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Production and immunogenicity of *Actinobacillus pleuropneumoniae* ApxIIA protein in transgenic rice callus

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ABSTRACT

Actinobacillus pleuropneumoniae is a major etiological agent that is responsible for swine pleuropneumonia, a highly contagious respiratory infection that causes severe economic losses in the swine production industry. ApxIIA is one of the virulence factors in *A. pleuropneumoniae* and has been considered as a candidate for developing a vaccine against the bacterial infection. A gene encoding an ApxIIA fragment (amino acids 439–801) was modified based on a plant-optimized codon and constructed into a plant expression vector under the control of a promoter and the 3′ UTR of the rice amylase 3D gene. The plant expression vector was introduced into rice embryogenic callus (*Oryza sativa* L. cv. Dongjin) via particle bombardment-mediated transformation. The integration and transcription of the $ApxIIA_{439–801}$ gene were confirmed by using genomic DNA PCR amplification and Northern blot analysis, respectively. The synthesis of $ApxIIA_{439–801}$ antigen protein in transgenic rice callus was confirmed by western blot analysis. The concentration of antigen protein in lyophilized samples of transgenic rice callus was 250 μg/g. Immunizing mice with protein extracts from transgenic plants intranasally elicited secretory IgA. These results demonstrate the feasibility of using a transgenic plant to elicit immune responses against *A. pleuropneumoniae*.

1. Introduction

Actinobacillus pleuropneumonia is a Gram-negative, fermentative, hemolytic, and facultative anaerobic encapsulated *coccobacillus* of the *Pasteurellaceae* family and causes porcine pleuropneumonia through respiratory infection [1]. *A. pleuropneumonia* has a broad spectrum worldwide with high levels of mortality, even with different prevalence for various serotypes [2,3]. *A. pleuropneumonia* has several virulence factors including capsules, Apx toxins, adhesins, transferrin binding proteins, outer membrane proteins, and secreted proteases that play important roles in pathogenesis [1]. Apx toxins are important virulence factors for porcine pleuropneumonia that are involved in evading the host's first line of defense. Apx toxins also form membrane pores in phagocytic and other target cells at high RTX (repeats in the structure toxin) toxin concentrations, resulting in osmotic swelling and ultimately cell death [1]. In particular, serovars producing the ApxI and ApxII exotoxins are the most virulent [4]. Thus, Apx toxins have strong immunogenicity and are the primary candidate for vaccines to protect against *A. pleuropneumoniae* infection [5–11].

The ApxIIA gene was cloned from a field strain of *A. pleuropneumoniae* serotype 2, which accounts for roughly half of the *A. pleuropneumoniae* cases in Korea. Antigen-specific and protective immune responses against *A. pleuropneumoniae* have been induced by oral vaccination with *Saccharomyces cerevisiae* and transgenic plants expressing recombinant ApxIIA protein [10,11]. However, the plants contained low amounts of ApxIIA antigen protein resulting in less efficient protection than that provided by injecting recombinant ApxIIA from *Escherichia coli* [11]. The low antigen expression levels in transgenic plants can induce low immune responses and is a drawback in developing plant-based vaccines. Antigen expression levels in transgenic plants have been improved by modifying target genes to optimize codon use for plants [12], chloroplast transformation [13], and transient

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expression with agrobacterium and viral expression systems [14].

Plant-based production systems have been used as an attractive platform to produce recombinant pharmaceutical proteins including enzymes, vaccines, and antibodies [15]. Development of plant expression systems has increased due to the need to develop edible or oral vaccines in plant molecular farming [16–18]. Plant expression systems include whole plants, plant tissue cultures, and cell suspension cultures and have several advantages over conventional protein expression systems, including no risk of pathogen contamination; easy production of vast amounts of recombinant protein, such as antigen proteins, at low cost; ability to perform eukaryotic post-translational modifications, such as glycosylation and disulfide bonding; and ability to assemble multimeric proteins, such as antibodies.

In this study, a fragment (amino acids 439–801) of the ApxIIA gene of *A. pleuropneumoniae* was modified to optimize codon use for plants and increase expression level in transgenic plants. The $ApxIIA_{439–801}$ gene was introduced into a plant expression vector under control of the rice amylase 3D promoter and was transformed into rice cells (*Oryza sativa* L. cv. Dongin) via particle bombardment-mediated transformation. $ApxIIA_{439–801}$ antigen proteins produced in transgenic rice callus elicited secretory IgA antibodies when used to immunize mice intranasally.

2. Materials and methods

2.1. Construction of plant expression vector containing the $ApxIIA_{439–801}$ gene

An ApxIIA gene fragment (amino acids 439–801) was amplified from the pMYO93 plasmid, which harbors the ApxIIA gene [11] of *A. pleuropneumoniae* serotype 2 isolated from Korean pigs, by using PCR with a primer set specific to the ApxIIA fragment gene. The forward primer was wApx5-BF, 5′-GGA TCC CAA GGT TAT GAT-3′ (the *Bam*HI restriction enzyme site is underlined), and the reverse primer was wApx5-KR, 5′-GGT ACC TGT AAT AGA ATC-3′ (the *Kpn*I restriction enzyme site is underlined). The PCR product was cloned into the pGEM®T-Easy vector (Promega, Madison, WI, USA), and its sequences were confirmed via sequence analysis. The $ApxIIA_{439–801}$ fragment was digested with *Bam*HI and *Kpn*I restriction enzymes and subcloned into the same sites of pMYV657 [19] under the control of the promoter and untranslated region of rice amylase 3D gene to yield pMYV655 (Fig. 1).

The $ApxIIA_{439–801}$ gene was modified to optimize codon use for plants (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=311553) and synthesized in COSMO GENETECH (Seoul, Korea) (Fig. 2) to target gene expression level in transgenic rice. The synthetic $ApxIIA_{439–801}$ gene ($sApxIIA_{439–801}$) was amplified with the forward primer sApx5-BF, 5′-GGA TCC CAG GGA TAC GAT-3′ (the *Bam*HI restriction enzyme site is underlined) and the reverse primer sApx5-KR, 5′-GGT ACC AGT AAT AGA ATC-3′ (the *Kpn*I restriction enzyme site is underlined) and cloned into the pGEM®T-Easy vector. The sequences were confirmed by sequence analysis. The $sApxIIA_{439–801}$ fragment was digested with the *Bam*HI and *Kpn*I restriction enzymes and subcloned into the same sites of pMYV657, yielding pMYV667 (Fig. 1).

2.2. Rice callus transformation

Rice embryogenic callus (*Oryza sativa* L. cv. Dongin) was prepared and transformed with plant expression vectors via particle bombardment-mediated transformation [19]. Briefly, dehusked and sterilized mature rice seeds were cultured on N6 salts and vitamins supplemented with 2,4-dichlorophenoxyalic acid (2,4-D) (2.0 mg/L), sucrose (30 g/L), kinetin (0.2 mg/L), and gelite (2.0 g/L) for 7 days to induce callus formation. After 5 days, the rice callus were transferred to N6 selection medium supplemented with 2,4 dichlorophenoxyacetic acid (2 mg/L), sucrose (30 g/L), proline (0.5 g/L), glutamine (0.5 g/L), casein enzymatic hydrolysate (0.3 g/L), gelite (2 g/L), and hygromycin B (35 mg/L) as the antibiotic to select for transgenic rice callus, for 2–3 weeks.

2.3. Genomic DNA PCR analysis of transgenic plants

For genomic DNA PCR, genomic DNA samples from putatively selected transgenic and wild-type rice callus were isolated with the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the supplier's instructions. Antigen genes in genomic DNA from transgenic callus were detected with a genomic DNA PCR with the specific primer set. The reactions contained 100 ng genomic DNA, 10 pmol gene specific primers, 200 μM dNTPs, 2 μl 10 × *Taq* polymerase buffer (10 mM Tris–HCl, pH 8.8, 50 mM KCl, and 0.1% Triton X-100), 1.5 mM $MgCl_2$, 2 U i-*Taq* polymerase (iNtRON Biotechnology, Seoul, Korea) in a total reaction volume of 20 μL. The PCR profile was as follows: denaturation at 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. This was followed by a final extension at 72°C for 5 min. The plant expression vectors were used as a positive control. The amplified PCR products were separated on 1.0% agarose gel electrophoresis followed by ethidium bromide staining (Sigma, St. Louis, Mo, USA).

2.4. Northern blot analysis

Total RNA was extracted from transgenic and wild-type rice callus after induction for 5 days without sugar by using the RNeasy Plant Mini Kit (Qiagen) according to the supplier's instructions. Thirty micrograms of total RNA was electrophoretically separated on 1.2% formaldehyde-containing agarose gels and then transferred

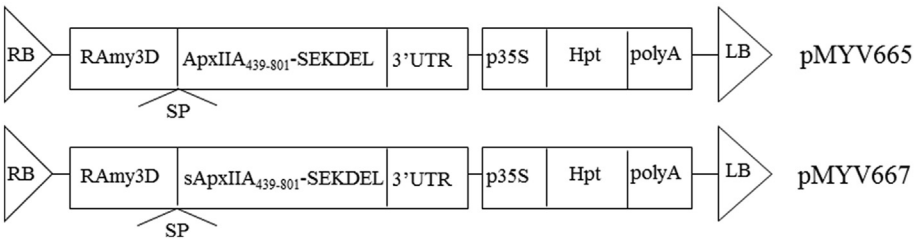

Fig. 1. Construction of plant expression vectors. Genes located within the T-DNA sequence flanked by the right and left borders (RB and LB) include a fragment of the *Actinobacillus pleuropneumoniae* ApxIIA gene (amino acids 439–801, $ApxIIA_{439–801}$) or modified ApxIIA ($sApxIIA_{439–801}$), with optimized plant codons under the control of the rice amylase 3D gene promoter and 3′ untranslated region (3′UTR). Signal peptide (SP) of rice amylase 3D gene and an ER retention sequence (SEKDEL) was used to accumulate antigen proteins into ER. An Hpt (hygromycin phosphotransferase) gene under the control of the Cauliflower Mosaic Virus 35S gene promoter (p35S) and terminator (polyA) was used for hygromycin selection. Plant expression vectors containing $ApxIIA_{439–801}$ and $sApxIIA_{439–801}$ were designated pMYV665 and pMYV667, respectively.