Hort. Environ. Biotechnol. 52(5):511-515. 2011.
DOI 10.1007/s13580-011-0007-x

Research Report

Expression of the Protective Antigen for PEDV in Transgenic Duckweed, *Lemna minor*

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Received January 5, 2011 / Accepted June 23, 2011

Abstract. Duckweeds are small, floating aquatic plants with a number of useful characteristics, including edibility, fast-growing, and a clonal proliferation. Duckweed is also fed to animals as a diet complement because of its high nutritional value. Porcine epidemic diarrhea virus (PEDV) is a major causative agent of fatal diarrhea in piglets and is a serious problem in the hog-raising industry. In this study, we assessed the feasibility of producing a protective antigen for the PEDV spike protein 1 using duckweed, *Lemna minor*. Stably transformed *Lemna* were obtained by co-cultivation with *A. tumefaciens* EHA105 harboring the PEDV spike protein gene. Transgene integration and expression of the PEDV spike protein 1 gene were confirmed by genomic PCR and RT-PCR and western blot analysis of transgenic *Lemna*, respectively. This is the first report of the expression of a vaccine antigen against an animal infectious disease in duckweed.

***Additional key words*:** aquatic plant, porcine diarrhea, spike protein, transformation, vaccine

Introduction

Plants have many potential advantages in the production of high-value proteins, such as therapeutic proteins, including a low risk of contamination with potential animal pathogens, possessing the proper protein modification machinery, and providing low-cost scalable system for commercial production. It may also be possible to use them as a vehicle for oral delivery of vaccine antigens, resulting in an 'edible plant vaccine' (Fischer et al., 2004; Koprowski and Yusibov, 2001; Ma et al., 2005).

Duckweeds are small, floating aquatic plants belonging to the Lemnaceae, a monocotyledonous family containing four genera: Spirodella, Lemna, Wolffia, and Wolfiella (Stomp, 2005). Useful characteristics of duckweed include being fast-growing with clonal proliferation, a small size and a simple growth habit, suggesting its use as a biomanufacturing platform for recombinant proteins (Stomp, 2005). Duckweed is also fed to animals and fish to complement their diets because of its high nutritional value (Leng, 1995). Duckweed has been used to successfully produce several high-value proteins, including human monoclonal antibody modified to lack plant-specific N-glycans (Cox et al., 2006), a bacterial endoglucanase (Sun et al., 2007), alpha-interferon (Gasdaska et al., 2003) and aprotinin, used in cardiac surgery (Rival et al., 2008).

Porcine epidemic diarrhea (PED) is a contagious, enteric disease clinical symptoms of which include vomiting, diarrhea and dehydration resulting in a loss of appetite and weight in adult pigs and death in neonatal piglets (Pensaert and de Bouck, 1978). Porcine epidemic diarrhea virus (PEDV) is a known causative agent of PED. PEDV is an enveloped RNA with a single, positive-stranded genome, belonging to the group I coronaviruses (Lai and Cavanagh, 1997). PEDV infects intestinal epithelial cells and causes loss of enterocytes with villous atrophy, resulting in diarrhea and dehydration (Coussement et al., 1982). Development of effective vaccination strategies against PEDV infection is important because PEDV-induced disease is a serious problem in the hog-raising industry. Edible vaccines delivered to mucosal surfaces may be good candidates for a vaccination system for inducing modulated

systemic immune responses without injection-related hazards. The antigen protein corresponding to the neutralizing epitope of the PEDV spike protein has been successfully expressed in a transgenic tobacco plant (Kang et al., 2005, 2006).

In this study, we report the stable transformation and expression of a protective antigen for PEDV in *Lemna minor* with potential for use as an effective complement to the diets of animals.

Materials and Methods

Plant Material

Lemna minor was collected from a rice field near Daejeon, Korea, and surface-sterilized with 1% sodium hypochlorite solution for 10 min. Fronds of *Lemna* were cultured on half-strength MS medium supplemented with 1 $mg \cdot L^{-1}$ BA, 0.4 $mg \cdot L^{-1}$ thiamine HCl, 100 $mg \cdot L^{-1}$ myo-inositol, 15 $g \cdot L^{-1}$ sucrose, and 4 $g \cdot L^{-1}$ gelrite (½MS1BA). The pH of the medium was adjusted to 5.8 using 1 N NaOH before autoclaving. All cultures were maintained at 26℃ under continuous fluorescent light (70 $\mu mol \cdot m^{-2} \cdot s^{-1}$, photoperiod 16/8 h).

Determination of the Phytotoxic Level of Antibiotics

To examine the phytotoxic level of antibiotics, fronds were cultured separately on ½MS1BA medium supplemented with kanamycin at 0, 50, 100, 200, 300, and 500 $mg \cdot L^{-1}$. Each treatment consisted of 20 fronds per dish with three replicates. Data for the mean percentage of productive fronds was calculated for each treatment.

Transformation of *Lemna minor*

A single cell colony of the bacteria was grown in 4 mL YEP liquid medium containing 50 $mg \cdot L^{-1}$ kanamycin for 12 h, and 2 mL were transferred to 50 mL YEP liquid medium containing 50 $mg \cdot L^{-1}$ kanamycin for another 12 h at 28℃. Bacterial cells were pelleted by centrifugation (4000 rpm, 15 min), and the pellet was dissolved in liquid ½MS1BA containing 100 μM acetosyringone. For transformation, fronds were injured with a pair of forceps and scalpel, and immersed in the bacterial suspension for 30 min. Fronds were then blotted onto sterile filter paper, and co-cultivated with *Agrobacterium tumefaciens* strain EHA105 harboring the PEDV spike protein 1 gene fused to a c-myc tag for 72 h on antibiotic-free ½MS1BA medium. After co-cultivation, the fronds were rinsed with liquid ½MS1BA supplemented with 500 $mg \cdot L^{-1}$ cefotaxime, and cultivated on solid ½MS1BA medium supplemented with 300 $mg \cdot L^{-1}$ cefotaxime and 200 $mg \cdot L^{-1}$ kanamycin. Kanamycin-resistant fronds were subcultured at 3 week intervals.

Genomic PCR Analysis

Total genomic DNA from the putative transgenic and untransformed wild-type *Lemna* was extracted according to Dellaporta et al. (1983). PCR was carried out using the specific forward/reverse primers designed for the PEDV spike protein 1 gene. The gene-specific primers used for genomic PCR were as follows: 5'-ATGGAACAGCCAATTTCTTT-3' and 5'-AAAAGATCTATGGTACAAGC-3'. After denaturation for 5 min at 95℃, samples were carried through 30 cycles using the following PCR conditions: 95℃ for 1 min, 55℃ for 1 min, and 72℃ for 1 min.

RT-PCR Analysis

Total RNA was extracted from the whole *Lemna* plants using the Trizol reagent (Invitrogen, USA). cDNA synthesis was carried out using 2 μg of DNase-digested total RNA with oligo (dT) primers using a M-MLV Reverse Transcriptase Kit (Promega, Madison WI) according to the manufacturer's protocol. The gene-specific primers used for RT-PCR were the same as those used for genomic PCR analysis. As a control, the actin gene was amplified using two degenerate primers (5'-TCTGGCATCACACCTTCTACAAC-3' and 5' CAGTGTGGCTGACACCATCACCA-3'). The following thermocycling conditions were employed: initial denaturation at 94℃ for 5 min, 25 cycles of 94℃ for 30 s, 55℃ for 30 s and 72℃ for 30 s, with a final extension at 72℃ for 7 min. The amplified products were separated on a 1.2% agarose gel and photographed.

Western Blot Analysis

Lemna tissue was ground in liquid nitrogen to a fine powder. The powder (0.1 g) was resuspended in 200 μL of extraction buffer consisting of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, and 2% PVPP. The extract was centrifuged (12,000 rpm, 30 min, 4℃), and the resulting supernatant was used for Western blot analysis. Extracted proteins were separated on a 10% Gradi-Gel II gradient Gel (Elpis Biotech, Korea) and transferred onto a nitrocellulose membrane (GE Healthcare, UK). After blocking with 5% non-fat dried milk, the blots were incubated with mouse anti-myc antibody (IGTHERAPY, Korea), followed by incubation with goat anti-mouse IgG coupled to horseradish peroxidase (Bio-Rad, Hercules, CA, USA). Immunoreactive signals were detected using PicoEPD Western Blot Detection Reagents (Elpis Biotech, Korea), according to the manufacturer's instructions.

Results and Discussion

Despite the useful characteristics of duckweed as a biomanufacturing platform for foreign proteins, so far there have only been a few reports on the production of recombinant pro-

teins, as described above. More protein trials and data collection are necessary to expand the industrial availability of duckweed as a biomanufacturing platform.

In the present study, we assessed the feasibility of producing a protective antigen for PEDV in *Lemna minor*, a member of the duckweed family. First, to determine the appropriate conditions for selecting *Lemna* transformants, the effect of increasing concentrations of kanamycin was assessed on *Lemna* fronds (Fig. 1A). Approximately 78% of the fronds were bleached on day 30 in culture medium containing 100 mg·L^{-1} kanamycin. The phytotoxic effect of kanamycin was noticeable at 200 mg·L^{-1} in the medium as fronds started to bleach between day 10 and day 14 of culture, resulting in a total loss of chlorophyll pigmentation (bleaching) by day 30. Thus, in subsequent experiments, we supplemented the ½ MS1BA medium with 200 mg·L^{-1} kanamycin to select the putative transformed fronds. Yamamoto et al. (2001) established efficient genetic transformation protocols for two duckweed species, L. gibba (geographic isolate G3) and *L. minor* (geographic isolates 8627 and 8744; strain number is designation in Lemnaceae Germplasm Collection). Only selection with 10 mg·L^{-1} kanamycin was used for the transformation of *L. gibba* G3 and *L. minor* 8744, while concentrations of up to 100 mg·L^{-1} were used for *L. minor* 8627 (Yamamoto et al., 2001). In our experiments, kanamycin at a comparatively high concentration (200 mg·L^{-1}) was used. This difference may be due to different sensitivity to antibiotics of various geographic isolates of *Lemna*. Moon et al. (1998) have been

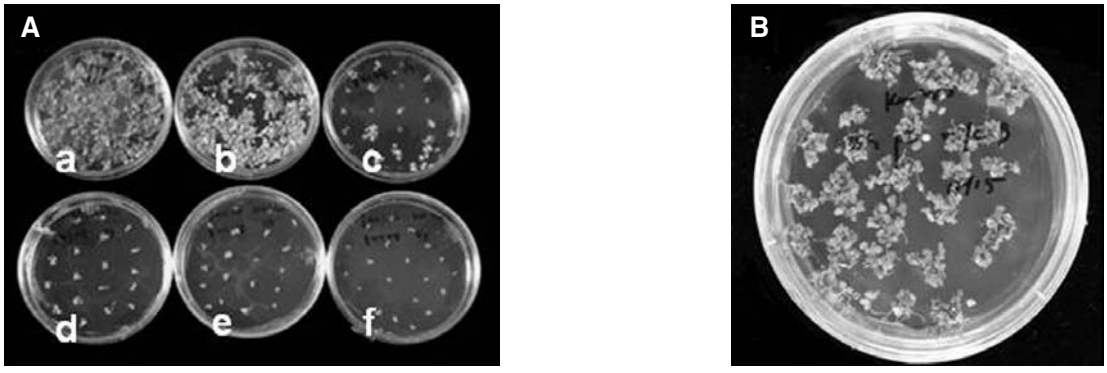

Fig. 1. Determination of kanamycin concentration (A) and putative *Lemna* transformants (B). *Lemna* fronds were cultured onto half-strength MS salts supplemented with 1 mg·L^{-1} BA, 3% sucrose, and 4 g·L^{-1} gelite. Several concentrations of kanamycin were used: 0 (a), 50 (b), 100 (c), 200 (d), 300 (e), and 500 mg·L^{-1} (f). For each treatment, there were three replicates.

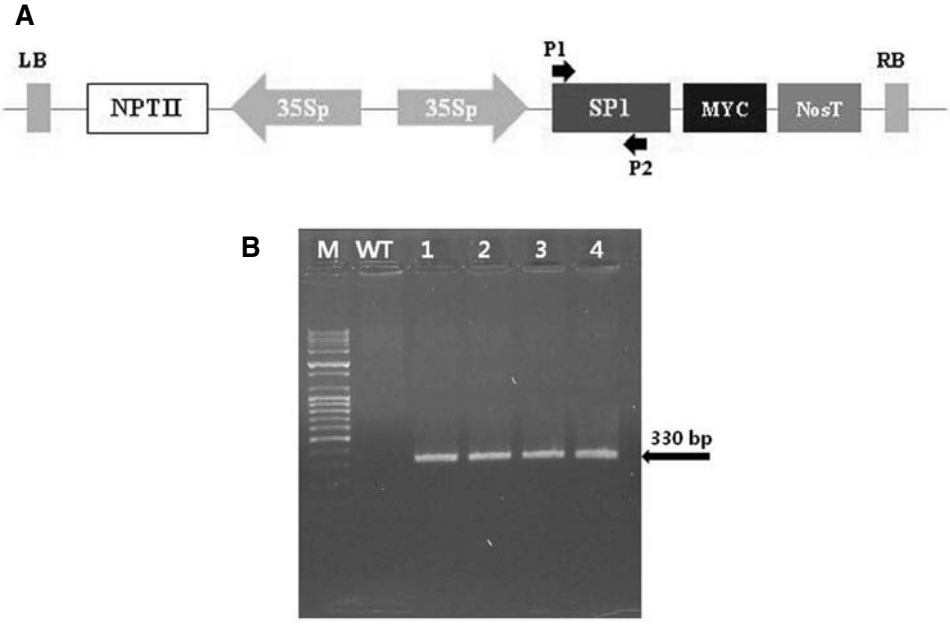

Fig. 2. Detection of the PEDVSP gene in putative *Lemna* transformants. (A) Plant transformation vector. 35Sp, CaMV 35S promoter; SP1, PEDV spike protein gene1; MYC, c-myc tag; NosT, nopaline synthase terminator; NPTII, neomycin phosphotransferase II; LB, left border; RB, right border. (B) Genomic PCR analysis. Genomic PCR was performed with specific primers, designated P1 and P2, for the PEDVSP1 gene. M: 1 kb DNA ladder; WT: untransformed wild-type plant; Lanes 1-4: putative *Lemna* transformants.

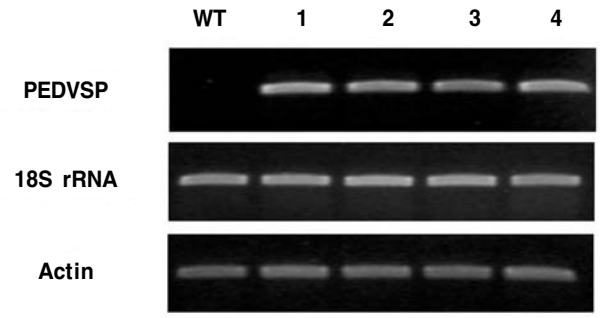

Fig. 3. RT-PCR analysis of *Lemna* transformants. RT-PCR was performed with the same primers as those used in genomic PCR analysis. The actin gene and 18S ribosomal DNA were used as internal controls. WT: untransformed wild-type plant; Lanes 1-4: putative *Lemna* transformants.

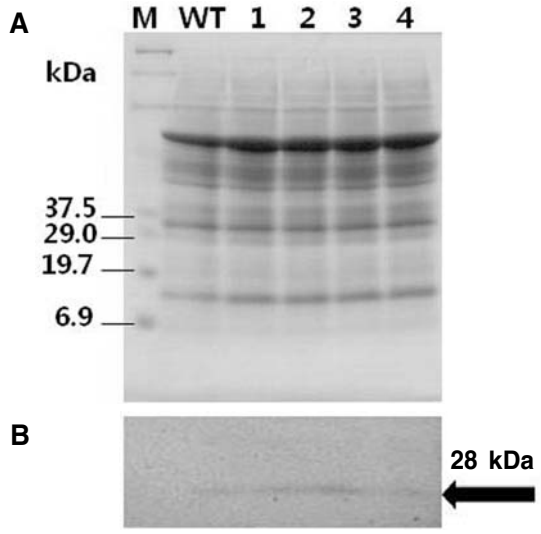

Fig. 4. Western blot analysis of *Lemna* transformants. Total soluble protein (30 μg) was separated by 10% SDS-gradient PAGE. Recombinant proteins were detected using a monoclonal c-myc primary antibody and HRP-labeled anti-mouse IgG as the secondary antibody. WT: untransformed wild-type plant; Lanes 1-4: *Lemna* transformants.

reported that proliferation of Lemna fronds varied six-fold across the 25 geographic isolates of Lemna collected at various geographical locations around the world. As described above, the *Lemna* used in this study was collected from a rice field in Daejeon, Korea.

The fronds of *Lemna* were transformed by co-cultivation with *A. tumefaciens* strain EHA105 harboring the PEDV spike protein1 gene fused to a c-myc tag. The c-myc tag sequence was introduced into the vector to facilitate purification of the target recombinant proteins. Kanamycin-resistant fronds were transferred to liquid medium containing kanamycin and subcultured under the high selection pressure. The liquid environment allows the antibiotic to contact the entire surface of the plant, thus ensuring reliable selection (Yamamoto et al., 2001). A liquid medium was used due to the differences in the tissue uptake of kanamycin in different media environments and different availability of kanamycin due to its binding to certain gelling agents (Chauvin et al., 1999; Yamamoto et al., 2001).

After an additional selection for 3 months under high kanamycin selection pressure, *Lemna* fronds resistant to kanamycin in liquid medium were obtained. The genomic integration of the PEDV spike protein 1 gene in the putative transformants was confirmed by genomic PCR analysis (Fig. 2). PCR products of the expected size (330 bp) corresponding to primers designed on the internal PEDV spike protein 1 gene were detected from kanamycin-resistant *Lemna*, whereas no DNA band corresponding to the target gene was detected in untransformed wild-type *Lemna*.

To confirm whether the PEDV spike protein 1 gene was transcribed in *Lemna* transformants, the genomic integration of the target gene was confirmed by genomic PCR analysis. A reverse transcription-PCR (RT-PCR) assay was performed with the specific primers used in genomic PCR analysis, corresponding to the PEDV spike protein 1 gene (Fig. 3). PCR products of the expected size corresponding to the specific primers were detected in the *Lemna* transformants, whereas no amplified PCR product was detected in untransformed wild-type *Lemna* (Fig. 3). The actin gene and 18S ribosomal DNA were used as internal controls and are shown in the lower panel. This result demonstrated that the PEDV spike protein 1 gene was correctly transcribed in the transgenic *Lemna*.

To further investigate the expression of the PEDVSP1-myc protein in the *Lemna* transformants in which transcription of PEDVSP1 mRNA was confirmed by RT-PCR, immunoblot analysis was performed using a monoclonal anti-myc primary antibody. The SDS-PAGE profile of total soluble proteins extracted from whole *Lemna* plants, including wild-type and transformants, and stained with Coomassie blue and immunoblotted is shown in Fig. 4. No specific band was detected in the Coomassie blue-stained gel, but immunoblotting with a monoclonal anti-myc revealed the specific band at a molecular weight of about 28 kDa (Fig. 4). This result indicated the PEDV spike1-myc fusion protein was produced in the transgenic *Lemna*.

In this study, we assessed the feasibility of producing a protective antigen for PEDV in *Lemna minor* that could be used as an effective complement in the diets of animals. This is the first report of the expression of a vaccine antigen for an animal infectious disease in duckweed, and may expand the industrial availability and development of effective edible vaccines.

Acknowledgements: We are grateful to Dr. Haeng-Soon Lee of KRIBB for providing the plant transformation vector. This research was supported by a grant from KRIBB Research Initiative Program and Priority Research Centers Program

through the National Research Foundation (NRF) funded by the Ministry of Education, Science and Technology (2010-00 29630).

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