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

H. Wu1, N.K. Singh2,*, R.D. Locy2, K. Scissum-Gunn3 & J.J. Giambrone1
Departments of 1 Poultry Science and 2 Biological Sciences, Auburn University, AL 36849, USA
3 Department of Biological Sciences, Alabama State University, AL 36104, USA
*Author for correspondence (Fax: (+334) 844-1645; E-mail: nksingh@acesag.auburn.edu)

Received 9 October 2003; Revisions requested 7 November 2003; Revisions received 8 March 2004; Accepted 9 March 2004

Key words: edible vaccines, infectious bursal disease virus, transgenic plant, VP2

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, Richter 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
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 Xhol site in Vh-1 and XbaI site in Vb-2, respectively, for directional cloning of amplified sequences: primer Vh-1 GCCCTCGAGATGGTTAGTAGAGATCCAGACA; primer Vb-2 GGCTCTAGATACACCTTCCCCAATTGCAT. 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 ampiclon was placed under control of super promoter vector between the restriction enzyme sites for Xhol and XbaI. The resulting recombinant construct had bar gene cassette for bialaphos (Phosphinothricin) 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 EcoRIV, 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 N2 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 OD405. Both control and transgenic plant extracts were analyzed similarly. The concentration of VP2 in transgenic plants was determined as % TSP and calculated as follows:

\[
VP2 = \frac{OD_{405}(leaf\ extract)}{OD_{405}(IBDV)} \times [VP2 - IBDV] \times 100%,
\]

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 XhoI restric-
Fig. 3. Southern blot analysis of VP2 in transgenic plants. Total DNA from leaves of control and transgenic plants were digested with EcoRIV, electrophoresed, transferred to nylon membrane, and hybridized to 32P 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 Vb-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.

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).
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), baculovirus (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.

**Acknowledgements**

This research was supported by a USDA-IFAFS grant (no. 00-52100-9705). We are grateful to Teresa Dormitorio and Chia-chen Weng for technical assistance.
References

Azad AA, Barrett SA, Fahey KJ (1985) The characterization and molecular cloning of the double-stranded RNA genome of an Australian strain of infectious bursal disease virus. Virology 143: 35–44.

Azad AA, Jagadish MN, Brown AM, Hudson PJ (1987) Deletion mapping and expression in Escherichia coli of the large genomic segment of a birnavirus. Virology 161: 145–152.

Bayliss CD, Peters RW, Cook JKA, Reece RL, Houes K, Binns MM, Boursnell MEG (1991) A recombinant fowl poxvirus that expresses the VP2 antigen of infectious bursal disease virus induces protection against mortality caused by the virus. Arch. Virol. 120: 193–205.

Bechtold N, Ellis J, Pelletier G (1993) In planta mediated gene transfer by infiltration of adult Arabidopsis thaliana plants. C.R. Acad. Sci. Paris, Life Sci. 316: 1194–1199.

Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72: 248–254.

Carrillo C, Wigdorovitz A, Oliveros JC, Zamorano PI, Sadir AM, Corley MM, Giambrone JJ, Dormitorio TV (2001) Detection of in-vitro expressed microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 272: 48–54.

Dybing JK, Jackwood DJ (1997) Expression of MD infectious bursal disease virus proteins in baculovirus. Avian Dis. 41: 617–626.

Daniell H, Streifeld JD, Wycoff K (2001) Medical molecular farming – production of antibodies, biopharmaceuticals and edible vaccines in plants. Trends Plant Sci. 5: 219–226.

Gömez N, Carrillo C, Salinas J, Purra F, Borca MV, Escribano JM (1998) Protective immune response to foot-and-mouth disease virus with VP1 expressed in transgenic plants. J. Virol. 72: 1688–1690.

Corley MM, Giambrone JJ, Dormitorio TV (2001) Detection of infectious bursal disease virus gene products in lymphoid tissues after in ovo vaccination of specific pathogen free embryos. Avian Dis. 45: 897–905.

Lee CC, Ko TP, Lee MS, Chou CC, Lai SY, Wang AH, Wang MY (2003) Purification, crystallization and preliminary X-ray analysis of immunogenic virus-like particles formed by infectious bursal disease virus (IBDV) structural protein VP2. Acta Crystallogr. D Biol. Crystallogr. 59: 1234–1237.

Mackenzie IG, Vaughan PR, Chapman AJ, McAndrew NJ, Jagadish MN, Heine HG, Ward CW, Fahey KJ, Azad AA (1990) Passive protection against infectious bursal disease virus by viral VP2 expressed in yeast. Vaccine 8: 549–552.

Mason HS, Bali JM, Shi JJ, Jiang X, Estes MK, Arntzen CJ (1996) Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice. Proc. Natl. Acad. Sci. USA 93: 5335–5340.

Mason HS, Haq TA, Clements JD, Arntzen CJ (1998) Edible vaccine protects mice against Escherichia coli heat-labile enterotoxin (LT). Vaccine 16: 1336–1343.

McGarvey PB, Hammond J, Dineelt MM, Hooper DC, Fu ZF, Dietzschold B, Koprwowski H, Michaels FH (1995) Expression of rabies virus glycoprotein in transgenic tomatoes. Biotechniques 13: 1484–1487.

Min N, Cui DC, Einstein J, Narasimhulu S, Vergara CE, Gelvin SB (1995) Strength and tissue specificity of chimeric promoters derived from the octopine and mannopine synthase genes. Plant J. 7: 661–676.

Ritchter LJ, Thanava Y, Arntzen CJ, Mason HS (2000) Production of hepatitis B surface antigen in transgenic plants for oral immunization. Nat. Biotechnol. 18: 1167–1171.

Sambrook J, Fritsh EF, Maniatis T (1989), Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Shalaby WS (1995) Development of oral vaccines to stimulate mucosal and systemic immunity: barriers and novel strategies. Clin. Immunol. Immunopathol. 74: 127–134.

Sheppard M, Wemer W, Tsitas E, McCoy R, Prouse S, Johnson M (1998) Fowl adeno-virus recombinant expressing VP2 of infectious bursal disease virus induces protective immunity against bursal disease. Arch. Virol. 143: 915–930.

Snyder DB, Vakharia VN, Savage PK (1992) Naturally occurring-neutralizing monoclonal antibody escape variants define the epidemiology of infectious bursal disease viruses in the United States. Arch. Virol. 127: 89–101.

Spies U, Muller H, Becht (1987) Properties of RNA polymerase activity associated with infectious bursal disease virus and characterization of its reaction products. Virus Res. 8: 127–140.

Tacket CO, Mason HS, Losonsky G, Clements JD, Levine MM, Arntzen CJ (1998) Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato. Nat. Med. 4: 607–609.

Tsukamoto K, Kojima C, Komori Y, Tanimura N, Mase M, Yamaguchi S (1999) Protection of chicken against very virulent infectious bursal disease virus (IBDV) and Marek’s disease virus (MDV) with a recombinant MDV expressing IBDV VP2. Virology 257: 352–362.

Wigdorovitz A, Carrillo C, Santos MJD, Trono K, Peralta A, Gomez MC, Rios RD, Franzone PM, Sadir AM, Escribano JM, Borca MV (1999) Induction of a protective antibody response to foot-and-mouth disease virus in mice following oral or potential immunization with alfalfa transgenic plants expressing the oral structural protein VP1. Virology 255: 347–353.