

Production of immunogenic VP6 protein of bovine group A rotavirus in transgenic potato plants

Brief Report

T. Matsumura¹, N. Itchoda², and H. Tsunemitsu³

¹National Institute of Advanced Industrial and Science Technology, Sapporo, Japan

²Hokkaido Green-Bio Institute, Naganuma, Hokkaido, Japan

³Shichinohe Research Unit, National Institute of Animal Health, Shichinohe, Aomori, Japan

Received August 28, 2001; accepted January 19, 2002
Published online April 26, 2002 © Springer-Verlag 2002

Summary. We report here the production of transgenic potato plants expressing the major capsid protein VP6 of bovine group A rotavirus (GAR). Transgenic plants under the control of a cauliflower mosaic virus 35S promoter, or a modified promoter linked to the tobacco mosaic virus 5'-untranslated sequence were positive for GAR antigens by ELISA. The expressed protein was consistent in size with VP6 of GAR by Western blot assay. The presence of the VP6 gene and its transcript was detected by PCR and RT-PCR. Adult BALB/c mice were immunized intraperitoneally with concentrated transgenic potato extracts emulsified in Freund's adjuvant. Sera collected after immunization showed the anti-VP6 response in ELISA and Western blot assay. These results suggest that the immunogenic VP6 protein expressed in plants could be useful for the preparation of diagnostic reagents.

*

Group A rotavirus (GAR) is one of the most important causes of severe viral diarrhea in humans and animals [8]. The major capsid protein VP6 that comprises approximate 51% by weight of the virion protein is located on the inner capsid and contains the common antigens in each rotavirus serogroup [8, 14]. Recent studies indicated that the VP6 has been defined as a protective antigen in an adult mouse rotavirus infection model [3]. The utilization of plants as bioreactors for the production of foreign proteins has been reported [1, 11, 13, 17]. Transgenic plants expressing recombinant proteins have many advantages, such as low-cost production and suitable form for edible vaccine [11, 17]. Recently,

several viral antigens have been produced in transgenic plants, which possessed immunogenicity in animals [2, 7, 12, 15, 20]. However, there are no reports on the production of transgenic plants expressing rotavirus capsid proteins although rotavirus enterotoxin has been expressed in transgenic plants [21], and the VP6 has been produced in plants using virus vectors [18]. We report here the production of transgenic potato plants expressing the immunogenic VP6 protein of bovine GAR.

The full-length VP6 gene was amplified by reverse transcription-PCR (RT-PCR) from viral RNA obtained from MA104 cells infected with bovine GAR 22R strain (G6, P[5]). This strain was isolated from a calf diarrhea in Japan, and the VP6 gene was 1356 nucleotides (nt) long (DDBJ accession no. AB040055). The amplified VP6 gene was cloned into the pGEM-T vector (Promega). The resulting clone, pGEM-T/VP6, was digested with *Sma*I and *Sac*I and subcloned into the plant expression vectors pBI121 (Clontech) to yield pBIVP6, and pBE2113 [16] to yield pBEVP6. The pBI121 is a binary vector for expression in plants using the cauliflower mosaic virus (CaMV) 35S promoter to drive transcription and the nopaline synthase terminator. The pBE2113 has the same promoter and terminator as the pBI121 but carries translational enhancers that are two tandem repeats of the 5'-upstream sequence of CaMV 35S promoter, and the 5'-untranslated sequence of tobacco mosaic virus. *Agrobacterium tumefaciens* LBA4404 was transformed with pBIVP6 or pBEVP6 and used for potato plants (*Solanum tuberosum* cv. *Mayqueen*) transformation as described previously [9]. The VP6 gene was also cloned into the baculovirus expression vector pVL1392 (PharMingen). Cotransfection of *Spodoptera frugiperda* (Sf9) cells with the recombinant pVL1392 and the Baculogold linearized baculovirus DNA (PharMingen) was performed by the Lipofectin (Gibco BRL) method in accordance with the manufacture's instructions.

For detection and quantification of recombinant VP6 in potato leaf or tuber extracts, a sandwich ELISA was performed. ELISA plates (Maxisorp; Nunc) were coated with hyperimmune or preimmune gnotobiotic pig anti-GAR (Lincoln strain) serum and incubated overnight at 4 °C. The plates were blocked with 5% BLOTTO [5% non-fat dry milk in phosphate-buffered saline (PBS) containing 0.05% Tween 20] at room temperature for 1 h. Potato leaf or tuber extracts obtained by homogenisation in PBS (pH 7.4) plus 50 mM sodium ascorbate, 1 mM EDTA, 1 mM phenylmethylsulfonyl and 0.2% Triton X-100 (5 ml/g fresh wt in leaves and 1 ml/g fresh wt in tubers) were added to the plates and incubated at 37 °C for 1 h. Hyperimmune rabbit anti-GAR (22R) serum and horseradish peroxidase-conjugated anti-rabbit IgG (KPL) were added in subsequent steps. ABTS [2,2'-azino-bis (3-ethyl-benzothiazole-6-sulfonic acid) diammonium salt] was added each well as substrate. The plates were developed at room temperature for 30 min and the reaction was terminated by the addition of 5% sodium lauryl sulfate. The absorbance of the samples was measured at 405 nm. Net optical density (OD) was calculated as the OD from hyperimmune antiserum-coated wells minus the OD from preimmune serum-coated wells. A sample was considered GAR antigens positive if a net OD had greater than 0.1. Each ELISA plate included positive controls containing 3 to 200 ng of baculovirus-expressed VP6. The level

of baculovirus-expressed VP6 was determined by comparing the band intensity of baculovirus-expressed VP6 with that of known concentration of bovine serum albumin (Sigma) following SDS-10% polyacrylamide gel electrophoresis and coomassie blue staining. The amount of VP6 in the medium of Sf9 cells infected with baculovirus recombinant 120 h postinfection was 100 $\mu\text{g/ml}$, which was similar to that reported previously [6]. The level of VP6 expressed in potato plants was determined by comparison of net ODs in ELISA between potato samples and the baculovirus-expressed VP6. The concentration of total soluble protein of potato samples was determined with the DC protein assay kit (Bio-Rad).

At the first trial of transformation, 70 transgenic potato plants in the pBIVP6 line were obtained and 16 of these potato leaf extracts were positive for GAR antigens by ELISA (Fig. 1A). Extracts from non-transgenic potato leaves or tubers were negative in the ELISA. The maximum levels of VP6 accumulation in the

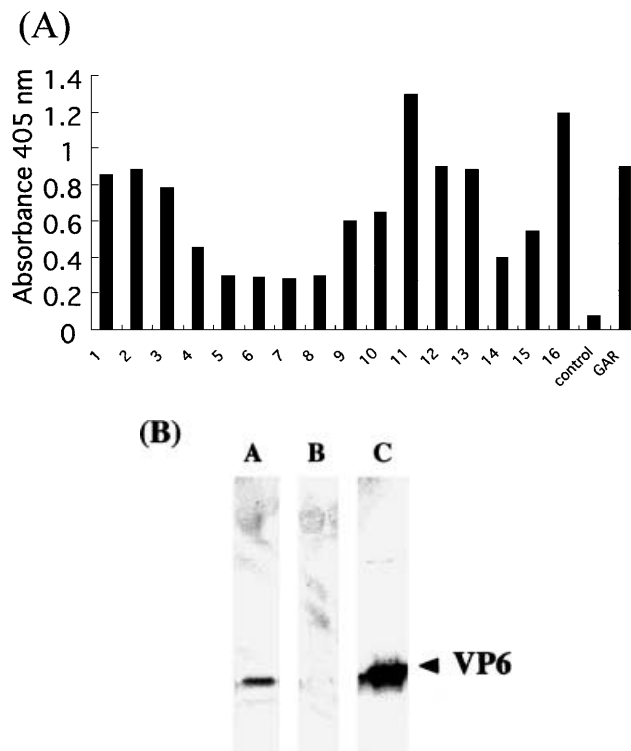


Fig. 1. Detection of GAR VP6 in transgenic potato extracts. **A** Presence of the GAR antigens in selected transgenic potato leaf extracts (1–16) by ELISA. *Control*, non-transgenic potato leaf extracts as a negative control. GAR, GAR 22R strain as a positive control. **B** Western blot detection of VP6 in a transgenic potato tuber extract. Concentrated transgenic or non-transgenic potato tuber extract was subjected to SDS-10% polyacrylamide gel electrophoresis, and blotted to an Immobilon P (Millipore) membrane. The membrane was incubated with diluted anti-22R guinea pig IgG. Bound antibodies were detected with horseradish peroxidase-conjugated anti-guinea pig IgG (KPL) and developed with ECL reagents. A concentrated transgenic potato tuber extract; B concentrated non-transgenic potato tuber extract; C purified 22R strain

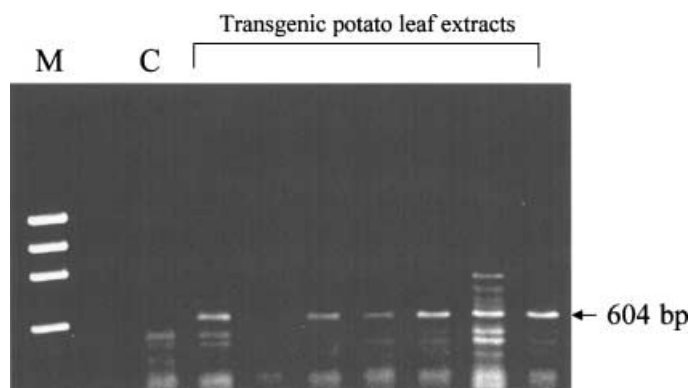


Fig. 2. Detection of VP6 gene transcripts in transgenic potato plants by RT-PCR. RNA was extracted from ELISA-positive potato leaf extracts, and RT-PCR was performed with primers designed to amplify 604 bp of VP6 gene transcripts. *C* non-transgenic potato leaf; *M* molecular weight marker

potato leaves and tubers were 0.006% and 0.002% of total soluble proteins, respectively.

To confirm the presence of the VP6 gene and its transcript, DNA and RNA were extracted with the TRIzol LS reagent (Life technologies) from the 16 ELISA-positive potato leaf extracts in the pBIVP6 line, and used for PCR and RT-PCR with a forward primer, 5'-AATGTATGCATGGACGAAAT-3' (nt 303 to 322 of the 22R VP6 gene) and a reverse primer, 5'-GTCATATTTGGTGGTCTCAT-3' (nt 906 to 925). The predicted RT-PCR and PCR products were 604 bp. All 16 transgenic potatoes were positive in these assays (Fig. 2). By contrast, non-transgenic potatoes were negative in these assays. The expressed protein in selected transgenic potato tuber extracts (No. 12) in the pBIVP6 line was consistent in size with VP6 (45 kDa) of GAR by Western blot assay using anti-22R guinea pig IgG, anti-guinea pig IgG and ECL reagents (Amersham Pharmacia Biotech) (Fig. 1B).

To investigate the immunogenicity of recombinant VP6 from transgenic potato plants, four adult BALB/c mice were injected intraperitoneally twice at an interval of 2 weeks with transgenic potato tuber extracts (No. 16) in the pBIVP6 line concentrated with the Pellicon XL (Millipore) (750 ng of VP6 per mouse) and emulsified in Freund's adjuvant (immunized mice). Four mice were injected with concentrated non-transgenic potato tuber extracts emulsified in Freund's adjuvant with the same manner as immunized mice (control mice). Sera were collected from these mice at each immunization and 2 weeks after the last booster and analyzed against the anti-VP6 response by ELISA and Western blot assay. In these assays, purified 22R strain was used as antigens. Before injection, GAR antibodies were not detected from sera of all mice by ELISA (Fig. 3A). After injection, sera from immunized mice showed the GAR antibody response in ELISA, but no antibody response was observed in control mice (Fig. 3A). Anti-VP6 antibodies were detected in immunized mice after injection (Fig. 3B) by Western blot assay.

Next, transformation with the pBEVP6 was conducted because the VP6 expression levels of the pBIVP6 transformants were relatively low. 108 transgenic

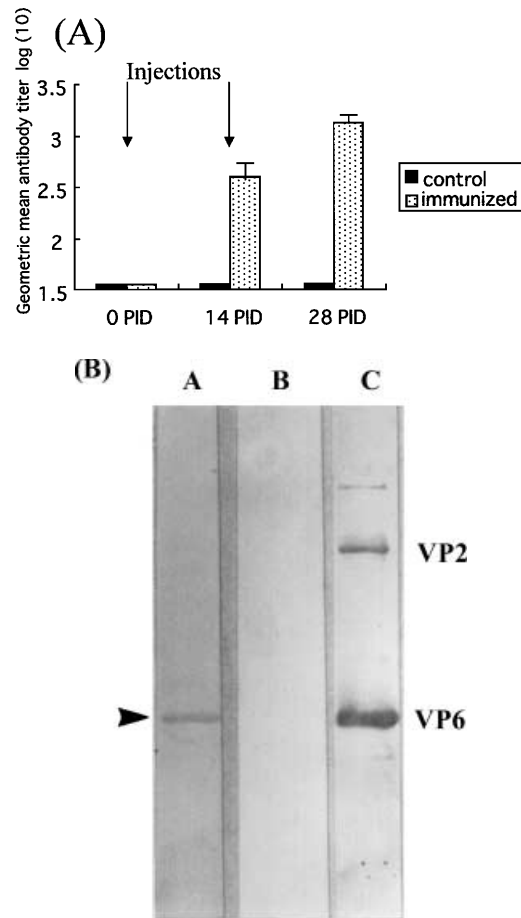


Fig. 3. Detection of Anti-VP6 antibodies in sera from mice immunized with transgenic potato tuber extracts. **A** Geometric mean ELISA titers of anti-VP6 antibodies in sera from 4 immunized and 4 control mice. Mice were inoculated intraperitoneally twice at an interval of 2 weeks with a transgenic potato extract (immunized mice) or a non-transgenic potato extract (control mice) emulsified in Freund's adjuvant. ELISA plates were coated with purified GAR 22R strain, and serial fourfold dilutions of sera started at 1:100 were added. Antibody titers were expressed as the reciprocal of the highest serum dilution which presented the absorbance at 405 nm above 0.1. Error bars represent one standard error of the mean. PID, postinoculation day from the first inoculation. **B** Anti-VP6 antibodies detected by Western blot in sera from a mouse immunized with transgenic potato tuber extracts. The purified GAR 22R strain was subjected to SDS-10% polyacrylamide gel electrophoresis, and blotted to Immobilon P (Millipore) membranes. The membranes were incubated with mouse sera diluted 1:100 or anti-22R guinea pig serum. Bound antibodies were detected with horseradish peroxidase-conjugated anti-mouse IgG or anti-guinea pig IgG (KPL) and developed with a TMB membrane substrate (KPL). *A* serum from a mouse at PID 28; *B* serum from a mouse at PID 0; *C* anti-22R guinea pig serum. Arrow head shows the position of VP6

potato plants in the pBEVP6 line were obtained and all of these potato leaf extracts were positive for GAR antigens by ELISA. The maximum level of VP6 accumulation in leaves of pBEVP6-transformed potatoes was 0.1% of the total soluble protein (30 $\mu\text{g/g}$ of fresh leaf tissue), which was approximately 17-fold increase in VP6 expression compared to the maximum level obtained from the pBIVP6 transformants. When compared with the amount of the baculovirus-expressed VP6 in culture supernatant, the maximum level of VP6 in pBEVP6-transformed potato leaves was approximately one third.

In general, production of transgenic plants needs 3 or 4 months after *Agrobacterium* infection. However, once transgenic plants are obtained, several advantages in expressing proteins in plants exist, compared to those of other expressing systems. The storage of genes and gene products in plants are very stable. Plants have a scale-up production, in which virtually limitless amounts of recombinant protein could be grown at minimal cost [11]. The cost of producing recombinant proteins in plants could be 10- to 50-fold lower than producing the same protein by *Escherichia coli* fermentation [10]. Cultivation of plants is easier than those of bacteria or animal cells, which does not require specialist, media, or equipment [11].

We could demonstrate the expression of antigenic and immunogenic GAR VP6 protein in transgenic plants. This is the first report on the expression of one of capsid proteins of rotavirus in transgenic plants. The VP6 contains the common antigens in each rotavirus serogroups, therefore, the recombinant VP6 expressed in plants could be useful for the preparations of diagnostic reagents for GAR infections. Recent studies have indicated that the VP6 is able to elicit protection against rotavirus shedding in mouse model [3]. A CD4⁺ T-cell epitope in VP6 might be effective for protection against rotavirus shedding [4]. There is the potential for VP6 expressed in transgenic plants to be developed into a rotavirus subunit vaccine.

Increase of the expression levels of foreign proteins in transgenic plants is a key for development of practical biopharmaceuticals, especially subunit edible vaccine. So far, the expression levels of viral protein in transgenic plants reported were from 0.001% of total soluble protein for rabies virus glycoprotein [15] to 0.37% for Norwalk virus capsid protein [12]. Several approaches have been reported to increase expression levels in transgenic plants, for example, modification of plant promoters for transcription of the genes and insertion of plant 5'- or 3'-untranslated regions for increase of mRNA stability and translatability [17, 19]. Mitsuhashi et al. [16] have reported that a CaMV 35S promoter with duplicated enhancer linked to the tobacco mosaic virus 5'-untranslated leader sequence enhanced expression levels of the β -glucuronidase gene. In the present study, the same promoter cassette increased the VP6 expression levels in transgenic potatoes to the maximum level of 0.1% of the total soluble protein. Evaluation of protective immunity of the VP6 in the transgenic potato tubers by feeding mouse is in progress. In addition, production of transgenic potatoes expressing VP2 is now under way for our first goal to produce self-assembled rotavirus-like particles by crossbreeding of VP2 and VP6 transgenic potato plants, which were produced by a baculovirus expression system [5].

Acknowledgement

This research was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan.

References

1. Arakawa T, Chong DK, Merritt JL, Langridge WH (1997) Expression of cholera toxin B subunit oligomers in transgenic potato plants. *Transgenic Res* 6: 403–413
2. Castanon S, Marin MS, Martin-Alonso JM, Boga JA, Casais R, Humara JM, Ordas RJ, Parra F (1999) Immunization with potato plants expressing VP60 protein protects against rabbit hemorrhagic disease virus. *J Virol* 73: 4452–4455
3. Choi AH, Basu M, McNeal MM, Clements JD, Ward RL (1999) Antibody-independent protection against rotavirus infection of mice stimulated by intranasal immunization with chimeric VP4 or VP6 protein. *J Virol* 73: 7574–7581
4. Choi AH, Basu M, McNeal MM, Flint J, VanCott JL, Clements JD, Ward RL (2000) Functional mapping of protective domains and epitopes in the rotavirus VP6 protein. *J Virol* 74: 11574–11580
5. Crawford SE, Labbe M, Cohen J, Burroughs MH, Zhou YJ, Estes MK (1994) Characterization of virus-like particles produced by the expression of rotavirus capsid proteins in insect cells. *J Virol* 68: 5945–5952
6. Estes MK, Crawford SE, Penaranda ME, Petrie BL, Burns JW, Chan WK, Ericson B, Smith GE, Summers MD (1987) Synthesis and immunogenicity of the rotavirus major capsid antigen using a baculovirus expression system. *J Virol* 61: 1488–1494
7. Gomez N, Carrillo C, Salinas J, Parra F, Borca MV, Escribano JM (1998) Expression of immunogenic glycoprotein S polypeptides from transmissible gastroenteritis coronavirus in transgenic plants. *Virology* 249: 352–358
8. Kapikian AZ, Chanock RM (1990) Rotaviruses. In: Fields BN, Knipe DM, Chanock RM, Hirsch MS, Melnick JL, Monath TP, Roizman B (eds) *Virology*, 2nd edn. Raven Press, New York, pp 1353–1404
9. Kondo T, Matsumura T, Uyeda I, Hataya T, Shikata E (1999) Restricted virus multiplication in potato plants transformed with the putative replicase component gene of potato leafroll virus. *J Faculty Agriculture Hokkaido University* 69: 143–150
10. Kusnadi A, Nikolov ZL, Howard JA (1997) Production of recombinant proteins in transgenic plants: practical considerations. *Biotechnol Bioeng* 56: 473–484
11. Ma JK-C, Vine ND (1999) Plant expression systems for the production of vaccines. *Curr Top Microbiol Immunol* 236: 275–292
12. Mason HS, Ball JM, Shi J, Jiang X, Estes MK, Arntzen CJ (1996) Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity. *Proc Natl Acad Sci USA* 93: 5335–5340
13. Mason HS, Lam DM, Arntzen CJ (1992) Expression of hepatitis B surface antigen in transgenic plants. *Proc Natl Acad Sci USA* 89: 11745–11749
14. Mattion NM, Cohen J, Estes MK (1994) The rotavirus protein. In: Kapikian AZ (ed) *Viral infections of the gastrointestinal tract*, 2nd edn. Marcel Dekker, New York, pp 169–249
15. McGarvey PB, Hammond J, Dienelt MM, Hooper DC, Fu ZF, Dietzschold B, Koprowski H, Michaels FH (1995) Expression of the rabies virus glycoprotein in transgenic tomatoes. *Biotechnology* 13: 1484–1487
16. Mitsuhashi I, Ugaki M, Hirochika H, Ohshima M, Murakami T, Gotoh Y, Katayose Y, Nakamura S, Honkura R, Nishimiya S, Ueno K, Mochizuki A, Tanimoto H, Tsugawa H, Otsuki Y, Ohashi Y (1996) Efficient promoter cassettes for enhanced expression of

- foreign genes in dicotyledonous and monocotyledonous plants. *Plant Cell Physiol* 37: 49–59
17. Mor TS, Gomez-Lim MA, Palmer KE (1998) Perspective: edible vaccines – a concept coming of age. *Trends Microbiol* 6: 449–453
 18. O'Brien GJ, Bryant CJ, Voogd C, Greenberg HB, Gardner RC, Bellamy AR (2000) Rotavirus VP6 expressed by PVX vectors in *Nicotiana benthamiana* coats PVX rods and also assembles into viruslike particles. *Virology* 10: 444–453
 19. Richter LJ, Thanavala Y, Arntzen CJ, Mason HS (2000) Production of hepatitis B surface antigen in transgenic plants for oral immunization. *Nat Biotechnol* 18: 1167–1171
 20. Wigdorovitz A, Carrillo C, Dus Santos MJ, 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 parenteral immunization with alfalfa transgenic plants expressing the viral structural protein VP1. *Virology* 255: 347–353
 21. Yu J, Langridge WH (2001) A plant-based multicomponent vaccine protects mice from enteric diseases. *Nat Biotechnol* 6: 548–552

Author's address: Dr. T. Matsumura, National Institute of Advanced Industrial and Science Technology, Sapporo 062-8517, Japan; e-mail: matsumura-t@aist.go.jp