterium* strain inoculated into plant tissues through the hypertonic effect of surfactant Silwet L-77 and sucrose (16,19,20). This method directly targets the whole plant, and can result in transformant seeds without need for an explant ex vivo regeneration system. Furthermore, it avoids the cumbersome process of tissue culture, and allows the possibility of genetic transformation for plants that are difficult to manipulate in tissue culture. We noted that 2 critical points should be carefully considered for the successful implementation of this method: the selection of the optimal timing of transformation and addition of surfactant Silwet L-77 at the appropriate concentration.

Promoter optimization is an important aspect of constructing efficient expression vectors. Currently, the plant expression system mainly adopts the

cauliflower mosaic virus (CaMV) 35S constitutive promoters. Under the control and regulation of constitutive promoters, there is no significant difference in the gene expression in different tissues and organs and in different developmental stages, and the expression level is low. Moreover, some adverse effects on the normal growth of plants may occur as plant constitutive promoter genes guide target genes to express in all tissues of plants, which will consume endogenous material and energy within the plants. Regulation of gene expression by tissue-specific promoters not only can enhance gene expression levels in specific organs or tissue locations, minimizing biological energy consumption, but also is conducive to the separation of the expression products. The plants used in the production of vaccine antigens often require a specific time to express the target protein in specific tissues or organs. Therefore, it is necessary to adopt tissue-specific expression promoters or inducible promoters. Richter et al. (21) successfully expressed hepatitis B surface antigen and Norwalk virus capsid protein with the use of tissue-specific expression patatin promoter in potato stem tuber tissue. Ruf et al. (22) successfully established a tomato fruit plastid expression system, which enabled over-expression of exogenous genes in tomato fruit, with recombinant protein accounting for 20% of the total soluble protein. This technology platform established a high-level expression system for vaccine proteins in higher plant fruits. The embryos of crop seeds are rich in soluble proteins, with a high expression of recombinant proteins, and able to maintain long-term preservation. Moreover, protein from these seeds can be easily separated and purified, which facilitates the concentration of recombinant antigen protein. Therefore, the seeds are ideal carriers for recombinant protein production (23). Our results also indicated that under the guidance of the 7S promoter, the VP1 gene could be expressed in transgenic *A. thaliana*. However, the VP1 antigen expression in transgenic plants was far below the amount of diluted FMDV antigen. We speculate that the low positive rate of ELISA detection may be related to the purification technology, and the crude protein extracted from plants may mask or degrade some of the expressed target proteins. Alternatively, the existing detection methods may not be sufficiently sensitive, which requires further exploration. It will be useful to try

different expression vectors, including the use of stronger promoters (24), plant-optimized synthetic genes (25,26), signal peptides that target the protein for retention in intercellular compartments (27), and a chloroplast expression system (28,29). It will also be important to develop sensitive methodologies that allow the screening of a large number of individual plants so as to identify and select those that express the highest levels of transgenic protein.

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