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Expression of immunogenic VP2 protein of infectious bursal disease virus in *Arabidopsis thaliana*

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Abstract

VP2 protein is the major host-protective immunogen of infectious bursal disease virus (IBDV) of chickens. Transgenic lines of *Arabidopsis thaliana* expressing recombinant VP2 were developed. The *VP2* gene of an IBDV antigenic variant E strain was isolated, amplified by RT-PCR and introduced into a plant expression vector, pE1857, having a strong promoter for plant expression. A resulting construct with a *Bar* gene cassette for bialaphos selection in plant (rpE-VP2) was introduced into *Agrobacterium tumefaciens* by electroporation. *Agrobacterium* containing the rpE-VP2 construct was used to transform *Ar. thaliana* and transgenic plants were selected using bialaphos. The presence of *VP2* transgene in plants was confirmed by PCR and Southern blot analysis and its expression was confirmed by RT-PCR. Western blot analysis and antigen-capture ELISA assay using monoclonal anti-VP2 were used to determine the expression of VP2 protein in transgenic plants. The level of VP2 protein in the leaf extracts of selected transgenic plants varied from 0.5% to 4.8% of the total soluble protein. Recombinant VP2 protein produced in plants induced antibody response against IBDV in orally-fed chickens.

Introduction

Infectious bursal disease virus (IBDV) causes infectious bursal disease (IBD), an important disease of commercial chicken flocks worldwide. Control of IBD currently employs biosecurity, sanitation, and vaccination. Commercial vaccines are not totally effective and less attenuated products can cause IBD (Jackwood *et al.* 1987, Snyder *et al.* 1992).

IBDV is a member of the Birnaviridae family, which is characterized by a bisegmented double-stranded RNA genome. The smaller genome, segment B (2.8 kb), encodes VP1, a 90-kDa multi-functional protein with polymerase and capping enzyme activities (Azad *et al.* 1985, Spies *et al.* 1987). The larger genome, segment A (3.2 kb), encodes a polyprotein that is cleaved by auto proteolysis to form mature viral proteins VP2, VP3, and VP4. VP2 is the major host-protective antigen with antigenic regions responsible for induction of neutralizing antibodies (Azad *et al.* 1987, Jagadish *et al.* 1998).

Since the introduction of edible plant-based vaccines by Mason *et al.* (1992), several laboratories have used transgenic plants for expression of viral and bacterial antigens (Carrillo *et al.* 1998, Daniell *et al.* 2001, Gomez *et al.* 1998, Haq *et al.* 1995, Kong *et al.* 2001, Lauterslager *et al.* 2001, Mason *et al.* 1992, 1996, 1998, McGarvey *et al.* 1995, Ritchter *et al.* 2000, Tacket *et al.* 1998, Wigdorovitz *et al.* 1999). Although oral vaccination can protect against infectious agents entering the body via mucosal surfaces of the host, the mechanisms of ensuing mucosal immunogenicity are not well understood (Shalaby *et al.* 1995).

Despite enormous potential, exploitation of plants as oral vaccines has been hampered by low immunogenicity, induction of immunotolerance, proteolytic degradation of antigens during passage through the gastrointestinal tract, and exposure to acidic conditions in the stomach (Lauterslager *et al.* 2001). Here

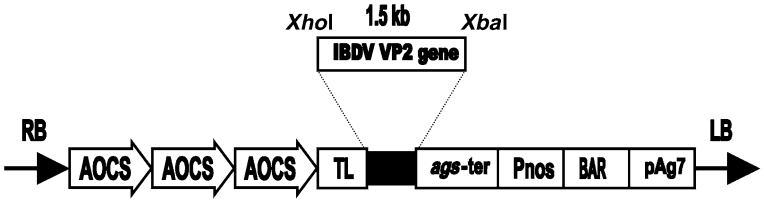

Fig. 1. T-DNA region of rpE-VP2 construct. *VP2* from TA vector with *Xho*I and *Xba*I was cloned in the plant-binary vector, pE1857 under control of super promoter. The resulting plasmid, rpE-VP2 has a *Bar* gene cassette as plant selection marker and confers resistance to bialaphos. LB and RB indicate the left and right borders of the T-DNA region. AOCS represents the octopine synthetase promoter. TL represents translational leader sequence. *ags*-ter represents the agropine synthase terminator region.

we demonstrate high level expression of immunologically active VP2 protein in transgenic *Arabidopsis thaliana*.

Materials and methods

Propagation and extraction of viral RNA

The infectious bursal disease virus (IBDV) antigenic variant E strain was propagated in 5-week-old specific pathogen free chickens. Birds were kept in Plexiglass isolation units maintained with filtered air under negative pressure. Birds were given a corn-soybean diet and water *ad libitum*. Care and handling of chicken was according to the Auburn University's Institutional Care and Use Committee. Bursae of Fabricius (the target organ for IBDV infection) were taken from infected birds at 3 days' post-infection. They were dissected and homogenized in TNE buffer (10 mM Tris/HCl, 100 mM NaCl, 10 mM EDTA, pH 8) at a ratio of 1 g bursa to 10 ml TNE buffer. After freezing and thawing three times, homogenates were centrifuged at 17 000 *g* for 15 min at 4 °C and the supernatant collected for viral RNA extraction with a Trizol RNA extraction kit (Gibco).

Design of VP2 *plant expression cassette*

The *VP2* cDNA was prepared from RNA using an RT-PCR preamplification system (Gibco). Primers flanking the *VP2* sequence were designed according to information in the GenBank. Primers were designed with *Xho*I site in Vh-1 and *Xba*I site in Vb-2, respectively, for directional cloning of amplified sequences: primer Vh-1 GGC**CTCGAG**AATGGTTAGTAGAGATCAGACA; primer Vb-2 GGC**TCTAGA**TACACCTTCCCCAATT GCAT. The plant expression vector pE1857 (Min *et al.* 1995) was obtained from a patent transfer agreement from S. Gelvin (personal communication). Briefly, the vector was derived from kanamycin-resistant pGPTV containing the patented super-promoter, *TEV* translational leader, polylinker derived from pBluescript, and *ags* terminator in pUC119. The *VP2* DNA amplicon was placed under control of super promoter vector between the restriction enzyme sites for *Xho*I and *Xba*I. The resulting recombinant construct had *bar* gene cassette for bialophos (Phosphoinothricin) selection in plants, and was designated as rpE-VP2 (Figure 1).

Selection of transgenic Arabidopsis thaliana *plants*

The rpE-VP2 expression cassette and pE1857 control vector were introduced into *Agrobacterium tumefaciens* strain C58C1 by electroporation and used for transformation of *Ar. thaliana* by vacuum infiltration (Betchtold *et al.* 1993). Seeds were harvested from the self-pollinated primary transformants and used to generate plants for screening as described below. Plants resistant to bialaphos were selected. Seedlings germinating in Promix potting medium were sprayed daily for 3 d with a solution of 50 mg bialaphos l^{-1} (Sigma). After 5 d, the procedure was repeated for an additional 3 d. Seeds from surviving plants were harvested and bialaphos selection performed for 3 additional generations to obtain homozygous transgenic lines.

PCR and Southern blot analysis

VP2 in transgenic plants was demonstrated by polymerase chain reaction (PCR) and Southern blotting. Total DNA from leaves was isolated using plant DNAzol reagent (Gibco). The *VP2* DNA was amplified with Vh-1 and Vb-2 primers and a 1.5 kb fragment was obtained. This fragment was labeled with ^{32}P-dCTP for use as a probe in Southern hybridization (Sambrook *et al.* 1989). Total DNA from transgenic and control plants was digested with *Eco*RIV, sep-

arated by electrophoresis on 1% agarose gels, and transferred to a nylon membrane. The membrane was hybridized to the *VP2* probe at 42 °C for 4 h in presence of 6 SSC and 50% (v/v) formamide. Blots were washed three times with 0.1 SSC and 0.5% SDS at 37 °C for 10 min each, and exposed to an autoradiography film at −80 °C for 24 h.

Analysis of VP2 expression in transgenic plants

Total RNA from transgenic and control *A. thaliana* plants were obtained from ∼1 g of leaves by an RNA isolation system (Omega Bio-tek, Inc.). A pre-amplification kit (Gibco) was utilized in RT-PCR to amplify *VP2* DNA with the gene-specific Vh-1 and Vb-2 primers**.**

Leaves were ground to a powder in liquid N_2 and added to 1 ml extraction buffer containing 10 mM 2-*N*-morpholino ethanesulfonic acid, pH 6, 10 mM NaCl, 5 mM EDTA, 0.6% Triton X-100, 0.25 M sucrose, 0.15 mM spermine, 0.5 mM spermidine, 10 mM DTT, 1 mM phenylmethyl sulfonyl fluoride. The tissue homogenate was centrifuged twice at 12 000 *g* for 15 min at 4 °C to remove insoluble debris, and resulting supernatant used for VP2 protein analysis.

Total soluble protein (TSP) concentration was determined using the Bradford (1976) protein assay. The concentration of VP2 protein in plants was determined by antigen-capture AC-ELISA (Corley *et al.* 2001). Plates were coated at 4 °C overnight with polyclonal anti-IBDV-chicken serum diluted at 1:1000 in PBS buffer pH 8; washed 3 times with PBST (phosphate-buffered saline with 0.05% Tween 20) buffer; and blocked with PBST buffer containing 5% skim milk at 37 °C for 3 h. Plates were washed 3 times with PBST and then dried and stored at 4 °C. Since VP2 is about 20% of the IBDV genome, and approximately the same proportion of the viral particle (Lee *et al.* 2003), the expression level of VP2 could be estimated from TSP measurements. Purified IBDV, 100 ml, was used as a standard to determine the concentration of VP2 in TSP by Bradford assay. VP2 concentration was estimated by TSP/5. VP2 antigen levels were estimated by AC-ELISA at OD_{405}. Both control and transgenic plant extracts were analyzed similarly. The concentration of VP2 in transgenic plants was determined as % TSP and calculated as follows:

$$\mathrm{VP2} = \mathrm{OD}_{405}(\mathrm{leafextract})/\mathrm{OD}_{405}(\mathrm{IBDV}) \times [\mathrm{VP2-IBDV}] \times 100\%,$$

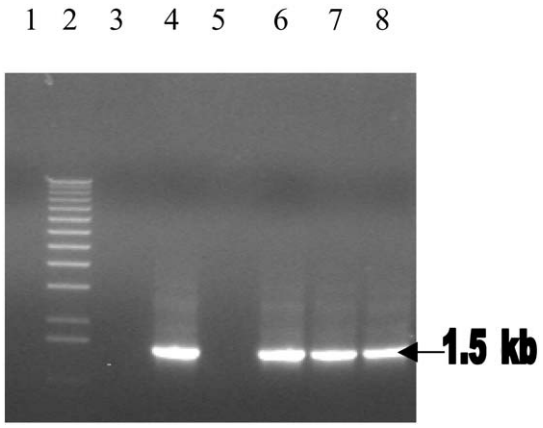

Fig. 2. Detection of *VP2* in transgenic plants by PCR. *VP2* was amplified from total DNA using Vh-1 and Vb-2 primers. Amplified DNA from transgenic lines V-4, V-3 and V-2 (lanes 4, 6 and 7, respectively), untransformed *Ar. thaliana* leaves (lanes 1 and 3), 1 kb DNA ladder (lane 2), plants transformed with control vector (lane 5), IBDV control (lane 8).

where OD_{405} (leaf extract) represents total soluble protein in leaf extracts detected by AC-ELISA, OD_{405} (IBDV) represents total soluble protein in purified IBDV detected by AC-ELISA, and [VP2-IBDV] represents VP2 protein in purified IBDV calculated from purified IBDV concentration divided by five.

Purified IBDV were subjected to SDS-PAGE followed by electro-blotting on to nitrocellulose membranes using Semi-dry Trans Blotter (Bio-Rad) according to manufacturer's instruction. Membranes were blocked with 3% (v/v) skim milk. After blotting, different lanes on nitrocellulose were separated. Each strip of nitrocellulose was independently probed with chicken serum orally immunized with leaf extracts from different transgenic lines and serum collected from chicken fed with untransformed plants and plants transformed with vector as negative controls. Monoclonal antibody against VP2 was used as positive control in preliminary experiment. The VP2 antibody produced in chicken in response to oral immunization and forming a complex with VP2 protein on the nitrocellulose was detected by horseradish peroxidase (HRP)-conjugated anti-chicken immunoglobulin G at a 1:1000 dilution following protocol of Jackson Immuno Research Laboratories.

Results and discussions

Full-length *VP2* cDNA was amplified from viral RNA by RT-PCR using primers that placed an *Xho*I restric-

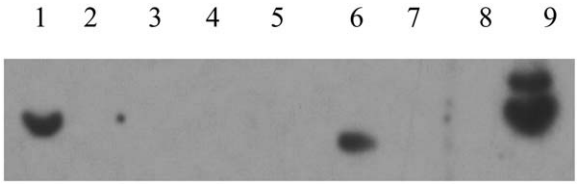

Fig. 3. Southern blot analysis of *VP2* in transgenic plants. Total DNA from leaves of control and transgenic plants were digested with *Eco*RIV, electrophoresed, transferred to nylon membrane, and hybridized to ^{32}P labeled *VP2* probe. Hybridization signals with *VP2* probe in transgenic *Ar. thaliana* lines V-2, V-3, V-4 (lanes 1, 6, and 9, respectively) are shown in the autoradiogram. DNA from plants transformed with pE1857 (lanes 2, 3, 4, and 5), and control plants (lanes 7 and 8) did not show hybridization with *VP2* probe.

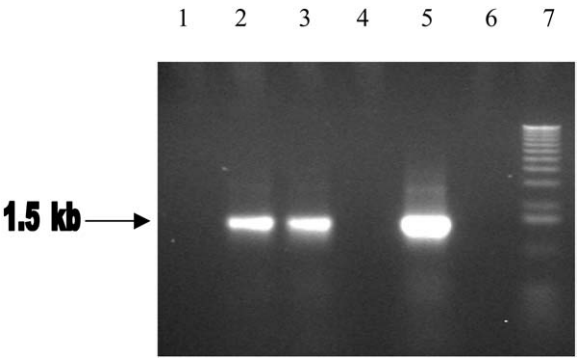

Fig. 4. RT-PCR of *VP2* RNA in transgenic plants. Total RNA was used to amplify the *VP2* transcript using Vh-1 and Vb-2 primers in an RT-PCR reaction. A 1.5 kb amplified DNA was detected in three transgenic lines V-2, V-3 and V-4 in lanes 2, 3 and 5, respectively. RNA from untransformed plants (lanes 1 and 4) and plants transformed with pE1857 (lane 6) did not show an amplified DNA product. A 1 kb DNA ladder is shown in lane 7.

tion site at the 5′ end and *Xba*I site at the 3′ end. The PCR product was ligated into the cloning site of the pGEM T-easy vector (TA vector), and the sequence of the cloned fragment verified. The *VP2* DNA was retrieved from the TA vector with the *Xho*I and *Xba*I restriction enzymes, and cloned in the plant expression vector pE1857. The recombinant expression vector, rpE-VP2 was used to transform *Ar. thaliana*.

Forty transgenic plants were selected after initial seedling screening with bialaphos. Three selected transgenic lines, V-2, V-3 and V- 4 were used in this study and demonstrated stable integration of the *VP2* by PCR amplification of total DNA from transgenic lines as template (Figure 2). Control plants transformed with pE1857 vector or untransformed plants did not show *VP2* DNA. Transgenic plants showing *VP2* amplification products also showed *VP2* positive hybridization signals on Southern blot (Figure 3). The *VP2* sequence was integrated into high molecular

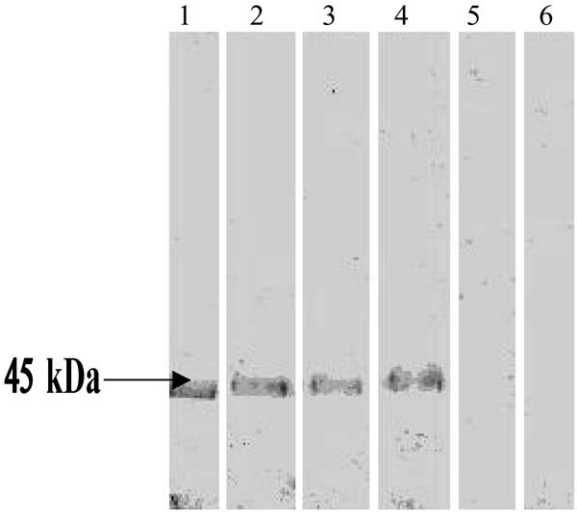

Fig. 5. Western blot analysis of antibodies present in sera from chicken orally immunized with leaf extract from transgenic plants. Equal amounts of denatured IBDV was used for SDS-PAGE, blotted on a membrane, and separately probed with VP2 monoclonal antibodies as a positive control (lane 1); serum from chicken immunized with transgenic lines V-2, V-3 and V-4 (lanes 2, 3, 4, respectively); serum from chicken fed with control vector transformed plants (lane 5), and serum from chicken fed with untransformed plants as negative control (lane 6).

weight chromosomal DNA. More than one copy of the *VP2* transgene was present in the transgenic *Ar. thaliana* lines, V-3 and V-4. Transgenic plants, expressing VP2 protein showed about 10–20% reduced growth compared to control plants or plants transformed with the pE1857 (data not shown).

VP2-specific amplified DNA product was obtained after RT-PCR of RNA from three selected transgenic plants. Non-transformed plants and transgenic plants transformed with control vector showed no *VP2* DNA (Figure 4).

Our effort to detect VP2 directly from plant extracts resulted in positive signals with smear across the lanes. Other groups working with VP2 protein of IBDV have experienced similar difficulties (Jagadish *et al.* 1998). It has been suggested that hydrophobic nature of VP2 proteins may be responsible for the conformation dependent distortion on SDS-PAGE. We used a modification of Western blot procedure with IBDV proteins for electrophoresis, and serum proteins from chickens fed with leaf extracts of transgenic plants to detect antibodies in chicken serum against VP2. A 45 kDa band was recognized in lanes where monoclonal anti-IBDV and serum from chicken fed with transgenic plant extracts was used for detection of cross reaction with VP2 protein from IBDV

Table 1. VP2 concentration in transgenic *Ar. thaliana*.

Transgenic *Arabidopsis* line	Total soluble protein (mg ml^{-1})	VP2 concentration (μg ml^{-1})	VP2/TSP (%)
V-2	0.5	2.86	0.5
V-3	0.067	3.27	4.8
V-4	0.1	2.85	2.85
Control (IBDV)	0.01	2.5	25

Three transgenic *Ar. thaliana* lines with high VP2 expression level were designated as V-2, V-3 and V-4, respectively. The ratio of VP2 to total soluble protein ranged from 0.5% to 4.8%.

(Figure 5). Chickens fed with the extracts of untransformed plants and plants transformed with control vector did not cross-react with IBDV proteins of blotted nitrocellulose.

Antigen capture ELISA was used to determine VP2 expression level in transgenic *Ar. thaliana*. The level of VP2 ranged from 0.5%–4.8% of TSP in different lines shown in Table 1. Transgenic line V-3 had the highest level of VP2 expression. The higher level of protein expression in V-3 was consistent with the presence of a higher copy number of the *VP2* transgene detected by Southern blot. This is the highest level of recombinant antigenic protein expression in plants, thus far. Other instances where recombinant antigens have been expressed in plants showed lower levels of expression, e.g. B subunits of *Escherichia coli* enterotoxin (0.01% TSP) (Azad *et al.* 1985), hepatitis B surface antigen (0.01% TSP) (Manson *et al.* 1992), and the gastroenteritis virus gS gene (0.06% TSP) (Gomez *et al.* 1998).

VP2 has been expressed in heterologous systems as recombinant protein in *E. coli* (Jagadish *et al.* 1998), yeast (Macreadie *et al.* 1990), bacculovirus (Dybing *et al.* 1997), fowl poxvirus (Bayliss *et al.* 1991), herpesvirus of turkey (Tsukamoto *et al.* 1999), and fowl adenovirus (Sheppard *et al.* 1998). These reports do not provide the concentration of VP2 protein in the experimental systems. Production of recombinant antigenic proteins in plants offer unique advantages over other model expression system. For example, it is economical to produce large quantities of protein in transgenic plants than by traditional industrial fermentation or bioreactor systems. Large scale harvesting and processing technology for plant proteins is readily available. Purification of the recombinant protein is not necessary when the plant tissue is used as animal feed. Plant cells can target proteins into intracellular compartments that are more stable and risks from contamination with pathogens are minimized.

VP2 expression in transgenic plants is an interesting model for development of edible vaccines for the control of viral diseases in poultry. Antigenic proteins of some pathogens, such as the gS gene of TGEV (Gomez *et al.* 1998) and VP1 of FMDV (Carrillo *et al*. 1998), are naturally resistant to degradation in gut when incorporated into the viral particle. Natural bioencapsulation of hepatitis B surface antigen expressed in plants provided protection from degradation in the digestive tract near an immune effector's site in the gut (Kong *et al.* 2001). VP2 protein is hydrophobic, and its antigenicity may be conformation-dependent (Kibenge *et al.* 1990). VP2 has not been investigated with respect to its resistant to gut degradation. However, our result shows recognition of IBDV by the serum of chickens fed with plant extracts expressing VP2 suggests that the recombinant VP2 produced in plants had the capacity to invoke immune response in chicken. This supported our view that VP2 protein is resistant to degradation in chicken gut and can elicit immune response against IBDV.

Transgenic plants offer a novel and safe system for vaccine production. Future demonstration of the efficacy of VP2 antigen produced in transgenic plants in the prevention of IBD will strengthen the concept of edible vaccine production for control of major pathogens of poultry and livestock.

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