Title: PRODUCTION METHOD OF RECOMBINANT ROTAVIRUS STRUCTURAL PROTEINS AND VACCINE COMPOSITION

Abstract: The present invention relates to a method for producing recombinant rotavirus structural proteins by culturing transformed plant cell, and to a vaccine composition comprising the same as an effective component. More specifically, the method comprises the steps of preparing the expression vector comprising cDNA fragment encoding rotavirus structural proteins; transforming plant cell with the expression vector; and recovering the vaccine composition including the rotavirus antigen from cell culture. The method for producing the recombinant rotavirus structural proteins has advantages in higher yield of more than 0.3 mg/L, lower cost and lower contamination hazard than those of the eukaryotic expression system such as baculovirus and animal cell, and can be used for producing edible vaccine. Also, the invention shows that the rotavirus structural protein can be produced with the high aspect rotary vessel reactor.
(S4) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). — before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments — with (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description

Published:
— with international search report

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PRODUCTION METHOD OF RECOMBINANT ROTAVIRUS STRUCTURAL PROTEINS AND VACCINE COMPOSITION

FIELD OF THE INVENTION

The present invention relates to a method for producing the structural proteins of the recombinant rotavirus by culturing a transformed plant cell, and the vaccine composition comprising such proteins as an effective component. More particularly, the method comprises the steps of producing the expression vector including the gene encoding the rotavirus structural protein, transforming the plant cell with the expression vector, cultivating the plant cell, and obtaining the vaccine composition including rotavirus antigen recovered from the culture solution.

BACKGROUND OF THE INVENTION

In 1973, Bishop discovered the Rotavirus in Australia firstly. The rotavirus is a double stranded RNA virus and belongs to the Reoviridae family. The rotavirus causes acute gastroenteritis in infant and is infected via fecal-oral route after an incubation period of about 1 to 3 weeks. The disease is severe in 6 to 24 week infant, but is mild or asymptomatic in neonate or most of adults. Thus, the acute infectious diarrhea by the rotavirus is a main cause of the death in the world. Moreover, it is estimated that about a million of patients are died of the infectious diarrhea by the rotavirus in the developing countries [see reference: Blacklow, N.R. & Greenburg, H.B., (1991) Viral gastroenteritis N. Engl. J. Med., 325:152-164, 1991]. Therefore, world health organization (WHO) considered more efficient suppression and prevention for the infection by the rotavirus the

The rotavirus is usually globular shape and is named after the outer and inner shells or double-shelled capsid structure of the same. The outer capsid is about 70 nm, and inner capsid is about 55 nm in diameter, respectively. The double-shelled capsid of the rotavirus surrounds the core including the inner protein shell and genome. The genome of the rotavirus consists of double stranded RNA segments encoding at least 11 rotavirus proteins. The inner capsid includes VP6 and VP2 proteins. VP4 and VP7 lie in outer side of the double-shelled capsid and constitute the outer capsid. Depending upon the antigenicity of VP6 which is a group-specific antigen, the rotavirus is divided into seven groups, A to G. VP2 protein is related to the synthesis of RNA. Group A rotavirus is further divided into the G-type (glycoprotein type) on the basis of the glycoprotein VP7, and P-type (protease-cleaved protein) on the basis of the VP4 which are associated with an important immunogenicity of the virus by forming a neutralizing antibody [see references: Estes M. K., et al., (1987) Synthesis and immunogenicity of the rotavirus major capsid antigen using a baculovirus expression system, J. Virol. 61:1488-1494; Estes M. K & Cohen J., (1989) Rotavirus gene structure and function. Microbiol. Rev. 53:410-419; Desselberger U & McCrae M. A., (1994) The rotavirus genome. Curr. Microbiol. Immuno. 185:31-66].

The methods to treat the acute diarrhea disease caused by the rotavirus were restricted to administer non-specific physical strength supplements such as water and electrolyte. Thus, the vaccine as an effective therapeutic agent has been required for completely preventing the human from the all serum-type of human rotavirus. As an attempt, a live vaccine of attenuated human rotavirus, animal rotavirus such as cow, or
reassortants comprised of RNA segments derived from different serotypes human and animal rotavirus have been used. As a result of such study, Wyeth Laboratories, a manufacturing company of vaccines, reported first rotavirus vaccine, RotaShield (trademark). The vaccine was produced by reassorting rotavirus of Rhesus monkey and human rotavirus of 3 serum types and was firstly approved by Food and Drug Administration (FDA) in the world in 1998. However, the FDA's approval to the vaccine was tentatively canceled in 1999, because of the side effects such as intussusception [MMWR Morb. Mortal Wkly. Rep., (1999) 5;48(43);1007].


However, it is difficult to produce the rotavirus on a large scale by cell culture, because the rotavirus is infected through the mucus cell. That is, because it is difficult to produce the virus protein particles similar to natural virus in prokaryotic expression systems such as E.coli expression system, the particle does not elicit antigenecity. In view of contamination hazard during culture process, purification problem, high cost and low yield, the mammalian expression system is not satisfactory.
Also, most of disease caused by rotavirus is commonly occurred in developing countries which are deficient in sanitary facilities and vaccine supplement. Even if technical success of the method for producing the virus structural proteins in other cells and the vaccine composition comprising the proteins is possible, long time and many studies are required for commercializing the method in view of high production cost and sale price. On the other hand, if the rotavirus structural proteins can be produced by using plant, especially edible plant and can be used for producing the vaccine, it is very economical in view of low production cost, no purification process, and efficiency in transport and storage.

Therefore, the present inventors found that rotavirus structural proteins capable of inducing the mucus and systemic immune response can be produced in the plant cell which is transformed with plant expression vector including rotavirus capsid gene, and then the plant cell is cultured under the suitable condition. Also, the vaccine composition comprising the rotavirus structural proteins can be obtained.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1a - 1d are cleavage maps showing the structure of binary vector used for transforming a plant cell, pILTAB357-VP6, pILTAB357-VP2, pILTAB357-VP4 and pILTAB357-VP7,

CsVMV promoter : Cassava vein mosaic virus promoter
NOS promoter : nopaline synthase promoter
NOS 3' : nopaline synthase transcription terminator region
NPT II : neomycin phosphotransferase II gene

Figs. 2a - 2d are the result of western blotting of normal and transformed tomato
cell. The arrows show recombinant rotavirus proteins, VP6, VP2, VP4 and VP7 respectively.

M: marker of molecular weight,

1: intracellular fraction of the normal cell,

2: medium fraction of the normal cell,

3: intracellular fraction of the transformed tomato cell,

4: medium fraction of the transformed tomato cell,

Fig. 3a is a graph showing cell concentration of transformed cell, and temporal change of the expression of recombinant rotavirus protein VP6.

Fig. 3b is a result showing the expression of recombinant rotavirus protein, VP6 by western blotting of the culture solution obtained on 12, 15, 18, and 21 after culture in the first and second work, respectively. Here, "C" in fifth lane shows VP6 fusion protein (0.2 $\mu$g) purified from E.coli.

Fig. 4a shows the transformed cell concentration in shaking flask culture, and a graph presenting the temporal change of the expression of the recombinant rotavirus protein, VP7 serotype G1.

Fig. 4b is a result showing the expression of recombinant rotavirus protein, VP7 having serotype G1 obtained by western blotting of the culture obtained on 2, 3, 4, 5, 6, and 7 after incubation in the first and second work, respectively.

Other features and advantages of the invention will be apparent from the following detailed description, and from the examples.
DETAILED DESCRIPTION OF THE INVENTION

The present invention provides the structural proteins of the recombinant rotavirus obtained by culturing transformed plant cells, and the vaccine composition comprising such proteins as an effective component.

The method for producing recombinant rotavirus structural proteins by using plant cell comprises the steps of:

1) preparing the expression plasmid comprising cDNA fragment encoding rotavirus structural proteins operably linked to the promoter regulating plant-specific expression, and further selection marker;

2) introducing the expression plasmid into plant cell;

3) inducing formation of callus from the transformed plant cells;

4) culturing the callus; and

5) recovering the recombinant rotavirus structural proteins expressed from the cDNA fragment in the culture solution.

The wide range of sources (e.g., human rotavirus and animal rotavirus such as cow) are available for the gene encoding rotavirus structural protein of step 1, as long as the gene encodes the rotavirus capsid protein. And, it is preferable to use the gene encoding inner capsid protein VP6, the gene encoding inner capsid protein VP2, the gene encoding outer capsid protein VP4, and the gene encoding outer capsid protein VP7. The rotavirus gene of the interest can be obtained by amplifying the gene from the feces of the subject having rotavirus-related disease with the known polymerase chain reaction (PCR). In a desired embodiment of the present invention, VP6 gene fragment can be available by excising the gene from the plasmid pGEM-VP6 (KCTC 8984P). In another embodiment of the invention, cDNA fragment encoding VP2, VP4, and VP7 serotype G1, VP7 serotype
G2, VP7 serotype G3 and VP7 serotype G4 can be used by isolating and amplifying the cDNA fragment from the faces of the subject having rotavirus-related disease.

Also, recombinant plasmid expressing the cDNA fragment encoding the rotavirus structural protein of the interest of step 1 can be constructed from the known plant expression vector as a basic vector. The binary vector, cointegration vector, or a general vector which is designed not to include T-DNA region but to be capable of being expressed in plant can be also available.

In the present invention, the examples of the desired binary vector include final binary vectors, for example, pILTAB357-VP6, pILTAB357-VP2, pILTAB357-VP4, pILTAB357-VP7, which are prepared by inserting the cDNA fragment encoding each rotavirus structural proteins, VP6, VP2, VP4 or VP7 into binary vectors comprising left border of T-DNA relating to infection of a foreign gene and right border of T-DNA for transforming a plant cell, cassava vein mosaic virus promoter between the left border and the right border, nopaline synthase promoter, transcription termination region of nopaline synthase, and selection marker for selecting transformants.

As the promoter regulating the plant-specific expression, all the known expression vectors maximally expressing the recombinant proteins in plant cell can be used, and the promoter includes ubiquitin promoter, cauliflower mosaic virus 35S promoter (CaMV 35S promoter), actin promoter, PG promoter or endosperm-specific promoter other than the cassava vein mosaic virus promoter.

In the transformation of the plant cell in step 2, when the binary vector or the cointegration vector is used, the plant cell transformation method mediated by agrobacterium will be employed. In such case, Agrobacterium tumefaciens or Agrobacterium rhizogenes can be used.
In the desired embodiment of the present invention, the final binary vector can be introduced into *Agrobacterium tumefaciens* via the known triparental mating. The triparental mating is a method of introducing the plasmid having the gene of interest to the agrobacterium by coculturing *E.coli* including helper plasmid for conjugation, *E.coli* including the recombinant plasmid having the gene of interest, and Agrobacterium for transforming the plant cell all together. More specifically, the parent *E.coli* used for triparent mating can be *E.coli* having the pILTAB357-VP6, and tomato seedling cotyledon can be transformed with the agrobacterium transformed by the triparental mating.

In case of transforming the monocotyledon plant with the vector which does not include T-DNA region, polyethylene glycol-mediated uptake, electroporation, or microparticle bombardment can be used and transformation using single DNA and simultaneous transformation using multiple DNA also can be used.

The transformed cell can be redifferentiated by using the standard technologies known to those skilled in the art. The plant cell which may be transformed by the above method includes dicotyledon plant such as lettuce, Chinese cabbage, radish, potato, and tomato, etc. and monocotyledon plant such as rice, barley, and banana, etc. Especially, in case of producing the rotavirus structural protein and the particle similar to the same by transforming edible plant, uptake of the transformed plant itself can induce directly the immune response, so it can be effectively used as a edible vaccine.

The selection marker to select the transformed plant cell is usually antibiotic resistant gene, but is not limited to the same. For examples, herbicide resistant gene, metabolism-related gene, luminous gene, green fluorescence protein (GFP), β-glucuronidase (GUS) gene, β-galactosidase (GAL) gene can be used as selection marker. Specifically, neomycin phosphotransferase II (NPTII) gene, hygromycin
phosphotransferase gene, phosphonomycin acetyltransferase gene, or dihydrofolate reductase gene can be used.

Meanwhile, the transformed plant cell of the present invention can be cultured by employing microgravity culture in High Aspect Rotating-Wall Vessel as well as the suspension culture. When the High Aspect Rotating-Wall Vessel is used, the cell growth rate is very slow compared to the general suspension culture method, because the lag phase is longer in order to fit the circumstance of the mimic microgravity. However, it is found that rotavirus protein yield per cell weight in the rotary reactor is similar to that in the suspension culture.

Also, the present invention provides rotavirus-like particle which can be made by folding the rotavirus structural proteins of the invention, VP2, VP6, VP4 and VP7. Herein, ‘rotavirus-like particle’ is intended to mean the antigenic particle obtained from an assembly of at least a capsid protein, or self-assembly of the same.

Furthermore, the present invention provides a vaccine composition useful for treatment and prevention from rotavirus-related disease comprising the rotavirus structural protein of the present invention as an effective component. More specifically, the vaccine composition comprises at least one selected form the group consisting of VP4, G1 serotype VP7, G2 serotype VP7, G3 serotype VP7, and G4 serotype VP7, or preferably comprises VP2 and VP6.

To prepare for the vaccine composition, the rotavirus structural proteins produced by the transformed plant cell can be isolated and purified from the cell culture by using the known purification method, but in case of transforming by using edible plant cell, it is more preferable to prepare the rotavirus structural proteins using plant cell itself, the part of redifferentiated transformed plant or the extract of plant without purification process in
view of the production cost and process management.

The vaccine useful for treatment and prevention for rotavirus-related disease can be administered orally or parenterally, and can be used in the usual form of drug.

Namely, the rotavirus vaccine composition can be prepared by containing optionally a pharmaceutically acceptable diluent or excipient such as filler, extender, binding agent, wetting agent, integrant, and surfactant. The solid preparation for oral administration includes tablet, pill, powder, granule, capsule, and can be prepared by mixing at least one of the excipient such as starch, calcium carbonate, sucrose, lactose, and gelatin with rotavirus vaccine composition. Also, lubricant such as magnesium stearate, talc as well as simple excipients can be used. The liquid preparation for oral administration can contain suspension, liquid drug, emulsion, and syrup, and include many excipient (e.g., wetting agent, sweetening agent, fragrant, preserving agent) other than commonly used simple diluents such as water, liquid and paraffin. The preparation for parenteral administration contains sterilized solution, non-aqueous solvent, suspension solvent, emulsifier, freeze-drying agent, or suppository. The non-aqueous solvent or suspension solvent can include propylene glycol, polyethylene glycol, vegetable oil such as olive oil, injectable ester such as ethyloleate. The basic material of suppository includes witepsol, macrogol, tween 61, cacao paper, laurin paper, glycerogelatin.

However, because most of rotavirus vaccine are administered to infant, especially 2 to 24 month-old infant, it is preferable to be administered as soon as possible before firstly exposed to the rotavirus, and the dosage form of the vaccine is more preferably oral preparation in liquid, injectable preparation, or suppository in order to easily administer to the infant.

As described below, the present invention will be more specifically illustrated by
examples of the expression system that cDNA encoding the recombinant rotavirus structural proteins is expressed in the transformed tomato cell in culture systems, and the expression system that the High Aspect Rotating-Wall Vessel designed by NASA is employed for optimally culturing the recombinant rotavirus structural proteins. However, modification or change of the method can be used, and thus the method does not limited to the following definite working examples.

**Preparation of vector including cDNA fragment encoding rotavirus structural proteins**

The gene cloning technology used herein can be easily performed by employing the method known to those skilled in the art. Such methods are well described in the following references such as Sambrook et al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y.; and Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associate and Wiley Interscience, N.Y..

A kind of binary vector used for transforming dicotyledon plant, especially tomato, is very various, and most of binary vectors can be available in international depository authority, for example, Center for the Application of Molecular Biology to International Agriculture (CAMBIA, GPO Box 3200, Canberra ACT2601, Australia). Basic binary vector which is variously modified to include selection marker, promoter, transcription terminator region in left border and right border region of transferred gene is used.

In the examples of the present invention, pGEM-VP6 (KCTC 0944BP) including cDNA encoding human rotavirus VP6 protein was used in order to constitute the binary vector. Also, the plasmids including cDNA fragment encoding human rotavirus VP2 of SEQ ID NO:1, cDNA fragment encoding human rotavirus VP4 of SEQ ID NO:2, cDNA
fragment encoding human rotavirus VP6 of SEQ ID NO:3, and cDNA fragment encoding human rotavirus VP7 of SEQ ID NO:4 to SEQ ID NO:7 having different serotype, respectively, were used. However, the vector suitable for the present invention is not limited to the above plasmids, and all range of the vectors which efficiently express the genes can be used. In the examples of the present invention, the plasmid transforming plant cell was constructed by inserting cDNA fragment to the cloning site of known plasmid, pILTAB357 (Scripps Research Institute, U.S.A.; Verdaguer et al, Isolation and expression in transgenic tobacco and rice plants, of the cassava vein mosaic virus promoter, Plant Molecular Biology 31: 1129-1139, 1996), using CsVMV promoter in T-DNA region of pILTAB357 and inserting neomycin phosphotransferase II (NPTII) gene as a selection marker (see Figs. 1a - 1d).

**Transformation procedure**

Transformation can be performed according to the method known to those skilled in the art which the present invention pertains to. The plant can be transformed via agrobacterium-mediated transformation which is illustrated in Paszkowsky et al., EMBO J 3:2717-2722 (1984). For example, agrobacterium-mediated transformation for the tomato is described in An et al., EMBO J 4:227-288 (1985). The transformation of monocotyledon plant can be achieved by directly implanting the gene into plasma via PEG or electroporation, or by introducing the gene into the callus tissue via particle bombardment. The transformed cell can be redifferentiated to the whole plant via the standard method known to those skilled in the art. The host cell for transformation can be usually Agrobacterium tumefaciens, and Agrobacterium tumefaciens strain LBA4404 which is well-known in the art was used in the desired embodiment of the present invention.
The binary vector of the present invention was introduced to Agrobacterium tumefaciens via trip parental mating. In such case, for example, E.coli MM294 including pILTAB357-VP6 which has the inserted cDNA fragment encoding human rotavirus VP6 protein was used as parent strain. Then, cotyledon excised from tomato (Lycopersicon esclentum Mill) seedling was transformed with recombinant Agrobacterium tumefaciens LBA 4404.

**Transformant culture procedure**

To culture the transformed tomato cells, suspension culture and High Aspect Rotating-Wall Vessel were used. To do this, sterilized tomato (Lycopersicon esclentum Mill) seed was inoculated on MS (Murashige and Skoog) medium, and was germinated, and then co-cultured with suspension culture solution containing Agrobacterium tumefaciens LBA 4404 including recombinant expression plasmid, and cultured successively. Also, to determine the culture system that can optimally produce recombinant rotavirus structural proteins, VP6, VP2, VP4 and VP7, the transformed tomato cell was cultured in a microgravity model system with High Aspect Rotating-Wall vessel designed by NASA.

**The expression of the recombinant rotavirus structural proteins**

The transformed tomato cell was established from the selected kanamycin-resistant callus, and was maintained in liquid medium added by kanamycin 200 mg/L for 3 months. The degree of gene expression was analyzed using the transformed tomato cell on two weeks after culture and normal tomato cell. As shown in Figs. 2a-2d, the recombinant rotavirus structural proteins, VP6, VP2, VP4 and VP7 could be detected in the transformed
tomato cell by western blotting.

For example, recombinant rotavirus protein VP6 is largely in intracellular fraction, and has molecular weight of about 44 kDa. VP2 has molecular weight of 94 kDa, and VP4 has a molecular weight of 87 kDa and VP7 has a molecular weight of 34 kDa. Such measured molecular weight of the proteins is almost consistent with those derived from the nucleotide sequencing. Recombinant rotavirus proteins, VP6, VP2, VP4 and VP7 were not detected in the non-transformed cell or fraction (Fig. 2a - 2d, see lane 1-2 in each figure). The fact means that VP6, VP2, VP4 and VP7 were expressed from pILTAB357-VP6, pILTAB357-VP2, pILTAB357-VP4 and pILTAB357-VP7 transformed in the tomato cell.

**Expression level change over time**

Fig. 3a shows the change of cell concentration and the expression of recombinant rotavirus protein VP6 with time while culturing the transformed tomato cell in shaking flask. When 40 g/L of cell was inoculated, maximum cell concentration was 192 g/L at live cell weight on 15 days after culture. As a result of western blotting analysis with fusion VP6 protein produced in *E.coli* having a molecular weight of 45.5 kDa as control, the production amount of recombinant rotavirus protein VP6 was 0.14, 0.21 and 0.19 mg/L in live cell weight on 15, 18 and 21 days after culture, respectively, when inoculating live cell of 14 g/L. When inoculating live cell of 40 g/L, the production amount of recombinant rotavirus protein VP6 was 0.29, 0.33, and 0.28 mg/L in live cell weight on 15, 18, and 21 days after culture, respectively. Maximum production amount of recombinant rotavirus proteins VP6 was 0.33 mg/L in live cell weight on 18 day after culture. It is found that the production amount of the recombinant protein produced from the transformed tomato cell according to the present invention, 0.33 mg/L, is higher than that of heavy chain of
recombinant monoclonal antibody produced from the transformed tobacco cell without adding polyvinylpyrrolidone according to LaCount' report, 0.19mg/L. [see LaCount W. et al., (1997) The effect of polyvinylpyrrolidone on the heavy chain monoclonal antibody production from plant suspension culture. Biotechnol. Lett. 19:93-96]

The invention will be further described in the following examples, which are not intended to limit the scope of the invention.

**EXAMPLE 1**

**Construction of binary vector for transformation**

The fragment produced by digesting pGEM-VP6 (KCTC 0944BP) including gene encoding human rotavirus VP6 protein with restriction enzyme EcoRI was inserted into the EcoRI restriction site of plasmid pILTAB357 (Scripps Research Institute, U.S.A.) to prepare recombinant plasmid pILTAB357-VP6 of 13.94 kb. Fig. 1a shows cleavage map of the pILTAB357-VP6. In the pILTAB357-VP6, the CsVMV promoter inserted between the left border and the right border of binary vector for transforming plant cell, pILTAB357, was used as a promoter for regulating the transcription of VP6 gene [Verdaguer B., et al., (1996) Isolation and expression in transgenic tobacco and rice plants, of the cassava vein mosaic virus (CsVMV) promoter. Plant Mol. Biol. 31:1129-1139], and neomycin phosphotransferase II (NPTII) was inserted as a marker for selecting the transformant in the growth medium containing kanamycin (Fig. 1a). By using restriction map and DNA sequencing, the location of VP6 gene in recombinant plasmid, pILTAB357-VP6, and whether reading frame of the VP6 was correctly inserted were identified. The prepared binary vector was introduced to *Agrobacterium tumefaciens* LBA 4404 (Cat. No. 18313-015, GibcoBRL, U.S.A.) via triparental mating [Ainsworth C. et al., (1996) *Techniques in*
Plant Molecular Biology, Wye, UK: Wye College]. In the trip parental mating, E.coli MM294 (KCCM 70079) including pLTAB357-VP6 was used as parent strain. Cotyledon cut from tomato (Lycopersicon esculentum Mill) was transformed with the recombinant Agrobacterium tumefaciens LBA 4404 according to agrobacterium-mediated leaf disc transformation [Horsch R.B. et al., Science 227: 1229-1231, 1985].

EXAMPLE 2

Transformation procedure

and establishment of suspension culture for the transformed cell

Tomato (Lycopersicon esculentum Mill) seed was immersed in 70% ethanol solution for 1 minute, sterilized with 1.2% sodium hypochloride (NaOCl) solution for 30 minutes, and then, was washed with sterilized distilled water many times. The sterilized seed was inoculated on MS medium [sucrose 30 g/l, 2,4-D (2,4-dichlorophenoxyacetic acid) 2 mg/l, kinetin 0.2 mg/l, timentin 150 mg/l, pH 5.7] containing agar 8 g/L. The seed applied on Magenta box was germinated in culture room in light cycle of 12 hours at 27°C. After about two weeks, germinated sterilized young seedling was used for transformation experiment. Cotyledon excised from the seedling was immersed in suspension culture of recombinant agrobacterium tumefaciens LBA 4404 including pLTAB357-VP6 for 3 minutes, and then extra microorganism was removed with filter paper. Then, explants were cultured with agrobacterium tumefaciens LBA 4404 on solid MS medium containing agar 8g for 3 days. The resultant explants were transferred onto MS medium containing kanamycin B 200 mg/L and timentin 150 mg/L. The explants were incubated in culture room at 27°C in light cycle of 12 hours for 4 weeks in order to induce callus. The induced callus was cut and then was maintained and amplified by passage culture at 4 weeks.
interval. The callus resistant to kanamycin was selected and transferred onto the liquid medium containing kanamycin 200 mg/L but not agar, and then, was cultured in suspension.

The transformed plant cell was cultured in suspension medium containing kanamycin B 200 mg/L in rotary shaking flask at 27°C, 100 rpm, and was maintained and amplified by passage culture at 3 weeks interval. The callus of normal tomato cell was induced as control according to all the process described above, except the process of transformation of agrobacterium, and cultured in suspension under the same conditions.

EXAMPLE 3

Suspension culture and gene expression analysis

1) suspension culture of the transformed plant cell and isolation of VP6

The transformed tomato cell was cultured at 27°C, 100 rpm in shaking flask containing MS liquid media 50 mg/L including kanamycin 200 mg/L. During the culture time, the cultured cell was collected and analyzed for the cell growth and expression of the recombinant protein at a regular interval.

The culture was centrifuged at 3,000 rpm for 5 minutes to separate the cell, and supernatant was used for analyzing extracellular recombinant protein. Cell fraction was freezed with liquid nitrogen, and then was grinded with mortar and pestle with adding protein extraction solution [Tris-HCl (pH 7.5) 50 mM, ethylenediaminetetraacetic acid (EDTA) 2 mM, ethylenebis (oxyethylenenitrilo) tetraacetic acid (EGTA) 0.5 mM, phenylmethylsulfonyl fluoride (PMSF) 1 mM, 1% Triton X-100]. Then the resultant was sonicated in the centrifugation tube at 4°C for 5 minutes 3 times. The cell extract was
centrifuged at 14,000 rpm at 4°C, for 15 minutes to remove cell debris, and supernatant was used for analyzing the intracellular recombinant protein. Unless especially defined, the mixture of intracellular fraction and extracellular fraction was used for analysis of protein production in total.

2) Western Blotting Analysis

The production amount of the recombinant rotavirus VP6 protein was measured by western blotting by using fusion VP6 protein of 44.5 kDa expressed in E.coli as control. The fusion VP6 protein has a molecular weight of 44.5 kDa. The fusion VP6 protein was expressed in E.coli using pET-15b (Novagen, U.S.A.) designed to express VP6 protein in E.coli and used by purifying with His-Bind kit (Novagen, U.S.A.). Polyhistidine tag sequence linked to cleavage site of thrombin is fused in N-terminal of fusion VP6 protein.

According to Laemmli’s method, SDS-PAGE electrophoresis was performed on the protein sample [Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685]. After electrophoresis, the resultant proteins were transferred onto nitrocellulose membrane and then, were combined with anti-rotavirus polyclonal antibody (Chung-Ang University, Seoul, Korea) obtained from Guinea pig, and were detected by rabbit anti-Guinea pig immunoglobulin G (Kirkegaard & Perry Laboratories, U. S. A.). After washing nitrocellulose membrane with buffer solution, the resultant was colored with BM purple AP substrate solution (Boehringer Mannheim, U. S. A.) and quenched by adding distilled water.

As shown in Fig. 2a, recombinant rotavirus protein VP6 was largely in intracellular fraction of the transformed plant and had molecular weight of about 44.5 kDa, which is consistent with that of VP6 expressed in E.coli. This shows that the transformed
plant cell of the present invention expressed successfully rotavirus protein VP6.

3) Analysis for expression amount of VP6 protein in suspension culture

Figs. 3a and 3b show the cell concentration of the transformed plant cell and the temporal change of the expression of recombinant rotavirus VP6 in suspension culture.

As shown by Figs. 3a and 3b, when inoculating live cell 40 g/L (first work: O), maximum cell concentration was 192 g/L at live cell weight on 15 days after culture. As a result of western blotting analysis of recombinant rotavirus proteins VP6 by using fusion VP6 proteins produced in E. coli having a molecular weight of 45.5 kDa as control, when inoculating 14 g/L of live cell (second work: ●), the production amount of recombinant rotavirus proteins VP6 was 0.14, 0.21 and 0.19 mg/L in live cell weight on 15, 18 and 21 days after culture, respectively. Maximum production amount of recombinant rotavirus proteins VP6 was 0.33 mg/L at live cell weight on 18 day after culture and this amount corresponds to 0.0018 mg/g, when converted into specific-production on the basis of cell weight. The figure is higher than the production amount of recombinant protein in plant cell so far, for example, that of heavy chain of recombinant monoclonal antibody produced from the transformed tobacco cell reported by LaCount et al (0.19 mg/L).

EXAMPLE 4

Microgravity culture

To test microgravity, culture solution of the transformed tomato cell was filled up in High Aspect Rotating-Wall vessel (HARV, Systhecon, Houston, TX) with the capacity of 10 ml to produce recombinant rotavirus protein VP6 and was cultured for 18 days. The
HARV was rotated with 10 rpm at 27°C. The result was showed in the following Table 1.

Table 1: The cell growth and recombinant protein production in HARV

<table>
<thead>
<tr>
<th></th>
<th>Cell growth</th>
<th>Recombinant VP6 production*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial cell density (g FW/L)</td>
<td>Final cell density (g FW/L)</td>
</tr>
<tr>
<td>Work 1</td>
<td>26</td>
<td>73</td>
</tr>
<tr>
<td>Work 2</td>
<td>36</td>
<td>76</td>
</tr>
</tbody>
</table>

*: The production of recombinant rotavirus protein VP6 was measured with western blotting by using purified VP6 fusion protein as a control

The cell growth of transformed cell in HARV was increased to 73 g/L and 76 g/L at live cell weight. As compared to the result of shaking flask culture shown in Figs. 3a and 3b, cell growth rate is slower in HARV than in the shaking flask. It is believed that the cell has longer lag phase to fit the circumstance of the simulated microgravity. The production amount of recombinant rotavirus protein VP6 in HARV was 0.13 mg/L and 0.15 mg/L, respectively. However, specific-production amount of VP 6 on the basis of cell was similar to that of shaking flask.

Although the further studies are required for well understanding of the cellular and molecular mechanism regarding plant cell culture in HARV, the present inventors found from the result that the circumstance of the HARV could maintain the plant cell growth and recombinant protein production.

**EXAMPLE 5**

Except that recombinant plasmid, pILTAB357-VP2 of 15.0 kb prepared by using pGEM-VP2 (KCTC 0947BP) including cDNA fragment of SEQ ID NO:1 encoding human rotavirus protein VP2, was used, instead of pGEM-VP6 (KCTC 0944BP) including cDNA
fragment encoding human rotavirus protein VP6, cotyledon excised from tomato seedling was transformed with *Agrobacterium tumefaciens* to which introduced binary vector pILTAB357-VP2 according to the same method of Examples 1-3. Then, transformed tomato cell was cultured in suspension. The recombinant rotavirus protein VP2 was isolated, and was analyzed with western blotting in order to identify that the transformed cell expresses rotavirus protein VP2 successfully (Fig. 2b). Maximum production amount of recombinant protein VP2 was 0.2 mg on 18 days after culture.

pGEM-VP2 including cDNA fragment encoding VP2 derived from human rotavirus was deposited on Feb. 8, 2001 to Korea Collection for Type Culture (KCTC) under Budapest treaty on the international recognition of the deposit of microorganism, and was acceded KCTC 0947BP as a deposit number. The cleavage map of recombinant plasmid, pILTAB357-VP2 was shown in Fig. 1b.

**EXAMPLE 6**

Except that recombinant plasmid, pILTAB357-VP4 of 14.8 kb prepared by using pGEM-VP4 (KCTC 0945BP) including cDNA fragment of SEQ ID NO:2 encoding human rotavirus protein VP4, was used, instead of pGEM-VP6 (KCTC 0944BP) including cDNA fragment encoding human rotavirus protein VP6, cotyledon excised from tomato seedling was transformed with *Agrobacterium tumefaciens* to which introduced binary vector pILTAB357-VP4 according to the same method of Examples 1-3. Then, transformed tomato cell was cultured in suspension. The recombinant rotavirus protein VP4 was isolated, and was analyzed with western blotting in order to identify that the transformed cell expresses rotavirus protein VP4 successfully (Fig. 2c). Maximum production amount of recombinant protein VP4 was 0.2 mg on 18 days after culture, as presented in example 5.
pGEM-VP4 including cDNA fragment encoding VP4 derived from human rotavirus was deposited on Feb. 8, 2001 to Korea Collection for Type Culture (KCTC) under Budapest treaty on the international recognition of the deposit of microorganism, and was acceded KCTC 0945BP as a deposit number. The cleavage map of recombinant plasmid, pILTAB357-VP4 was shown in Fig. 1c.

**EXAMPLE 7**

Except that recombinant plasmid, pILTAB357-VP7 of 13.7 kb prepared by using pGEM-VP7 (KCTC 0946BP) including cDNA fragment of SEQ ID NO:4 encoding human rotavirus protein VP7 serotype G1, was used, instead of pGEM-VP6 (KCTC 0944BP) including cDNA fragment encoding human rotavirus protein VP6, cotyledon excised from tomato seedling was transformed with *Agrobacterium tumefaciens* to which introduced binary vector pILTAB357-VP7 according to the same method of Examples 1-3. Then, transformed tomato cell was cultured in suspension. The recombinant rotavirus protein VP7 was isolated, and was analyzed with western blotting in order to identify that the transformed cell expresses rotavirus protein VP7 serotype G1 successfully (Fig. 2d). Maximum production amount of recombinant protein VP7 serotype G1 was 0.2 mg on 18 days after culture, as presented in example 5.

pGEM-VP7 including cDNA fragment encoding VP7 having serotype G1 derived from human rotavirus was deposited on Feb. 8, 2001 to Korea Collection for Type Culture (KCTC) under Budapest treaty on the international recognition of the deposit of microorganism, and was acceded KCTC 0946BP as a deposit number. The cleavage map of recombinant plasmid, pILTAB357-VP7 was shown in Fig. 1d.
EXAMPLE 8

The pCR2.1-VP7 (KCTC 0948BP) including cDNA fragment of SEQ ID NO:5 encoding human rotavirus protein VP7 serotype G2 was used, instead of pGEM-VP7(KCTC 0946BP) including cDNA fragment encoding human rotavirus protein VP7 serotype G1 as described in Example 7. It was confirmed that the transformed cell expressed rotavirus protein VP7 serotype G2 successfully by working according to the same method as example 7.

The pCR2.1-VP7 including cDNA fragment encoding VP7 serotype G2 derived from human rotavirus was deposited on Feb. 8, 2001 to Korea Collection for Type Culture (KCTC) under Budapest treaty on the international recognition of the deposit of microorganism, and was acceded KCTC 0948BP as a deposit Number.

EXAMPLE 9

The pCR2.1-VP7 (KCTC 0949BP) including cDNA fragment of SEQ ID NO:6 encoding human rotavirus protein VP7 serotype G3 was used, instead of pGEM-VP7 (KCTC 0946BP) including cDNA fragment encoding human rotavirus protein VP7 having G1 serotype as described in Example 7. It was confirmed that the transformed cell expressed rotavirus protein VP7 serotype G3 successfully by working according to the same method as example 7.

The pCR2.1-VP7 including cDNA fragment encoding VP7 serotype G3 derived from human rotavirus was deposited on Feb. 8, 2001 to Korea Collection for Type Culture (KCTC) under Budapest treaty on the international recognition of the deposit of microorganism, and was acceded KCTC 0949BP as a deposit Number.
EXAMPLE 10

The pCR2.1-VP7 (KCTC 0950BP) including cDNA fragment of SEQ ID NO:7 encoding human rotavirus protein VP7 serotype G4 was used, instead of pGEM-VP7 (KCTC 0946BP) including cDNA fragment encoding human rotavirus protein VP7 having G1 serotype as described in Example 7. It was confirmed that the transformed cell expressed rotavirus protein VP7 serotype G4 successfully by working according to the same method as example 7.

The pCR2.1-VP7 including cDNA fragment encoding VP7 serotype G4 derived from human rotavirus was deposited on Feb. 8, 2001 to Korea Collection for Type Culture (KCTC) under Budapest treaty on the international recognition of the deposit of microorganism, and was acceded KCTC 0950BP as a deposit Number.

INDUSTRIAL APPLICABILITY

According to the genetic recombination technology of the present invention, the method for producing rotavirus structural proteins in plant cell on a large scale and vaccine composition comprising the proteins as an effective component has advantages in low production cost and no need to purification. Also, when the protein is produced from edible plant, it is possible to use the protein as edible vaccine or oral vaccine by preparing vaccine comprising the isolated virus structural proteins and pharmaceutically acceptable additive, or by taking the plant itself directly. In particular, when it is important to induce mucus immune response such as rotavirus, the oral vaccine contributes desired mucous and systemic immune response. Thus, the protein can be used for vaccine composition useful for treatment and prevention from rotavirus-related disease. In addition, the method of the present invention is easy and efficient in aspect of transport and storages compared to other
culture system.
INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13bis)

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<td>Address of depositary institution (including postal code and country)</td>
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INDICATIONS RELATING TO DEPOSITED MICROORGANISM
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B. IDENTIFICATION OF DEPOSIT

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Korea Research Institute of Bioscience and Biotechnology (KRIBB)
#52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea

Date of deposit
2001, 2, 8.

Accession Number
KCTC 0948BP

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#52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea

Date of deposit: 2001. 2. 8.  
Accession Number: KCTC 0949BP

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What is claimed is:

1. A method for producing antigenic recombinant rotavirus structural proteins in plant cell comprising the steps of:
   1) preparing the expression plasmid comprising cDNA fragment encoding rotavirus structural proteins operably linked to the promoter regulating plant-specific expression, and further selection marker;
   2) introducing the expression plasmid of step 1 into plant cell;
   3) inducing formation of callus from the transformed plant cells of step 2;
   4) culturing the callus of step 3; and
   5) recovering the recombinant rotavirus structural proteins expressed from the cDNA fragment in the culture solution of step 4.

2. The method according to claim 1, wherein the rotavirus of step 1 is a rotavirus derived from human or a rotavirus derived from animals such as cow, Rhesus monkey, pig, dog, cat, birds, and mice.

3. The method according to claim 1, wherein the rotavirus structural proteins of step 1 are VP2, VP4, VP6 or VP7.

4. The method according to claim 1, wherein the cDNA fragment encoding rotavirus structural proteins has the sequence represented by SEQ ID NO: 1 encoding VP2 derived from human rotavirus.

5. The method according to claim 1, wherein the cDNA fragment encoding rotavirus
structural proteins has the sequence represented by SEQ ID NO: 2 encoding VP4 derived from human rotavirus.

6. The method according to claim 1, wherein the cDNA fragment encoding rotavirus structural proteins has the sequence represented by SEQ ID NO: 3 encoding VP6 derived from human rotavirus.

7. The method according to claim 1, wherein the cDNA fragment encoding rotavirus structural proteins has the sequence represented by SEQ ID NO: 4 encoding VP7 serotype G1 derived from human rotavirus.

8. The method according to claim 1, wherein the cDNA fragment encoding rotavirus structural proteins has the sequence represented by SEQ ID NO: 5 encoding VP7 serotype G2 derived from human rotavirus.

9. The method according to claim 1, wherein the cDNA fragment encoding rotavirus structural proteins has the sequence represented by SEQ ID NO: 6 encoding VP7 serotype G3 derived from human rotavirus.

10. The method according to claim 1, wherein the cDNA fragment encoding rotavirus structural proteins has the sequence represented by SEQ ID NO: 7 encoding VP7 serotype G4 derived from human rotavirus.

11. The method according to claim 1, wherein the promoter regulating plant-specific
expression of step 1 is cassava vein mosaic virus promoter, ubiquitin promoter, cauliflower mosaic virus 35S promoter (CaMV 35S promoter), actin promoter, PG promoter, or endosperm-specific promoter.

12. The method according to claim 1, wherein the selection marker of step 1 is neomycin phosphotransferase II (NPTII) gene, hygromycin phosphotransferase gene, phosphynotricin acetyltransferase gene, or dihydrofolate reductase gene.

13. The method according to claim 1, wherein the plasmid of step 1 comprises sequentially the right border of T-DNA, nopalín synthase promoter, neomycin phosphotransferase II (NPT II) gene, nopalín transcription termination region, CaMV 35S promoter, cDNA fragment encoding rotavirus structural protein, nopalín transcription termination region and the left border of T-DNA.

14. The method according to claim 13, the plasmid of step 1 is plasmid pILTAB357-VP6 as shown in the cleavage map of Fig. 1a, plasmid pILTAB357-VP2 as shown in the cleavage map of Fig. 1b, plasmid pILTAB357-VP4 as shown in the cleavage map of Fig. 1c, or plasmid pILTAB357-VP7 as shown in the cleavage map of Fig. 1d.

15. The method according to claim 1, wherein the introduction of expression plasmid into plant cell of step 2 is performed by culturing the plant cell and the agrobacterium transformed with recombinant binary vector or cointegration vector together.

16. The method according to claim 15, wherein the agrobacterium is agrobacterium
*tumefaciens* or *agrobacterium rizogenes*.

17. The method according to claim 1, wherein the introduction of expression plasmid into plant cell of step 2 is performed by PEG-mediated uptake, microparticle bombardment, or electroporation.

18. The method according to claim 1, wherein the plant cell of step 2 is edible plant cell selected from the group consisting of tomato, lettuce, Chinese cabbage, banana, potato, radish and rice.

19. A recombinant rotavirus structural protein produced by the method according to any one of claims 1 to 18.

20. A rotavirus-like particle prepared by assembling after folding recombinant rotavirus proteins, VP2, VP6, VP4, and VP7 produced by the method according to any one of claims 1 to 18.

21. A rotavirus-like particle prepared by assembling after folding recombinant rotavirus proteins, VP2, VP4, and VP7 produced by the method according to any one of claims 1 to 18.

22. A vaccine composition for prevention and treatment of rotavirus-related disease comprising the recombinant rotavirus structural proteins produced by the method according to any one of claims 1 to 18 as an effective component.
23. The vaccine composition according to claim 22, wherein the recombinant rotavirus structural protein is any one or more selected from the group consisting of VP2, VP4, VP6, VP7 serotype G1, VP7 serotype G2, VP7 serotype G3, or VP7 serotype G4.

24. The vaccine composition according to claim 22, wherein the recombinant rotavirus structural protein exists in the form of plant cell itself, transgenic plant redifferentiated from plant cell, or the extract of plant cell.

25. The vaccine composition according to claim 22, wherein the vaccine is administered orally or edibly for immunization.
FIG. 2C

FIG. 2D
FIG. 3A

CELL GROWTH (g FW/l)

TIME (DAYS)

FIG. 3B

DAYS AFTER CULTURE

12 15 18 21 C

FIRST WORK

SECOND WORK
SEQUENCE LISTING

CHUNG, In Sik
KIM, Won Yong

Production method of recombinant rotavirus structural proteins
and vaccine composition

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2000-02-12
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human rotavirus

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INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 5/00

According to International Patent Classification (IPC) or to both national classification and IPC.

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7 C12N 5/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

NCBI pubmed. USPTO database

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
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☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use of exhibition or other means

"P." document related to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&." document member of the same patent family

Date of the actual completion of the international search

13 JULY 2001 (13.07.2001)

Date of mailing of the international search report

16 JULY 2001 (16.07.2001)

Name and mailing address of the ISA/KR

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Form PCT/ISA/210 (second sheet) (July 1998)