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Research Note—

Immunization of Chickens with VP2 Protein of Infectious Bursal Disease Virus Expressed in *Arabidopsis thaliana*

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SUMMARY. Transgenic plants represent a safe, effective, and inexpensive way to produce vaccines. The immunogenicity of VP2 protein of an infectious bursal disease (IBD) virus variant E isolate expressed in transgenic *Arabidopsis thaliana* was compared with a commercial vaccine in specific-pathogen-free broiler chickens. The *VP2* coding sequence was isolated and integrated into *A. thaliana* genome by *Agrobacterium tumefaciens*–mediated transformation. Soluble VP2 expressed in transgenic plants was used to immunize chickens. Chickens receiving oral immunization with plant-derived VP2 at 1 and 3 wk of age had an antibody response using enzyme-linked immunosorbent assay and 80% protection against challenge infection at 4 wk. Chickens primed with a commercial vaccine at 1 wk followed by an oral booster with VP2 expressed in plants at 3 wk of age showed 90% protection. Chickens immunized with a commercial vaccine at 1 and 3 wk showed 78% protection. Results supported the efficacy of plant-produced VP2 as a vaccine against IBD.

RESUMEN. *Nota de Investigación*—Immunización de pollos con la proteína VP2 del virus de la enfermedad infecciosa de la bolsa expresada en *Arabidopsis thaliana*.

Las plantas transgénicas representan una manera segura, efectiva y económica de producir vacunas. La inmunogenicidad de la proteína VP2 expresada en *Arabidopsis thaliana* de un aislamiento de la variante E del virus fue comparada con una vacuna comercial en aves libres de patógenos específicos. La secuencia codificadora de la proteína VP2 se aisló e integró al genoma de *A. thaliana* por medio de la transformación mediada por *Agrobacterium tumefaciens*. La VP2 soluble expresada en plantas transgénicas fue utilizada para inmunizar pollos. Los pollos que recibieron a la primera y tercera semanas de edad la inmunización oral con VP2 derivada de plantas, mostraron una respuesta de anticuerpos detectada por el ensayo de inmunoabsorción con enzimas ligadas, además de una protección de un 80% contra el desafío a las cuatro semanas. Los pollos primovacunados con una vacuna comercial a la semana de edad seguido de un refuerzo a las tres semanas con VP2 expresada en plantas, mostraron 90% de protección. Los pollos inmunizados con una vacuna comercial a la primera y tercera semana mostraron 78% de protección. Los resultados confirmaron la eficacia de la proteína VP2 producida en plantas como vacuna contra el virus de la enfermedad infecciosa.

Key words: IBDV, edible vaccine, transgenic plants, oral immunization

Abbreviations: B2 = Bursine 2; ELISA = enzyme-linked immunosorbant assay; IBD = infectious bursal disease; IBDV = infectious bursal disease virus; mAb = monoclonal antibody; N = untransformed; PBS = phosphate-buffered saline; RT-PCR = reverse transcription–polymerase chain reaction; SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SPF = specific-pathogen-free; SQ = subcutaneously; T = V-3 plant

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Recombinant gene technology has revolutionized vaccine production (11,15). Plant-derived antigens of pathogens could be used in vaccines. Plants are natural bioreactors and are effective for production of recombinant proteins and antigens (6,7,10). Plants can express high levels of properly folded protein and can perform posttranslational modifications of introduced protein products (17). Plant-derived recombinant proteins can induce immune responses that provide protection against challenge (14,18,19,22,23). Utilization of plant-derived antigens for oral delivery in a diet provides a new dimension for control of pathogens compared to current vaccination administration methods such as injection, drinking water, or coarse spray.

Infectious bursal disease (IBD) is an acute, contagious chicken disease caused by a bisegmented double-stranded RNA virus infectious bursal disease virus (IBDV) (13). The larger segment A (~3.3 kbp) of IBDV encodes a polyprotein that is proteolytically cleaved into two structural proteins, VP2 and VP3, as well as the 28 kD viral protease VP4. Vp4 is responsible for processing the precursor polypeptide (2,12). VP2 protein is the major host-protective antigen of IBDV. VP2 protein expressed in prokaryotic and eukaryotic model expression systems has demonstrated immunogenicity of recombinant protein (3,8,9,12).

The *VP2* coding region of IBDV antigenic variant E strain was previously isolated and cloned into a plant expression vector, pE1857 (S. Gelvin, Purdue University) with a strong promoter for plant expression (22). A resulting construct (rpE-VP2) with *Bar* gene cassette for bialaphos selection in plant was introduced into *Agrobacterium tumefaciens* by electroporation. *Agrobacterium* containing the rpE-VP2 construct was used to transform *Arabidopsis thaliana*, and transgenic plants were selected using bialaphos. The *VP2* transgene was demonstrated by polymerase chain reaction (PCR) and Southern blot, and its expression was confirmed by reverse transcription–PCR (RT-PCR) and antigen-capture enzyme-linked immunosorbent assay (ELISA) using monoclonal anti-VP2. This present study determined the antigenicity of this plant-derived VP2 in chickens. Results demonstrated the feasibility of plant-derived VP2 as a vaccine against IBD.

MATERIALS AND METHODS

Animals. Specific-pathogen-free (SPF) broiler chickens from an Auburn University flock were used. Chickens were housed in modified Horsfall Bauer isolation units maintained with filter air under negative pressure. Chicken were fed a commercial corn soybean broiler diet and water *ad libitum*. Birds were reared and handled according to the guidelines of the Auburn University Animal Care and Use Committee.

Commercial IBDV vaccine and challenge virus. A live intermediate vaccine (Bursine-2 from Fort Dodge Animal Health Laboratories, Fort Dodge, KS) was used. The vaccine contained antigenic standard virus and was administered via eye and nasal drop. The antigenic Delaware variant E strain of IBDV (20) was the challenge virus and was administered at $10^{3.5}$ mean chicken infective doses by eye and nasal drop.

Plant-derived VP2. Antigen-capture ELISA (5) using monoclonal anti-VP2 determined the expression of VP2 protein in lines of transgenic *A. thaliana*. One line, V-3, with highest level (4.8% VP2 of total soluble protein), was selected (24). Western blot detected antibody in chickens against VP2 from IBDV. Equal amounts of IBDV protein determined by Bradford assay (4) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by electroblotting onto nitrocellulose membranes using Semi-dry Trans Blotter (Bio-Rad, Hercules, CA), according to manufacturer's instructions. Membranes were blocked with 3% skim milk. After blotting, lanes of nitrocellulose were cut into strips and were independently probed with serum from chickens orally immunized with leaf extracts from transgenic plants and with serum from chickens fed untransformed plants and plants transformed with vector as negative controls. Monoclonal antibody against VP2 was used as a positive control.

VP2 antibody produced in chickens orally immunized with VP2 was detected on the nitrocellulose by horseradish peroxidase–conjugated anti-chicken immunoglobulin G at a 1:1000 dilution following the protocol of Jackson Immuno Research Laboratories (West Grove, PA).

Determination of antibody titer and protection against IBDV infection. Three independent replicates with 60 chickens in each set of experiments were used. Sixty chickens were separated into six groups. Each group was housed in a separate unit. Plant vaccines were orally administered by gavage feeding or subcutaneously (SQ) with a syringe. Leaves from transgenic and control plants were dried, ground to fine powder, and were reconstituted in water. Concentration of VP2 in each dose was determined by antigen-capture ELISA (24). At 1 wk of age, vaccination regimens were started. Group 1 chickens were given orally sterile saline; group 2 chickens received an oral dose of the untransformed (N) plant extract; group 3 was given commercial vaccine, Bursine 2 (B-2); group 4 was primed with B-2 followed by an oral booster with V-3 plant (T) extract; group 5 was given T plant extract mixed with Freund complete adjuvant by SQ; and group 6 received T extract orally. Chickens

in the oral feeding groups were given five oral doses at 3-day intervals. Each bird received 11.44 μg total soluble protein per chicken. This protein concentration was maintained to simulate the protein concentration received by chickens receiving VP2 in feed.

At 3 wk of age, birds were boosted with a dose similar to the first, except for groups 5 and 6, which were boosted with the T extract SQ and T extract orally, respectively. Blood samples were collected at weekly intervals. Titers of pooled serum in each group were determined with an ELISA kit (Affinitech Ltd., Bentonville, AR). At 4 wk of age, chickens in all groups except group 1 were challenged. Each chick was banded and weighed prior to challenge. At 7 days postchallenge, all birds were euthanatized and bursae weighed.

Statistical analysis. Bursa-to-body ratio was calculated using the following formula: bursa weight in grams/body weight in grams × 1000. Mean and standard deviations were calculated for groups using statistical analysis system (SAS Institute, Inc., Cary, NC). A bird with a bursa–body weight ratio that was two standard deviations below the mean of the control group was scored as lacking protection (atrophied bursa). Percentage of birds protected was calculated for each group, and chi-square was used to differentiate groups.

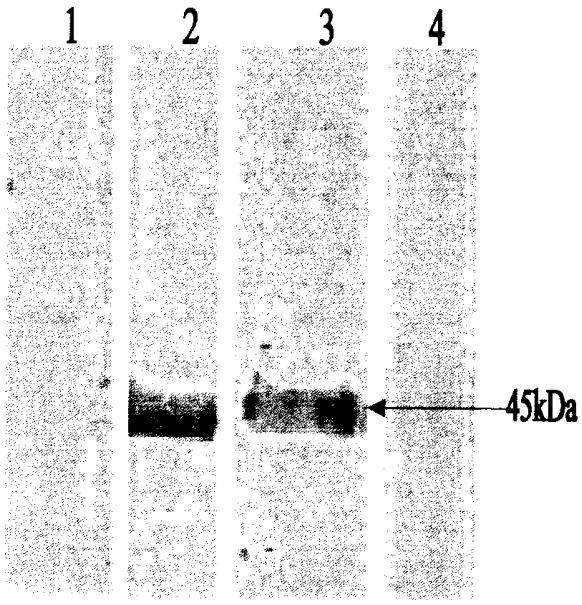

Fig. 1. Western blot analysis of antibody in chickens orally immunized with transgenic V-3 plants. Equal amounts of denatured IBDV were used for SDS-PAGE, blotted on a membrane, and probed with serum from chickens fed untransformed plants (lane 1); VP2 monoclonal antibody (lane 2); serum from chickens immunized with transgenic line V-3 (lane 3); and serum from chickens fed control vector-transformed plants (lane 4).

RESULTS

Characterization of VP2 expressed in transgenic plants. A 45-kD band was recognized in lanes in which monoclonal anti-IBDV and serum from chickens fed with transgenic plant extracts were used for detection of VP2 protein (Fig. 1). Chickens fed extracts of untransformed plants and plants transformed with a control vector did not react with IBDV proteins.

Serology test of SPF chickens. Antibody was evident at 1 wk postvaccination for all vaccinated groups except the negative control (NT-plant extract) (Fig. 2). Titers decreased at 2 wk postvaccination in all groups and then went up following the booster dose with all vaccinations, except for group 2 (NT-plant extract). Titers increased after challenge in all groups, except for group 4 (T-plant extract, SQ) and group 5 (B-2+T-plant extract, oral).

Efficacy of VP2 expressed in plants. Immunization and protection results of chicken are summarized in Table 1. All groups receiving vaccine, except group 2 (NT-plant extract), had significant protection against IBDV (Table 1). The best protection (90%) was in chickens immunized with the T-plant extract by SQ (group 4). The lowest protection was in group 5 (60%), which received B-2 followed by the T-plant extract orally. Group 6, which received only the T-plant extract orally, had 80% protection.

DISCUSSION

VP2 expression in *A. thaliana* was previously reported (24). VP2 from plant extracts showed positive signals with background smeared across the lane. Others experienced similar difficulties and suggested that the hydrophobic nature of VP2 may cause conformation-dependent distortion on SDS-PAGE (8,12). For detection of VP2 protein, we modified the Western blot procedure. This modification detected antibody in chickens fed the plant extract expressing VP2. Plant-derived VP2 given by SQ or orally induced antibody and protection from challenge. This is the first report showing protection in chickens with an antigen expressed in plants.

Resistance of VP2 to gut degradation has not been reported. Results demonstrated IBDV in chickens fed

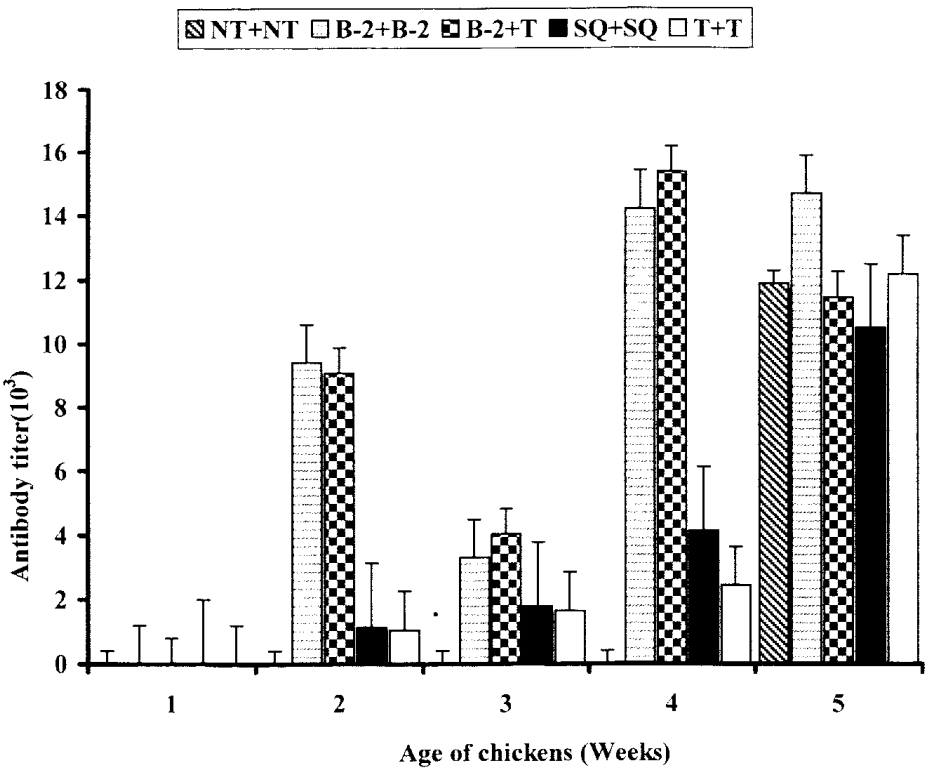

Fig. 2. Antibody response of SPF chickens to VP2. Vaccination of SPF chickens was performed at 1 and 3 wk of age and challenge at 4 wk of age, except in the case of the group 1 control. Groups 3, 4, and 5 received one booster at week 3, whereas groups 1, 2, and 6 were boosted five times at 3-day intervals. Each data point represented antibody of pooled serum. NT = untransformed plant; B-2 = Bursine 2; T = transformed plant; SQ = subcutaneous treatment; O = oral administration. Group 1, which received saline and no challenge, was excluded from the figure.

plant extracts expressing VP2. Since the recombinant plant-derived VP2 invoked an immune response, it was resistant to intestinal degradation.

Antibody is important in protection against IBDV (16). Two administrations with the plant-derived VP2 showed the highest antibody and best protection against variant E IBDV. Furthermore, this group did not show an increase in titer after challenge, indicating that the birds were refractory to viral infection.

In ovo vaccination for IBDV with high-speed robotic machine is widely used in the United States

Table 1. Efficacy of VP2 produced in plants and administered to SPF chickens.[A]

Treatment group		Variant E virus challenge	
First vaccination	Second vaccination	Protected birds/total birds	% Protection
# 1 Saline	+ Saline (NC)		—
# 2 NT-plant oral	+ NT-plant oral	0/30	0
# 3 B-2	+ B-2	24/30	78
# 4 B-2	+ T-plant oral	27/30	90
# 5 T-plant SQ	+ T-plant SQ	18/30	60
# 6 T-plant oral	+ T-plant oral	24/30	80

[A]NT = untransformed; B-2 = Bursine 2; T = transformed; SQ = subcutaneous; NC = no challenge. Groups 3, 4, and 5 received only one booster at week 3, whereas groups 1, 2, and 6 were boosted five times at 3–4-day intervals.

and in other countries where labor costs are high. Results showed that priming with a commercial vaccine followed by booster with the plant-derived VP2 produced 90% protection (group 4) against the variant E IBDV. The protection in this group 4 was higher than that afforded by the commercial vaccine (containing only antigenic standard IBDV) (group 3). This is not surprising, since the B-2 vaccine contains only a standard IBDV.

Single immunization may not provide protection against IBDV during the entire growth period of broilers. Hence, it may be beneficial to use a plant-derived VP2 as a booster vaccine in chickens that receive an *in ovo* live primer vaccination. Future studies will compare feeding of transgenic VP2 to conventional vaccines for boosting birds that have been initially vaccinated *in ovo.*

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