JOURNAL OF VIROLOGY, Aug. 2003, p. 9090–9093 Vol. 77, No. 16
0022-538X/03/$08.00+0 DOI: 10.1128/JVI.77.16.9090–9093.2003

Expression of Immunogenic S1 Glycoprotein of Infectious Bronchitis Virus in Transgenic Potatoes

Ji-Yong Zhou,[1] Jian-Xiang Wu,[1] Li-Qin Cheng,[1] Xiao-Juan Zheng,[1] Hui Gong,[1] Shao-Bin Shang,[1] and En-Min Zhou[2]*

Institute of Preventive Veterinary Medicine, Zhejiang University, Hangzhou 310029, China,[1] and Department of Veterinary Diagnostic and Productive Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, Iowa 50011[2]

Received 19 February 2003/Accepted 19 May 2003

The expression of infectious bronchitis virus (IBV) S1 glycoprotein in potatoes and its immunogenicity in mice and chickens were investigated. Potato plants were genetically transformed with a cDNA construct encoding the IBV S1 glycoprotein with the *Agrobacterium* system. Genomic DNA and mRNA analyses of the transformed plantlets confirmed the integration of the foreign cDNA into the potato genome, as well as its transcription. Mice and chickens vaccinated with the expressed IBV S1 glycoprotein produced antibodies that neutralized IBV infectivity. After three immunizations, vaccinated chickens were completely protected from virulent IBV infection. These results demonstrate that transgenic potatoes expressing IBV S1 glycoprotein can be used as a source of recombinant antigen for vaccine production.

Infectious bronchitis virus (IBV), one of the prototype viruses of the *Coronaviridae* family, is the causative agent of an acute, highly contagious respiratory, renal, and urogenital disease characterized by high mortality rates in affected flocks. The IBV genome encodes three major structural proteins, the nucleocapsid, membrane, and spike (S) proteins. The S protein is posttranslationally cleaved into the N-terminal S1 and C-terminal S2 glycoproteins. The S1 glycoprotein induces virus-neutralizing and hemagglutination-inhibiting antibodies (5, 15). Therefore, the S1 glycoprotein may be useful as a vaccine component. The commercial live and inactivated vaccines that have been used to control IBV-associated disease in chickens (4) have some disadvantages. Inactivated vaccines are safe but are costly and less effective than live attenuated vaccines, while attenuated vaccines may be associated with the emergence of variant strains of the virus (11, 21, 22). Thus, there is a need for vaccines with higher efficacy and fewer side effects.

The expression of recombinant proteins in transgenic plants was first reported in 1995 by Haq et al. (10). Since then, several viral and bacterial antigens have been efficiently expressed in transgenic plants (1, 2, 8, 9, 12, 13, 14, 18, 19). In this paper, we report the expression of the S1 glycoprotein of IBV in transgenic plants and demonstrate its immunogenicity in mice. We also show that chickens immunized with the transgenic plants are protected against a challenge with virulent IBV.

A pair of specific primers, based on the cDNA sequence of the S protein-encoding gene of IBV strain ZJ971, was designed to amplify the S1 gene of IBV (22). Strains ZJ971, H52, and M41 have the same serotype and have 97.1 to 99.7% S1 gene identity. The upstream primer 5′-GCTCTAGAATGTTGGT AACACCTCTT-3′ contained the *Xba*I site, and the downstream primer 5′-CGGGATCCTTAATAACTAACATAAGG GCA-3′ contained the *Bam*HI site. The open reading frame of the S1 gene of IBV, consisting of 1,654 bp, was obtained by PCR. The amplified cDNA sequence encoding the IBV S1 glycoprotein was cloned downstream of the cauliflower mosaic virus (35S) promoter in the binary vector pBI121 (Clontech, Franklin Lakes, N.J.), followed by the nopaline synthase terminator (Fig. 1). The pBI121 vector containing the S1 cDNA (designated pBI121-S1) was introduced into *Agrobacterium tumefaciens* EHA105 (Clontech) from *Escherichia coli* by triparental mating as described by Ditta et al. (7). *A. tumefaciens* EHA105 was used to transfer the constructs into potato plants (*Solanum tuberosum* cv. Dongnong303) as described previously (3).

The transgenic plants resistant to the selection medium appeared similar in morphology to the nontransgenic potato plants. Eighty-three different transgenic plants containing the coding sequence of the IBV S1 gene were obtained and grown under controlled laboratory conditions. Genomic DNA was extracted from the transgenic plantlets as described by Gomez et al. (9). These plantlets were screened by PCR, and 90% contained the 1.7-kb recombinant S1 gene (data not shown). To confirm the PCR results, the genomic DNA of six lines of PCR-positive transgenic plantlets was digested with *Eco*RI. The PCR products (plantlets 2, 3, 5, 7, 8, and 9) were separated by electrophoresis in a 0.8% agarose gel, transferred to a nylon membrane (Amersham Pharmacia Biotech AB, Uppsala, Sweden), and hybridized with the ^{32}P-labeled IBV S1 probe with the Prime-Gene DNA Label Kit (Promega, Madison, Wis.) as described by Sambrook and Russell (16). As shown in Fig. 2, one to three copies of the cDNA sequence of the IBV S1 gene were inserted into several different sites of the tetraploid potato genome. Four of six transgenic plantlets contained two cDNA copies (numbers 2, 5, 7, and 8 [lanes 1, 3, 4, and 5, respectively]), and the other two transgenic plantlets contained one (number 9 [lane 6]) and three (number 3 [lane 2]) cDNA

* Corresponding author. Mailing address: Department of Veterinary Diagnostic and Production Animal Medicine, 2630 Vet. Med. Building, College of Veterinary Medicine, Iowa State University, Ames, IA 50011. Phone: (515) 294-4699. Fax: (515) 294-3654. E-mail: ezhou@iastate.edu.

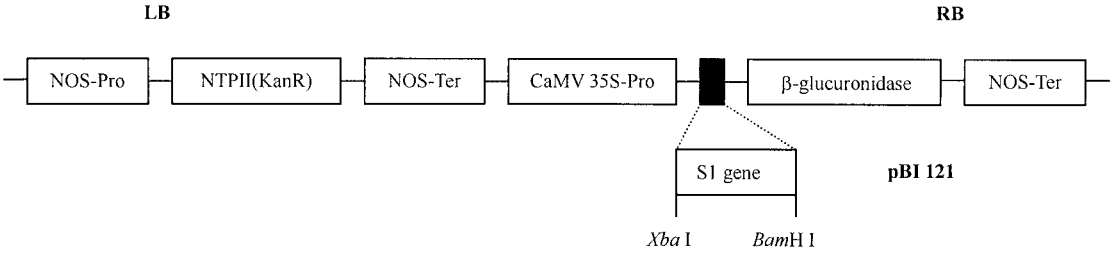

FIG. 1. Schematic structure of binary plasmid pBI121, which was used for *Agrobacterium*-mediated plant transformation. The cDNA sequence encoding the S1 glycoprotein of IBV was cloned downstream of the cauliflower mosaic virus 35S promoter (CaMV 35S-Pro) in recombinant pBI121, followed by the nopaline synthase terminator (NOS-Ter). NOS-Pro, nopaline synthase promoter; LB, left border; RB, right border.

copies. These results indicated that the IBV S1 gene was integrated into the genome of the transgenic plants.

Fresh transgenic plantlets were frozen in liquid nitrogen and ground to a powder with a mortar and pestle. Total RNA from each of the six transgenic plantlets was extracted with TRIzol reagents (GIBCO BRL, New York, N.Y.), separated individually by electrophoresis in a 1.2% agarose-formaldehyde gel, and transferred onto a nylon membrane (Amersham Pharmacia Biotech AB). The transferred RNA was then hybridized with a ^{32}P-labeled IBV S1 gene probe as described previously (16). Figure 3 shows that different levels of IBV S1 glycoprotein-specific RNA were observed among the six independent transformants. Transgenic plantlets 1, 3, 8, C1, and D1 (lanes 10, 3, 6, 8, and 9) had stronger hybridization signals than plantlets 5, 7, and 9 (lanes 4, 5, and 7). Transgenic plantlet 2 (lane 2) had no hybridization signal. The amount of S1 glycoprotein expressed in the potato microtubers was quantified by enzyme-linked immunosorbent assay (ELISA) as described by Zhou et al. (23). As shown in Fig. 4, the S1 glycoprotein was detected in the protein extracts from 17 selected plants. The transgenic plants expressed different amounts of S1 protein. The maximum amount of S1 protein expressed in the tubers represents 0.07 to 0.22% (3.64 to 11.44 μg) of the transgenic potato tuber soluble protein. Plant C1 expressed the most S1 glycoprotein, and plant 2 expressed the least.

To determine the immunogenicity of the recombinant S1 glycoprotein, 4-week-old specific-pathogen-free BALB/c mice (Shanghai Laboratory Animal Center, Chinese Academy of Sciences, Shanghai, China) were divided into five groups, with six mice per group, and immunized with different doses of S1 protein or with a commercial IBV vaccine. Group 1 and 2 mice received preparations containing, respectively, 1.0 g (11.44 μg of S1 glycoprotein) and 0.5 g (5.72 μg of S1 glycoprotein) of the tuber extracts from a transgenic line (plantlet C1). Group 3 mice received the commercial modified live IBV vaccine (H52 strain; Hangzhou Jianliang Veterinary Bioproducts Co. Ltd., Hangzhou, China). Group 4 mice received 1.0 g of wild-type tuber extracts, and group 5 mice received the extraction buffer. All antigens were administered by gastric intubation (gavage) on days 0, 7, and 14. Serum samples were collected via the tail vein at day 0 and 1 week after each immunization. The presence of anti-S1 glycoprotein antibodies in serum samples from the mice was detected with a virus neutralization (VN) test with the tracheal organ culture (TOC) assay (6). As shown in Table 1, mice that received 1.0 g of potato microtuber had higher VN titers (1:621) after three immunizations than did mice that received 0.5 g of potato microtuber (1:300). In comparison, mice that received a commercial modified live IBV vaccine also developed anti-S1 antibodies, but the mean titer was lower (1:57). No anti-S1 glycoprotein antibodies were detected in the control mice.

The immunogenicity of the transgenic S1 glycoprotein was also tested in chickens. Specific-pathogen-free Leghorn chicken eggs obtained from Beijing Merial Vital Laboratory Animal Technology Co. Ltd., Beijing, China, were hatched in our laboratory, and the chicks were maintained in strict isolation throughout the study. Fourteen-day-old chicks were divided into seven groups with six chicks per group. Group 1 and 2 chicks were orally immunized with, respectively, 5 g (57.2 μg of S1 glycoprotein) and 2.5 g (28.6 μg of S1 glycoprotein) of tuber from transgenic lines. Group 3 and 4 chicks were vaccinated intramuscularly with 5 and 2.5 g of tuber extract, respec-

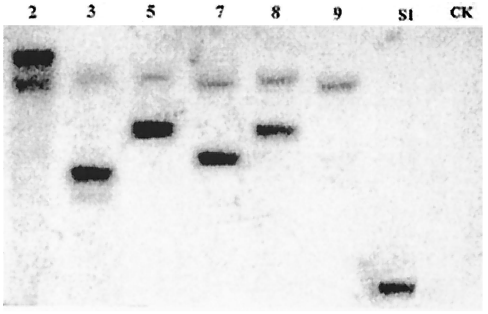

FIG. 2. IBV S1 glycoprotein gene of the genomic DNA in representative transgenic potato plants hybridized with ^{32}P-labeled IBV-S1 probe. Each lane represents an S1 gene product expressed in an individual transgenic potato plant. S1, PCR product of S1 glycoprotein cDNA sequence; CK, nontransgenic plants.

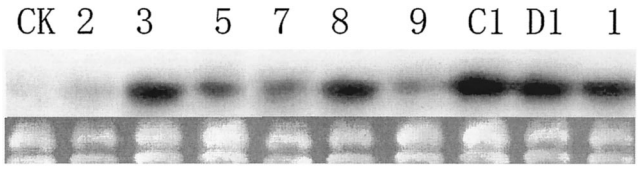

FIG. 3. Northern blot assay of RNA. RNA was probed with an IBV S1 gene coding sequence demonstrating specific mRNAs of IBV S1 glycoprotein expressed in transgenic potatoes. CK, no hybridized signal of nontransgenic potato plant. Each lane represents an individual transgenic plantlet.

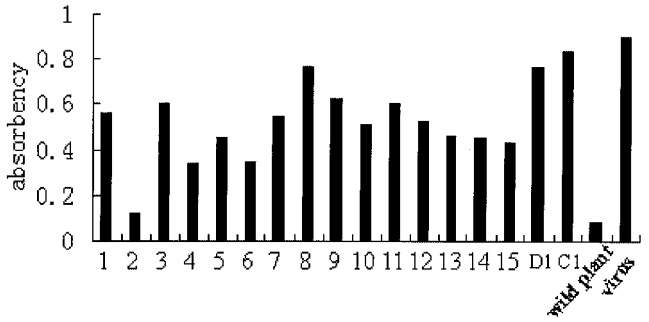

FIG. 4. Detection of S1 glycoprotein from 17 transgenic potato plants by ELISA. The mean absorbance ± the standard error was obtained from three independent assays. The wild plant and the virus were used as negative and positive controls, respectively.

tively. The fifth group received the commercial modified live IBV vaccine intranasally in accordance with the manufacturer's instructions (H52 strain; Hangzhou Jianliang Veterinary Bioproducts Co. Ltd.). The sixth group was fed with wild-type tuber at a dose of 5 g per chick. Chicks in the seventh group were injected intramuscularly with the extraction buffer. All immunizations were administrated at days 0, 7, and 14. Serum samples were collected via wing vein at day 0 and 1 week after each immunization. The VN antibody titer against IBV was measured with the TOC assay (6). As shown in Table 2, chickens that received transgenic potato tuber intramuscularly had higher VN antibody titers (1:2,187) after three immunizations than did chickens that received transgenic potato tuber orally (1:1,650). After three immunizations, the VN titer of chickens vaccinated with the commercial vaccine (1:1,412) was similar to that of the chickens vaccinated orally with potato tuber. No anti-IBV antibodies were detected in chickens vaccinated with wild type potato or buffer.

All vaccinated chicks were challenged intranasally with 50 μl of virulent IBV (M41 strain; titer, $10^{4.89}$ 50% embryo lethal doses/0.1 ml) 7 days after the third immunization and observed daily for up to 7 days. The criterion for protection was absence of respiratory signs of infectious bronchitis as described elsewhere (4). The chickens vaccinated with 5.0 g of transgenic potato or the commercial vaccine were completely protected (six of six) against a challenge with virulent IBV (Table 2). Five (83.3%) of the six chickens that received 2.5 g of transgenic potato were also protected against a challenge. No virus was isolated in trachea and feces samples collected from these chickens at 7 days postchallenge. No protection was seen in placebo chickens that received wild-type potato or buffer.

TABLE 1. Murine VN antibody responses against potato plant-derived IBV S1 glycoprotein

Group	Immunization[a]	No. of mice	VN titer[b]
1	Transgenic plant I (1 g)	6	1:621
2	Transgenic plant II (0.5 g)	6	1:300
3	IBV live vaccine	6	1:57
4	Nontransgenic potato	6	0
5	Buffer	6	0

[a] Mice were immunized three times by grastric intubation on days 0, 7, and 14.
[b] The VN titer is the geometric mean titer obtained 7 days after the third vaccination.

TABLE 2. Chicken anti-IBV VN antibody response and protection from challenge with virulent IBV

Group	Immunization[a]	Dose (g)	Route	Serum VN titer[c]		No. protected/ no. challenged
				1st immunization	3rd immunization	
1	TP	5	Oral	11.4	1,650	6/6
2	TP	2.5	Oral	10.4	1,270	5/6
3	TP	5	i.m.[b]	16.9	2,187	6/6
4	TP	2.5	i.m.	12.6	1,357	5/6
5	LAV	1	i.n.[b]	13	1,412	6/6
6	WTT	5	Oral	0	0	0/6
7	Buffer		Oral	0	0	0/6

[a] Each group consisted of six chickens that were immunized three times with transgenic potatoes (TP), live attenuated vaccine (LAV), wild-type tuber (WTT), or buffer.
[b] i.m., intramuscular; i.n., intranasal.
[c] The VN titer is the geometric mean titer.

In a previous report, mice vaccinated with the S glycoprotein of IBV, expressed in recombinant vaccinia virus, produced antibodies that recognized the S antigen in an ELISA and neutralized IBV infectivity, as shown by TOC assay (20). Song et al. have shown that recombinant S1 glycoproteins expressed in a baculovirus induced protective immunity against a challenge with virulent IBV in chickens (17). To the best of our knowledge, however, this is the first report of the expression of an IBV antigen in a transgenic system. Our studies demonstrate that an IBV antigen expressed in transgenic potatoes can induce protection against wild-type IBV infection in chickens and could be an effective candidate for an IBV vaccine.

This work was supported by National Natural Science Foundation of China grant 30070570.

We thank David J. Larson, Donald L. Reynolds, and Ann Spicker for critical suggestions and comments.

REFERENCES

1. **Arakawa, T., D. K. X. Chong, and W. H. R. Langridge.** 1998. Efficacy of a food plant-based oral cholera toxin B subunit vaccine. Nat. Biotechnol. **16:**292–297.
2. **Carrillo, C., A. Wigdorovitz, J. C. Oliveros, P. I. Zamorano, A. M. Sadir, N. Gomez, J. Salinas, J. M. Escribano, and M. V. Borca.** 1998. Protective immune response to foot and mouth disease virus with VP1 expressed in transgenic plants. J. Virol. **72:**1688–1690.
3. **Castanon, S., M. S. Marin, J. M. Martin-Alonso, J. A. Boga, R. Casais, J. M. Humara, R. J. Ordas, and F. Parra.** 1999. Immunization with potato plants expressing VP60 protein protects against rabbit hemorrhagic disease virus. J. Virol. **73:**4452–4455.
4. **Cavanagh, D., and S. A. Naqi.** 1997. Infectious bronchitis, p. 511–526. *In* B. W. Calnek, H. J. Barnes, C. W. Beard, L. R. McDougald, and Y. M. Saif (ed.), Diseases of poultry, 10th ed. Iowa State University Press, Ames.
5. **Cavanagh, D., P. J. Davis, J. H. Darbyshire, and R. W. Peters.** 1986. Coronavirus IBV: virus retaining spike glycopolypeptide S2 but not S1 is unable to induce virus-neutralizing or haemagglutination inhibiting antibody, or induce chicken tracheal protection. J. Gen. Virol. **67:**435–1442.
6. **Cook, J. K. A., J. H. Darbyshire, and R. W. Peter.** 1976. The use of chicken trachea organ culture for the isolation and assay of infectious bronchitis virus. Arch. Virol. **50:**109–118.
7. **Ditta, D., S. Stanfield, D. Corbin, and D. R. Helinskim.** 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77:**7347–7351.
8. **Gil, F., A. Brun, A. Wigdorovitz, R. Catala, J. L. Martinez-Torrecuadrada, I. Casal, J. Salinas, M. V. Borca, and J. M. Escribano.** 2001. High-yield expression of a viral peptide vaccine in transgenic plants. FEBS Lett. **488:**13–17.
9. **Gomez, N., C. Carrillo, J. Salinas, F. Parra, M. V. Borca, and J. M. Escri-**

bano. 1998. Expression of immunogenic glycoprotein S polypeptides from transmissible gastroenteritis *Coronavirus* in transgenic plants. Virology **249:** 352–358.
10. **Haq, T. A., H. S. Mason, J. D. Clements, and C. J. Arntzen.** 1995. Oral immunization with a recombinant bacterial antigen produced in transgenic plants. Science **268:**714–716.
11. **Jia, W., K. Karaca, C. R. Parrish, and S. A. Naqi.** 1995. A novel variant of avian infectious bronchitis virus resulting from recombination among three different strains. Arch. Virol. **140:**259–271.
12. **Mason, H. S., T. A. Haq, J. D. Clements, and C. J. Arntzen.** 1998. Edible vaccine protects mice against *Escherichia coli* heat-labile enterotoxin (LT): potatoes expressing a synthetic LT-B gene. Vaccine **16:**1336–1343.
13. **Mason, H. S., J. M. Ball, J. J. Shi, X. Jiang, M. K. Estes, and C. J. Arentzen.** 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.
14. **Modelska, A., B. Dietzschold, N. Sleysh, Z. F. Fu, K. Steplewski, D. C Hooper, H. Koprowski, and V. Yusibov.** 1998. Immunization against rabies with plant-derived antigen. Proc. Natl. Acad. Sci. USA **95:**2481–2485.
15. **Moore, K. M., M. W. Jackwood, and D. A. Hilt.** 1997. Identification of amino acids involved in a serotype and neutralization specific epitope within the S1 subunit of avian infectious bronchitis virus. Arch. Virol. **142:**2249–2256.
16. **Sambrook, J., and D. W. Russell.** 2001. Southern blotting, p. 6.39–6.58, and Northern hybridization, p. 7.21–7.75. *In* Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
17. **Song, C. S., Y. J. Lee, C. W. Lee, H. W. Sung, J. H. Kim, I. P. Mo, Y. Izumiya, H. K. Jang, and T. Mikami.** 1998. Induction of protective immunity in chickens vaccinated with infectious bronchitis virus S1 glycoprotein expressed by a recombinant baculovirus. J. Gen. Virol. **79:**719–723.
18. **Streatfield, S. J., J. M. Jilka, E. E. Hood, D. D. Turner, M. R Bailey, J. M. Mayor, S. L. Wooddard, K. K. Beiffuss, M. E. Horn, D. E. Delaney, I. R. Tizard, and J. A. Howard.** 2001. Plant-based vaccines: unique advantages. Vaccine **19:**2742–2748.
19. **Thanavala, Y., Y. F. Yang, P. Lyons, H. S. Mason, and C. J. Arntzen.** 1995. Immunogenicity of transgenic plant-derived hepatitis B surface antigen. Proc. Natl. Acad. Sci. USA **92:**3358–3361.
20. **Tomley, F. M., A. P. A. Mockett, M. E. G. Boursnell, M. M. Binns, J. K. A. Cook, T. D. K. Brown, and G. L. Smith.** 1987. Expression of the infectious bronchitis virus spike protein by recombinant vaccinia virus and induction of neutralizing antibodies in vaccinated mice. J. Gen. Virol. **68:**2291–2298.
21. **Wang, L., D. Junker, and E. W. Collisson.** 1993. Evidence of natural recombination within the S1 gene of infectious bronchitis virus. Virology **192:**710–716.
22. **Zhou, J. Y., L. Q. Cheng, and X. Y. Shen.** 2002. Cloning and sequencing of S gene of novel variant of infectious bronchitis virus ZJ971 isolates in China. Agric. Sci. China **1:**101–107.
23. **Zhou, X. P., Y. L. Liu, L. Calvert, C. Munoz, W. Otim-Nape, D. J. Robinson, and B. D. Harrison.** 1997. Evidence that DNA-A of a geminivirus associated with severe cassava mosaic disease in Uganda has arisen by interspecific recombination. J. Gen. Virol. **78:**2101–2111.