

ROTAVIRUS-LIKE PARTICLE PRODUCTION IN PLANTS

FIELD OF INVENTION

5 [0001] This invention relates to producing rotavirus structural proteins in plants. More specifically, the present invention relates to producing virus-like particles comprising rotavirus structural protein in plants.

BACKGROUND OF THE INVENTION

10 [0002] Rotavirus infection is a global problem mainly affecting children under the age of five. It results in severe gastroenteritis and in worst cases death.

[0003] Rotaviruses are members of the Reoviridae family of viruses (genus Rotavirus) that affect the gastrointestinal system and respiratory tract. The name is derived from the wheel like appearance of virions when viewed by negative contrast
15 electron microscopy (Figure 1a; prior art). 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
20 stranded RNA segments encoding at least 11 rotavirus proteins.

[0004] The dsRNA codes for six structural proteins (VP) and six non-structural proteins (NSP) (Figure 1c; prior art). The structural proteins comprise VP1, VP2, VP3, VP4, VP6 and VP7 (Figure 1b; prior art). Three concentric layers are formed by the assembly of VP2, VP6 and VP7 respectively, with VP4 forming “spikes” on the
25 surface of the virus structure. The NSPs are synthesized in infected cells and function in various parts of the replication cycle or interact with some of the host proteins to influence pathogenesis or the immune response to infection (Greenberg and Estes, 2009).

[0005] VP2 is a 102 kDa protein and is the most abundant protein of the viral core. It
30 forms the inner-most structural protein layer and provides a scaffold for the correct

assembly of the components and transcription enzymes of the viral core (Lawton, 2000). VP1, the largest viral protein at 125 kDa, acts as an RNA-dependent polymerase for rotavirus, creating a core replication intermediate, and associates with VP2 at its icosahedral vertices (Varani and Allain, 2002; Vende et al., 2002). VP3, a 98 kDa protein, is also directly associated with the viral genome, acting as an mRNA capping enzyme that adds a 5' cap structure to viral mRNAs. Together, VP1 and VP3 form a complex that is attached to the outer 5-fold vertices of the VP2 capsid layer (Angel, 2007). VP6 is a 42 kDa protein which forms the middle shell of the viral core, is the major capsid protein and accounts for more than 50% of the total protein mass of the virion (González et al., 2004; Estes, 1996). It is required for gene transcription and may have a role in encapsulation of the rotavirus RNA by anchoring VP1 to VP2 in the core, as seen in bluetongue virus, another member of the Reoviridae family. It also determines the classification of rotaviruses into five groups (A to E) with group A most commonly affecting humans (Palombo, 1999). VP6 in rotavirus group A has at least four subgroups (SG), which depend on the presence or absence of SG specific epitopes: SG I, SG II, SG (I+II) and SG non-(I+II). Groups B and C lack a common group A antigen but are also known to infect humans, while group D only affects animals e.g chickens and cows (Thongprachum, 2010).

[0006] The two outer capsid proteins VP7, a 37 kDa glycoprotein (G) and the 87 kDa protease sensitive VP4 (P), define the virus' serotypes. These two proteins induce neutralizing antibody responses and are thus used to classify rotavirus serotypes into a dual nomenclature system, depending on the G-P antigen combination (e.g. G1 P[8] or G2 P[4]) (Sanchez-Padilla et al., 2009). The VP4 protein dimerizes to form 60 spikes on the outer shell of the virus, which are directly involved in the initial stages of host cell entry. The spike protein contains a cleavage site at amino acid (aa) position 248. Upon infection, it is cleaved by the protease trypsin to produce VP5 (529 aa, 60 kDa) and VP8 (246 aa, 28 kDa) (Denisova et al., 1999). This process enhances virus infectivity (cell attachment and invasion of host cell) and stabilizes the spike structure (Glass, 2006). The VP7 glycoprotein forms the third or outside layer of the virus. At present, 27 G and 35 P genotypes are known (Greenberg and Estes, 2009). VP4 and VP7 are the major antigens involved in virus neutralization and are important targets for vaccine development (Dennehy, 2007).

[0007] In infected mammalian cells, rotaviruses undergo a unique mode of morphogenesis to form the complete triple-layered VP2/6/4/7 viral particles (Lopez et al., 2005). The triple-layer capsid is a very stable complex which enables faecal–oral transmission and delivery of the virus into the small intestine where it infects non-dividing differentiated enterocytes near the tips of the villi (Greenberg and Estes, 2009). Firstly, the intact virus attaches to sialic acid-independent receptors via 60 VP4 dimer spikes on the surface of the virus (Lundgren and Svensson, 2001). The 60 VP4 dimer spikes on the surface of the virus allow the virus to attach to these cell receptors. VP4 is susceptible to proteolytic cleavage by trypsin which results in a conformational change that exposes additional attachment sites on the surface of the glycoprotein for interaction with a series of co-receptors.

[0008] The multi-step attachment and entry process is, however, not clearly understood but the virus is delivered across the host's plasma membrane. The VP7 outer capsid shell which is also involved in the entry process, is removed in the process and double-layered particles (DLP) are delivered into the cell cytoplasm in vesicles (Figure 2; prior art). The DLP escapes from the vesicle and goes into non-membrane bound cytoplasmic inclusions. Early transcription of the genome by VP1 begins in particles so that dsRNA is never exposed to the cytoplasm. RNA replication and core formation takes place in these non-membrane-bound cytoplasmic inclusions. The nascent (+) RNAs are then transported into the cytoplasm and serve as templates for viral protein synthesis. VP4 is produced in the cytosol and transported to the rough endoplasmic reticulum (RER), and VP7 is secreted into the RER. VP2 and VP6 are produced and assemble in the cytosol in virosomes and subsequently bud into the RER compartments, receiving a transient membrane envelope in the process (Lopez et al., 2005; Tian et al., 1996). In the RER, the transient envelopes of the viral particles are removed and replaced by VP4 and VP7 protein monomers, with critical involvement of rotaviral glycoprotein NSP4 (Tian et al., 1996; Lopez et al., 2005; Gonzalez et al., 2000). NSP4 functions as an intracellular receptor in the ER membrane and binds newly made subviral particles and probably also the spike protein VP4 (Tian et al., 1996). NSP4 is also toxic to humans and is the causative agent of the diarrhea. The complete, mature particles are subsequently transferred from the RER through the Golgi apparatus to the plasma membrane for secretion (Lopez et al., 2005).

[0009] A variety of different approaches have been taken to generate a rotavirus vaccine suitable to protect human populations from the various serotypes of rotavirus. These approaches include various Jennerian approaches, use of live attenuated viruses, use of virus-like particles, nucleic acid vaccines and viral sub-units as immunogens. At present there are two oral vaccines available on the market, however, these have low efficacy in some developing countries due to strain variation and presence of other pathogens.

[0010] U.S. Pat. Nos. 4,624,850, 4,636,385, 4,704,275, 4,751,080, 4,927,628, 5,474,773, and 5,695,767, each describe a variety of rotavirus vaccines and/or methods of preparing the same. A commonality shared by the members of this group is that each of these vaccines relies on the use of whole viral particles to create the ultimate rotavirus vaccines. Given the long standing need for an effective, multivalent vaccine, it is clear that this body of work has been only partially successful in addressing the need for such a vaccine.

[0011] Departing from traditional methods of vaccine generation, advances in the field of molecular biology have permitted the expression of individual rotavirus proteins. Crawford et al. (J Virol. 1994 September; 68(9): 5945–5952) cloned VP2, VP4, VP6, and VP7 coding for the major capsid protein into the baculovirus expression system and expressed each protein in insect cells. Co-expression of different combinations of the rotavirus major structural proteins resulted in the formation of stable virus-like particles (VLPs). The co-expression of VP2 and VP6 alone or with VP4 resulted in the production of VP2/6 or VP2/4/6 VLPs, which were similar to double-layered rotavirus particles. Co-expression of VP2, VP6, and VP7, with or without VP4, produced triple-layered VP2/6/7 or VP2/4/6/7 VLPs, which were similar to native infectious rotavirus particles. The VLPs maintained the structural and functional characteristics of native particles, as determined by electron microscopic examination of the particles, the presence of non-neutralizing and neutralizing epitopes on VP4 and VP7, and hemagglutination activity of the VP2/4/6/7 VLPs.

[0012] Vaccine candidates generated from virus-like particles of different protein compositions have shown potential as subunit vaccines. O'Neal et al. ("Rotavirus Virus-like Particles Administered Mucosally Induce Protective Immunity," J. Virology, 71(11):8707-8717 (1997)) showed that VLPs containing VPs 2 and 6 or

VPs 2, 6, and 7 when administered to mice with and without the addition of cholera toxin induced protective immunity in immunized mice, although protection was more effective when the VLPs were administered with cholera toxin (CT).

[0013] Core-like particles (CLP) and VLPs have also been used to immunize cows
5 Fernandez, et al., ("Passive Immunity to Bovine Rotavirus in Newborn Calves Fed Colostrum Supplements From Cows Immunized with Recombinant SA11 rotavirus core-like particle (CLP) or virus-like particle (VLP) vaccines," Vaccine, 16(5):507-516 (1998)). In this study the ability of CLPs and VLPs to create passive immunity was studied. This group concluded that VLPs were more effective than CLPs in
10 inducing passive immunity.

[0014] Plants are increasingly being used for large-scale production of recombinant proteins. For example US 2003/0175303 discloses the expression of recombinant rotavirus structural protein VP6, VP2, VP4 or VP7 in stable transformed tomato plants.

[0015] Saldana et al. expressed VP2 and VP6 in the cytoplasm of tomato plants using a cauliflower mosaic virus (CaMV) 35S promoter and recombinant *A. tumefaciens*
15 (Saldana et al., 2006). Electron microscopy studies showed that a small proportion of the particles had assembled into 2/6 VLPs. A protective immune response was detected in mice and this may have to some extent been contributed by the non-assembled VPs. Individual proteins have been shown to elicit immune responses in
20 mice, as in the case of VP8 and VP6 (Zhou et al., 2010).

[0016] Matsumura et al., (2002) were first to report bovine rotavirus A VP6 expression and assembly in transgenic potato plants. In their study, they used transgenic potato plants regulated by a cauliflower mosaic virus (CaMV) 35S
25 promoter and recombinant *Agrobacterium tumefaciens* carrying the VP6 gene. The protein was expressed, purified and immunogenic studies performed. Immune-response in adult mice showed presence of VP6 antibodies in the sera. However, they did not show evidence of assembled VP6 proteins. It may have been simple monomers or trimers that could elicit an immune response in mice. Another group's
30 work showed VP6 assembly in *Nicotiana benthamiana* using a potato virus X (PVX) vector (O'Brien et al., 2000). When the VP6 protein was expressed in plants, it was discovered that it only assembled when fused to the PVX protein rods. Once cleavage

occurred, VP6 assembled into icosahedral VLPs as seen in a similar study of HIV-PVX by Marusic et al., (2001). This result probably suggests that rotavirus proteins may require an additional factor or enhancement in order to form VLPs.

[0017] Production of VLP is a challenging task, as both the synthesis and assembly of one or more recombinant proteins are required. This is the case for VLP of rotavirus which is an RNA virus with a capsid formed by 1860 monomers of four different proteins. For VLP production the simultaneous expression and assembly of two to three recombinant proteins is necessary. These comprise 120 molecules of VP2 (inner layer), 780 molecules of VP6 (middle layer) and 780 molecules of the glycoprotein VP7 (outer layer), ultimately forming a double or triple-layered particle. In addition, the production of most VLP requires the simultaneous expression and assembly of several recombinant proteins, which – for the case of rotavirus like particle (RLP) – needs to occur in a single host cell.

[0018] A more recent study showed the successful expression of codon-optimized human rotavirus VP6 in *Chenopodium amaranticolor* using a Beet black scorch virus (BBSV) mediated expression system. The protein was engineered as a replacement to the coat protein open reading frame of BBSV. Oral immunization of female BALB/c mice with the plant based VP6 protein induced high titers of anti-VP6 mucosal IgA and serum IgG (Zhou et al., 2010). The group, however, did not mention whether the VP6 proteins assembled into VLPs or particles.

[0019] Rotavirus VP7 has also been successfully expressed in tobacco plants and was shown to maintain its neutralizing immune response in mice (Yu and Langridge, 2001). Another study using transgenic potato plants to express VP7 showed that the VP7 gene was stable over 50 generations in the transformed plants. VP7 protein from the 50th generation induced both protective and neutralizing antibodies in adult mice (Li et al., 2006).

[0020] Yang et al. (Yang Y M, Li X, Yang H, et al. 2011) co-expressed three rotavirus capsid proteins VP2, VP6 and VP7 of group A RV (P[8]G1) in tobacco plants and expression levels of these proteins, as well as formation of rotavirus-like particles and immunogenicity were studied. VLPs were purified from transgenic tobacco plants and analyzed by electron microscopy and Western blot. Yang et al.

results indicate that the plant derived VP2, VP6 and VP7 protein self-assembled into 2/6 or 2/6/7 rotavirus like particle with a diameter of 60-80 nm.

SUMMARY OF THE INVENTION

5 [0021] The present invention relates to producing rotavirus structural proteins in plants. More specifically, the present invention also relates to producing virus-like particles comprising rotavirus structural protein in plants.

[0022] According to the present invention there is provided a method (A) of producing a rotavirus-likeparticle (RLP) in a plant comprising:

10 a) introducing a first nucleic acid comprising a first regulatory region active in the plant operatively linked to a first nucleotide sequence encoding a first rotavirus structural protein, a second nucleic acid comprising a second regulatory region active in the plant operatively linked to a second nucleotide sequence encoding a second rotavirus structural protein and a third nucleic acid comprising a third regulatory
15 region active in the plant operatively linked to a third nucleotide sequence encoding a third rotavirus structural protein into the plant, portion of a plant or plant cell,

b) incubating the plant, portion of a plant or plant cell under conditions that permit the transient expression of the first, second and third nucleic acid, thereby producing the RLP.

20 [0023] Furthermore a fourth nucleic acid comprising a fourth regulatory region active in the plant and operatively linked to a fourth nucleotide sequence encoding a fourth rotavirus structural protein may be introduced into the plant, portion of a plant or plant cell in step a), and is expressed when incubating the plant, portion of a plant or plant cell in step b).

25 [0024] In the method (A) as described above the first rotavirus structural protein may be VP2, the second rotavirus structural protein may be VP6 and the third rotavirus structural protein may be VP4 or VP7. Furthermore, the fourth rotavirus structural protein may be VP7 or VP4. The VP4 may be processed or cleaved to produce VP5 and VP8. Cleavage of VP4 may be performed using a protease, for example, trypsin, a
30 trypsin-like protease, a serine protease, a chymotrypsin-like protease, or a subtilisin. The protease may be co-expressed within the plant.

[0025] The present invention also provides a method (B) of producing a rotavirus-like particle (RLP) comprising:

a) introducing a nucleic acid comprising a regulatory region active in the plant operatively linked to a first nucleotide sequence encoding one or more rotavirus structural protein, into the plant, portion of a plant or plant cell,

b) incubating the plant, portion of a plant or plant cell under conditions that permit the transient expression of the first nucleic acid, thereby producing the RLP.

[0026] The method (B) as described above may further comprise introducing in (step a) a second nucleic acid comprising a second regulatory region active in the plant and operatively linked to a second nucleotide sequence encoding one or more rotavirus structural protein and expressing the a second nucleic acid when incubating the plant, portion of a plant or plant cell in step b).

[0027] The method (B) as described above may further comprise introducing in (step a) a third nucleic acid comprising a third regulatory region active in the plant and operatively linked to a third nucleotide sequence encoding one or more rotavirus structural protein is introduced into the plant, portion of a plant or plant cell in step a), and expressing the third nucleic acid when incubating the plant, portion of a plant or plant cell in step b).

[0028] Furthermore, in method (A) or (B) an additional nucleic acid may be expressed in the plant, portion of a plant or plant cell, and wherein the additional nucleic acid comprises a regulatory region active in the plant operatively linked to a nucleotide sequence encoding a suppressor of silencing.

[0029] The codon usage of the nucleotide sequence may be adjusted to preferred human codon usage, increased GC content or a combination thereof.

[0030] The rotavirus structural protein may comprise a truncated, native or a non-native signal peptide. The non-native signal peptide may be a protein disulfide isomerase signal (PDI) peptide.

[0031] The first, second, third or fourth nucleotide sequence or a combination thereof may be operatively linked to a Cowpea Mosaic Virus (CPMV) regulatory region.

[0032] The method (A) or (B) as described above may further comprise the steps of:

c) harvesting the plant, portion of a plant or plant cell, and

d) purifying the RLPs from the plant, portion of a plant or plant cell.

[0033] During the step of harvesting or purifying in method (A) or (B), VP4 may be processed or cleaved to produce VP5 and VP8 using trypsin, a trypsin-like protease, a serine protease, a chymotrypsin-like protease, subtilisin.

5 [0034] The RLPs may range size from 70-100 nm and may purified in the presence of calcium.

[0035] The present invention provides a RLP produced by the methods (A) or (B) as described above. The RLP produced may comprise at least aVP4 rotavirus structural protein. The VP4 may be cleaved into VP5 and VP8 using a protease for example,
10 trypsin, a trypsin-like protease, a serine protease, a chymotrypsin-like protease, subtilisin. The protease may be co-expressed within the plant or added during harvesting, purification, or both. Furthermore the RLP produced by the method (A) or (B) may be a double layered RLP and/or a triple layered RLP.

[0036] Furthermore, nucleotide sequences are provided. The nucleotide sequence
15 encoding VP2 may comprise from 80% to 100% identity with a nucleotide sequence as defined by SEQ ID NO:13, SEQ ID NO:14, or SEQ ID NO: 45. The nucleotide sequence encoding VP6 may comprise from 80% to 100% identity with a nucleotide sequence as defined by SEQ ID NO:17, SEQ ID NO:18 or SEQ ID NO:46. The nucleotide sequence encoding VP7 may comprise from 80% to 100% identity with a
20 nucleotide sequence as defined by SEQ ID NO: 19, 20, 48, 49, 52, 53, 54 or 57. And the nucleotide sequence encoding VP4 may comprise from 80% to 100% identity with a nucleotide sequence as defined by SEQ ID NO: 15, 16, 47, 50, or 51. In addition VP2 may be encoded by an amino acid sequence comprising from 80% to 100% identity with the amino acid sequence defined by SEQ ID NO:1 or SEQ ID
25 NO: 25. VP6 may be encoded by an amino acid sequence comprising from 80% to 100% identity with the amino acid sequence defined by SEQ ID NO:3 or SEQ ID NO: 31. VP7 may be encoded by an amino acid sequence comprising from 80% to 100% identity with the amino acid sequence defined by SEQ ID NO: 4, 39, 43 or 59. VP4 may be encoded by an amino acid sequence comprising from 80% to 100%
30 identity with the amino acid sequence defined by SEQ ID NO: 2 or SEQ ID NO: 36. 33. The one or more rotavirus structural protein may be VP2, VP4, VP6 and/or VP7. The VP4 may be processed to VP5 and VP8. The one or more rotavirus structural

protein may be selected from rotavirus strain G9 P[6], rotavirus A WA strain, rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] strain, and rotavirus SA11 strain.

[0037] In method (A) as described above the first, second or third nucleic acid sequence or a combination thereof may comprise a regulatory region active in the plant operatively linked to one or more than one comovirus enhancer, to one or more than one amplification element and to a nucleotide sequence encoding a rotavirus structural protein, and wherein a fourth nucleic acid encoding a replicase may be introduced into the plant, portion of a plant or plant cell.

[0038] In method (B) as described above the first, second, third or fourth nucleic acid sequence or a combination thereof may comprise a regulatory region active in the plant operatively linked to one or more than one comovirus enhancer, to one or more than one amplification element and to a nucleotide sequence encoding a rotavirus structural protein, and wherein a fifth nucleic acid encoding a replicase may be introduced into the plant, portion of a plant or plant cell.

[0039] In addition, according to the present invention there is provided a method (C) of producing a rotavirus-likeparticle (RLP) in a plant comprising:

a) introducing a nucleic acid comprising a regulatory region active in the plant operatively linked to a nucleotide sequence encoding one or more rotavirus structural protein, into the plant, or portion of the plant,

b) incubating the plant, portion of the plant under conditions that permit the transient expression of the first nucleic acid, thereby producing the RLP.

[0040] Furthermore, a second nucleic acid may be introduced into the plant or portion of the plant, the second nucleic acid comprises a second regulatory region that is active in the plant and operatively linked to a second nucleotide sequence encoding one or more rotavirus structural protein and wherein the second nucleic acid is expressed when incubating the plant or portion of the plant in step b).

[0041] Furthermore a third nucleotide sequence may be introduced into the plant or portion of the plant, the third nucleic acid comprises a third regulatory region that is active in the plant and operatively linked to a third nucleotide sequence encoding one or more rotavirus structural protein and wherein the third nucleic acid is expressed when incubating the plant or portion of the plant in step b).

[0042] The method (C) as described above may further comprising a step of harvesting the plant and extracting the RLPs.

[0043] The one or more rotavirus structural protein of method (C) may be rotavirus protein VP2, VP4 or VP6. The one or more rotavirus structural protein encoded by the first or second nucleotides sequence may be VP2 or VP6. The one or more rotavirus structural protein encoded by the third nucleotides sequence may be VP4. The VP4 may be cleaved to produce VP5 and VP8.

[0044] The first, second or third nucleotide sequence may further encode, comprise, or encode and comprise, one or more than one compartment targeting sequence and/or an amplification element. The one or more compartment targeting sequence directs the one or more rotavirus structural protein to the endoplasmatic reticulum (ER), chloroplast, plastid or apoplast of the plant cell.

[0045] The present invention also provides a method (D) of producing a rotavirus-likeparticle (RLP) comprising,

a) providing a plant or portion of a plant comprising a nucleic acid comprising a regulatory region active in the plant operatively linked to a nucleotide sequence encoding one or more rotavirus structural protein;

b) incubating the plant, portion of the plant or plant cell under conditions that permit the transient expression of the nucleic acid, thereby producing the RLP.

[0046] Furthermore the plant or portion of the plant of method (D) may further comprise;

i) a second nucleic acid comprising a second regulatory region active in the plant and operatively linked to a second nucleotide sequence encoding one or more rotavirus structural protein or,

ii) a second and third nucleic acid, wherein the second nucleic acid comprises a second regulatory region active in the plant and operatively linked to a second nucleotide sequence encoding one or more rotavirus structural protein and the third nucleic acid comprises a third regulatory region active in the plant and operatively linked to a third nucleotide sequence encoding one or more rotavirus structural protein,

wherein the second or the second and third nucleic acids are expressed when incubating the plant or portion of the plant in step b).

[0047] The one or more structural protein in method (D) may be rotavirus protein VP2, VP4 or VP6. The one or more rotavirus structural protein encoded by the first or second nucleotides sequence may be VP2 or VP6. The one or more rotavirus structural protein encoded by the third nucleotides sequence may be VP4. The VP4 may be cleaved into VP5 and VP8 using a protease for example, trypsin, a trypsin-like protease, a serine protease, a chymotrypsin-like protease, subtilisin. The protease may be co-expressed within the plant or added during harvesting, purification, or both.

[0048] The present invention provides a RLP produced by the methods (A), method (B), method (C), method (D), or a combination thereof, as described above. The RLP may comprise one or more rotavirus structural protein that may comprises comprise plant-specific N-glycans, or modified N-glycans.

[0049] The present invention includes a composition comprising an effective dose of the RLP made by the method (A), method (B), method (C), method (D), or a combination thereof as just described, for inducing an immune response, and a pharmaceutically acceptable carrier.

[0050] The present invention also includes a method of inducing immunity to a rotavirus infection in a subject, comprising administering the RLP as just described, to the subject. The RLP may be administered to a subject orally, intradermally, intranasally, intramuscularly, intraperitoneally, intravenously, or subcutaneously.

[0051] The present invention also provides plant matter comprising a RLP produced by the method (A), method (B), method (C), method (D), or a combination thereof, as described above. The plant matter may be used in inducing immunity to a rotavirus virus infection in a subject. The plant matter may also be admixed as a food supplement.

[0052] In the methods as described above (methods A, B, C or D) the plant or portion of the plant may further be administered with, or may further comprise, another nucleic acid sequence encoding a suppressor of silencing.

[0053] Furthermore, the present invention also provides a method (E) of producing rotavirus structural protein in plant comprising

a) introducing a nucleic acid comprising a regulatory region active in the plant operatively linked to a nucleotide sequence encoding one or more rotavirus structural protein, into the plant, or portion of the plant;

b) incubating the plant or portion of the plant under conditions that permit the transient expression of the nucleic acid, thereby producing the one or more rotavirus structural protein.

[0054] This summary of the invention does not necessarily describe all features of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0055] These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings wherein:

[0056] **FIGURE 1** shows rotavirus structure and gene-protein assignment . (A) Transmission electron microscopy of rotavirus particles (bar represents 100nm). (B) Organization of the virus capsid proteins comprising inner, intermediate and outer. (C) Rotavirus dsRNA segments arranged as per size and function. The dsRNA can be separated polyacrylamide gel electrophoresis (D). Proteins in (C) are indicated by dsRNA segments in (D). Images from Crawford et al.,1997 (A), Swiss Institute of Bioinformatics, 2008 (B) and Greenberg and Estes, 2009 (D).

[0057] **Figure 2** shows rotavirus cell entry and replication. When rotavirus enters a cell, VP4 and VP7 are lost, forming a double layered particle (DLP). Transcription of the dsRNA commences resulting in translation of VP2, VP4, VP6 and VP7. Progeny cores with replicase activity are produced in virus factories (also called viroplasms). Late transcription occurs in these progeny cores. At the periphery of virus factories, these core are coated with VP6, forming immature DLPs that bud across the membrane of the endoplasmic reticulum, acquiring a transient lipid membrane which is modified with the ER resident viral glycoproteins NSP4 and VP7; these enveloped particles also contain VP4. As the particles move towards the interior of the ER cisternae, the transient lipid membrane and the nonstructural protein NSP4 are lost, while the virus surface proteins VP4 and VP7 rearrange to form the outermost virus protein layer, yielding mature infectious triple-layered particles (see Swiss Institute of

Bioinformatics (ViralZone): viralzone.expasy.org/viralzone/all_by_species/107.html)

[0058] **FIGURE 3** shows Agrobacterium vectors pTRAc, pTRAc-*rbcs1*-cTP and pTRAc-ERH. P35SS, CaMV 35S promoter with duplicated transcriptional enhancer;; CHS, chalcone synthase 5' untranslated region; pA35S, CaMV 35S polyadenylation signal; SAR, scaffold attachment region of the tobacco Rb7 gene; LB and RB, the left and right borders for T-DNA integration; ColE1ori, origin of replication for *E. coli*; RK2ori, origin of replication for Agrobacterium; bla, ampicillin/carbenicillin-resistance bla gene; LPH, signal-peptide sequence from the murine mAb24 heavy chain; his6, 6 × His tag sequence; SEKDEL, ER-retention signal sequence; *rbcs1*-cTP, chloroplast-transit peptide sequence of a Rubisco small-subunit gene (*rbcs1*) from *Solanum tuberosum*; npt II, kanamycin resistance npt II gene; Pnos and pAnos, promoter and polyadenylation signal of the nopaline synthase gene (Maclean et al., 2007).

[0059] **Figure 4** shows an overview of rotavirus cloning and infiltration procedure

[0060] **Figure 5** shows an apoplast protein extraction procedure. (A) Illustration of the plant cell and location of the apoplast. VP proteins are expressed in the cytosol and targeted to the apoplast (red arrow). (B) - After time trial, plant leaf is vacuum infiltrated with PBS (1) and placed in a perforated spin column (2) then centrifuged in a 2ml Eppendorf tube to collect sap (3).

[0061] **Figure 6** shows a western blots of expression of rotavirus VP6 protein in plant leaf cell compartments over 7 days. Mouse anti-rotavirus VP6 antibody (1:5000) was used to probe the membranes. (+) and (-) indicates expression with or without silencing suppressor respectively. The red lines indicate the position of VP6 proteins in the various samples analysed (~ 40 kDa). Expression and extraction efficiency of VP6 was best in the cytoplasm.

[0062] **Figure 7** shows a western blot showing the individual expression of his-tagged rotavirus proteins at day 3 in the cytoplasm of *N. benthamiana* plant leaves. +ve – bacterial expressed rotavirus VP2; M – molecular weight marker; VP – rotavirus capsid protein. VP7 infiltration resulted in yellowing of leaves (b).

[0063] **Figure 8** shows expression of rotavirus VP2 (a) and VP4 (b) targeted to various *N. benthamiana* plant leaf cell compartments at day 3. The respective chicken

anti-rotavirus serum (1:2000) for VP2 and VP4 were used for probing the proteins. cTp - chloroplasts; ER – endoplasmic reticulum; pTRAc – cytoplasm; A – apoplast; Negative control (-ve) – plants infiltrated with silencing suppressor only; Positive control (+ve) in (a) – bacterial expressed VP2, (b) – bacterial expressed VP4; (- and +) with or without silencing suppressor; M – molecular weight marker. The arrows indicate the position of the protein bands in question.

[0064] **Figure 9** shows western blot analysis of day 3 extracts of co-expressed VP2/6/4 in the cytoplasm of *N. benthamiana* leaves. Proteins were probed with a mixture of chicken anti-rotavirus serum (anti-VP2 (1/5000) and anti-VP4 (1/5000)) and mouse anti-VP6 antibody (1:5000). Infiltration of recombinant *Agrobacterium* was done with silencing suppressor. Negative control (-ve) – whole plants infiltrated with silencing suppressor only; M – molecular weight marker.

[0065] **Figure 10** shows electron micrographs of day 3 cytoplasm extracted rotavirus proteins stained with uranyl acetate. (a) Negative protein sample extract with silencing suppressor; (b) VP6 protein extract; (c) VP2/6 protein extract and (d) VP2/6/4 protein extract. Bars = 200 nm. All RLPs detected were between 70 - 100 nm in diameter. Arrow in (b) indicates VP6 sheath/mat. Arrow in (c) indicates an example of aRLP. All proteins were expressed in the presence of a silencing suppressor. All were captured with mouse-anti VP6 antibody (1:2000).

[0066] **Figure 11** shows sucrose gradient purification of co-expressed VP2/6 and VP2/6/4 (a). Dot blots of sucrose gradient purified VP2/6 (b) and VP2/6/4 (c). Protein extracts were loaded on a sucrose gradient (10 – 60 %) and ultracentrifuged. Fractions were analysed by probing with (b) mouse anti-VP6 antibody (1:5000) and (c) chicken anti-VP2 and VP4 serum (1:5000).

[0067] **Figure 12** shows western blot analysis of VP2/6 fractions (a), SDS-PAGE coomassie stained gel photograph of fractions VP2/6 fractions 16 and 17 (b), and western blot analysis of fractions 16 and 17 (c). Mouse anti-VP6 (1:5000) and chicken anti-VP2 serum (1:5000) was used in the western blot (a) and only mouse anti-VP6 (1:5000) in (c). Negative control (- ve) in (a) and (c) – bacterial expressed VP4, and in (b) – plants infiltrated with silencing suppressor and sucrose gradient purified; crude – non-purified VP2/6 extract; Positive control (+ve) in (a) – bacterial expressed VP2,

(b) and (c) – plant expressed VP6; VP6-SF9 – VP6 protein of known concentration expressed in SF9 insect cells. Arrows indicate protein bands of interest.

[0068] **Figure 13** shows total soluble protein assay on fractions of sucrose density gradient purified VP2/6. (a) – IgG standard curve, (b) - absorbance readings of fractions taken at 750 nm. Points of interest: fractions 16 to 19.

[0069] **Figure 14** shows sucrose density gradient analysis of cytoplasm co-expressed VP2/6/4 fractions. Raw absorbance readings at 750nm were taken to verify the protein peaks previously detected on the dot blot of VP2/6/4.

[0070] **Figure 15** shows transmission electron micrographs of sucrose density gradient purified VP2/6 particles. Both (a) and (b) show two different sections viewed on the copper grid. All RLPs detected were between 70 - 100 nm in diameter. Samples were captured with mouse-anti VP6 antibody (1:2000). Bars represent 200 nm.

[0071] **Figure 16a** shows amino acid sequence (SEQ ID NO:1) and nucleotide sequences of Rotavirus VP2 (SEQ ID NO:13 and 14). **Figure 16b** shows amino acid sequence (SEQ ID NO:2) and nucleotide sequences of Rotavirus VP4 (SEQ ID No: 15 and 16). **Figure 16c** shows amino acid sequence (SEQ ID NO: 3) and nucleotide sequences of Rotavirus VP6 (SEQ ID NO: 17 and 18). **Figure 16d** shows amino acid sequence (SEQ ID NO:4) and nucleotide sequences of Rotavirus VP7 (SEQ ID NO: 19 and 20).

[0072] **Figure 17A** shows nucleotide sequence of primer IF-WA_VP2(opt).s1+3c (SEQ ID NO: 21). **Figure 17B** shows nucleotide sequence of primer IF-WA_VP2(opt).s1-4r (SEQ ID NO: 22). **Figure 17C** shows a schematic representation of construct 1191. SacII and StuI restriction enzyme sites used for plasmid linearization are annotated on the representation.

[0073] **Figure 18** shows nucleotide sequence (SEQ ID NO: 23) of construct 1191 from left to right t-DNA borders (underlined). 2X35S/CPMV-HT/NOS with Plastocyanine-P19-Plastocyanine silencing inhibitor expression cassette.

[0074] **Figure 19** shows nucleotide sequence encoding VP2(opt) from Rotavirus A WA strain (SEQ ID NO:45).

[0075] **Figure 20** shows amino acid sequence of VP2 from Rotavirus A WA strain (SEQ ID NO: 25).

[0076] **Figure 21** shows a schematic representation of construct number 1710.

[0077] **Figure 22A** shows a schematic representation of construct 193. SacII and StuI restriction enzyme sites used for plasmid linearization are annotated on the representation. **Figure 22B** shows nucleotide sequence of construct 193 (SEQ ID NO: 26). Construct 193 is shown from left to right t-DNA borders (underlined). 2X35S/CPMV-HT/NOS into BeYDV(m)+Replicase amplification system with Plastocyanine-P19-Plastocyanine silencing inhibitor expression cassette.

[0078] **Figure 23** shows nucleotide sequence of expression cassette 1710 (SEQ ID NO: 27). Expression cassette number 1710 is shown from 2X35S promoter to NOS terminator. VP2(opt) from Rotavirus A WA strain is underlined.

[0079] **Figure 24** shows a schematic representation of construct number 1711.

[0080] **Figure 25A** shows nucleotide sequence of primer IF-WA_VP6(opt).s1+3c (SEQ ID NO:28). **Figure 25B** shows nucleotide sequence of primer IF-WA_VP6(opt).s1-4r (SEQ ID NO: 29). **Figure 25c** shows expression cassette number 1713 from 2X35S promoter to NOS terminator (SEQ ID NO: 30). VP6(opt) from Rotavirus A WA strain is underlined. Figure 25d shows nucleotide sequence encoding VP6(opt) from Rotavirus A WA strain (SEQ ID NO: 46)

[0081] **Figure 26** shows the amino acid sequence of VP6 from Rotavirus A WA strain (SEQ ID NO: 31).

[0082] **Figure 27** shows the schematic representation of construct number 1713.

[0083] **Figure 28** shows the nucleotide sequence of expression cassette number 1714 from 2X35S promoter to NOS terminator (SEQ ID NO:32). VP6(opt) from Rotavirus A WA strain is underlined.

[0084] **Figure 29** shows a schematic representation of construct number 1714.

[0085] **Figure 30A** shows the nucleotide sequence of primer IF-Rtx_VP4(opt).s1+3c (SEQ ID NO: 33). **Figure 30B** shows the nucleotide sequence of primer IF-Rtx_VP4(opt).s1-4r (SEQ ID NO: 34).

[0086] **Figure 31A** shows the nucleotide sequence of expression cassette number 1731 from 2X35S promoter to NOS terminator (SEQ ID NO: 35). VP4(opt) from Rotavirus A Rotarix strain is underlined. **Figure 31B** shows optimized coding sequence of Rotavirus A VP4 from strain RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P1A[8] (SEQ ID NO: 47). **Figure 31C** shows the nucleotide sequence of expression cassette number 1730 from 2X35S promoter to NOS terminator (SEQ ID NO: 44). VP4(opt) from Rotavirus A Rotarix strain is underlined.

[0087] **Figure 32** shows amino acid sequence of VP4 from Rotavirus A Rotarix strain (SEQ ID NO: 36).

[0088] **Figure 33A** shows a schematic representation of construct number 1730. **Figure 33B** shows a schematic representation of construct number 1731.

[0089] **Figure 34A** shows the nucleotide sequence of primer IF-Rtx_VP7(opt).s1+3c (SEQ ID NO: 37). **Figure 34B** shows the nucleotide sequence of primer IF-Rtx_VP7(opt).s1-4r (SEQ ID NO: 38). **Figure 34C** shows the nucleotide sequence of expression cassette number 1733 from 2X35S promoter to NOS terminator. VP7 from Rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] strain is underlined (SEQ ID NO: 24). **Figure 34D** shows nucleotide sequence encoding VP7 from Rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] strain (SEQ ID NO: 48). **Figure 34E** shows the optimized coding sequence of Rotavirus A VP7 from strain RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P1A[8] (SEQ ID NO 54).

[0090] **Figure 35** shows the amino acid sequence of VP7 from Rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] strain (SEQ ID NO: 39).

[0091] **Figure 36** shows a schematic representation of construct number 1733.

[0092] **Figure 37** shows the nucleotide sequence of primer IF-Rtx_VP7(opt).s2+4c (SEQ ID NO: 40).

[0093] **Figure 38** shows a schematic representation of construct 1192. SacII and StuI restriction enzyme sites used for plasmid linearization are annotated on the representation.

[0094] **Figure 39** shows the nucleotide sequence of construct 1192 from left to right t-DNA borders (underlined) (SEQ ID NO: 41). 2X35S/CPMV-HT/PDISP/NOS with Plastocyanine-P19-Plastocyanine silencing inhibitor expression cassette are shown.

[0095] **Figure 40A** shows the nucleotide sequence of expression cassette number 1735 from 2X35S promoter to NOS terminator (SEQ ID NO: 42). PDISP/VP7(opt) from Rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] strain is underlined. **Figure 40B** Nucleotide sequence encoding PDISP/VP7(opt) from Rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] strain (SEQ ID NO: 49).

[0096] **Figure 41** shows amino acid sequence of PDISP/VP7 from Rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] strain (SEQ ID NO: 43).

[0097] **Figure 42** shows a schematic representation of construct number 1735.

[0098] **Figure 43A** shows the coding sequence of Rotavirus A VP4 from strain RVA/Simian-tc/ZAF/SA11-H96/1958/G3P5B[2] (SEQ ID NO: 50). **Figure 43B** shows the optimized coding sequence of Rotavirus A VP4 from strain RVA/Simian-tc/ZAF/SA11-H96/1958/G3P5B[2] (SEQ ID NO : 51). **Figure 43C** shows the coding sequence of Rotavirus A VP7 from strain RVA/Simian-tc/ZAF/SA11-H96/1958/G3P5B[2] (SEQ ID NO : 52). **Figure 43D** shows optimized coding sequence of Rotavirus A VP7 from strain RVA/Simian-tc/ZAF/SA11-H96/1958/G3P5B[2] (SEQ ID NO :53).

[0099] **Figure 44A** shows the nucleotide sequence of primer IF-TrSp+Rtx_VP7(opt).s1+3c (SEQ ID NP: 55). **Figure 44B** shows the nucleotide sequence of primer IF-Rtx_VP7(opt).s1-4r (SEQ ID NO: 56). **Figure 44C** shows the nucleotide sequence of optimized coding sequence of Rotavirus A VP7 from strain RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P1A[8] (SEQ ID NO: 57). **Figure 44D** shows the nucleotide sequence of expression cassette number 1734 from 2X35S promoter to NOS terminator (SEQ ID NO 58). VP7 from Rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] strain is underlined. **Figure 44E** shows the amino acid sequence of TrSp-VP7 from Rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] strain (SEQ ID NO: 59). **Figure 44F** shows the schematic representation of construct number 1734.

[00100] **Figure 45** shows purification of rotavirus-like particles comprising VP2 and VP6 by iodixanol density gradient centrifugation. **Figure 45A** Coomassie-stained SDS-PAGE analysis of the load prior to centrifugation and fractions 1 to 10 (fraction 1 being at the bottom of the tube). Position of the rotavirus antigens are

shown by arrows. **Figure 45B** Western blot analysis of the same fractions as in (A) using a rabbit polyclonal anti-rotavirus antibody. **Figure 45C** Western blot analysis of the same fractions as in (A) using a rabbit polyclonal anti-VP2 antibody.

[00101] **Figure 46** shows purification of rotavirus-like particles comprising
5 VP2, VP6 and VP7 by iodixanol density gradient centrifugation. **Figure 46A**
Coomassie-stained SDS-PAGE analysis of the load prior to centrifugation and
fractions 1 to 10 (fraction 1 being at the bottom of the tube). Position of the rotavirus
antigens are shown by arrows. **Figure 46B** Western blot analysis of the same fractions
as in (A) using a rabbit polyclonal anti-rotavirus antibody. **Figure 46C** Western blot
10 analysis of the same fractions as in (A) using a rabbit polyclonal anti-VP7 antibody.

[00102] **Figure 47** shows purification of rotavirus-like particles comprising
VP2, VP4, VP6 and VP7 by iodixanol density gradient centrifugation. **Figure 47A**
Coomassie-stained SDS-PAGE analysis of the load prior to centrifugation and
fractions 1 to 10 (fraction 1 being at the bottom of the tube). Position of the rotavirus
15 antigens are shown by arrows. **Figure 47B** Western blot analysis of the same fractions
as in (A) using a rabbit polyclonal anti-rotavirus antibody. **Figure 47C** Western blot
analysis of the same fractions as in (A) using a rabbit polyclonal anti-VP7 antibody.

[00103] **Figure 48** shows assessment of VP4 content in purified rotavirus-like
particles comprising VP2, VP4, VP6 and VP7 by anti-VP4 specific ELISA.

20 [00104] **Figure 49** shows cryo-electron microscopy image of purified
rotavirus-like particles comprising VP2 and VP6 (left panel) and VP2, VP4, VP6 and
VP7 (right panel).

DETAILED DESCRIPTION

[00105] The following description is of a preferred embodiment.

25 [00106] The present invention relates to virus-like particles (VLPs) comprising
one or more rotavirus structural protein (i.e. a rotavirus like particle, rotavirus VLP or
RLP), and methods of producing rotavirus-like particle (RLPs) in plants. The
rotavirus like particle (RLP) may therefore comprise one or more rotavirus structural
protein. The RLP may be double layered or triple layered.

30 [00107] The present invention in part provides a method of producing a
rotavirus-like particle (RLP) in a plant. The method may comprise introducing one or

more nucleic acid comprising a regulatory region active in the plant operatively linked to a nucleotide sequence encoding one or more rotavirus structural protein into the plant, or portion of the plant. Followed by incubating the plant or portion of the plant under conditions that permit the transient expression of the nucleic acids, thereby producing the RLP.

[00108] Furthermore, the present invention in part provides a method of producing a rotavirus-like particle (RLP) vaccine candidate in a plant. The method may comprise introducing a first nucleic acid comprising a first regulatory region active in the plant operatively linked to a first nucleotide sequence encoding a first rotavirus structural protein, a second nucleic acid comprising a second regulatory region active in the plant operatively linked to a second nucleotide sequence encoding a second rotavirus structural protein and a third nucleic acid comprising a third regulatory region active in the plant operatively linked to a third nucleotide sequence encoding a third rotavirus structural protein into the plant, portion of the plant or plant cell. Followed by incubating the plant, portion of the plant or plant cell under conditions that permit the transient expression of the first, second and third nucleic acid, thereby producing the RLP. The RLP may be single, double or triple layered.

[00109] The “rotavirus structural protein” may refer to all or a portion of a rotavirus structural protein sequence isolated from rotavirus, present in any naturally occurring or variant rotavirus strain or isolate. Thus, the term rotavirus structural protein and the like include naturally occurring rotavirus structural protein sequence variants produced by mutation during the virus life-cycle or produced in response to selective pressure (e.g., drug therapy, expansion of host cell tropism or infectivity, etc.). As one of skill in the art appreciates, such rotavirus structural protein sequences and variants thereof may be also produced using recombinant techniques.

[00110] Furthermore, structural proteins may include capsid proteins such for example VP2 and VP6 and/or surface proteins such for example VP4. The structural protein may further include for example VP7.

[00111] Non-limiting examples of rotavirus structural protein are rotavirus protein VP2, VP4, VP6 and VP7, and a fragment of VP2, VP4, VP6 and VP7. Non-limiting examples of VP2, VP4, VP6 and VP7, or fragments of VP2, VP4, VP6 and VP7 protein that may be used according to the present invention include those VP2,

VP4 VP6 and VP7 protein from rotavirus strain G9 P[6], rotavirus A WA strain, rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] strain and rotavirus SA11 strain.

[00112] An example of a VP2 structural protein, which is not to be considered limiting, is set forth in the amino acid sequence of SEQ ID NO: 1 and SEQ ID NO:25. Furthermore, the VP2 structural protein may comprise the sequence set forth in SEQ ID NO: 1, SEQ ID NO:25, or a sequence having at least about 90-100% sequence similarity thereto, including any percent similarity within these ranges, such as 91, 92, 93, 94, 95, 96, 97, 98, 99% sequence similarity thereto. In addition, a VP2 structural protein may be encoded by a nucleotide sequence as set forth in SEQ ID NO:13, 14, 25, or 45 or a sequence having at least about 80-100% sequence similarity thereto, including any percent similarity within these ranges, such as 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99% sequence similarity thereto.

[00113] An example of a VP4 structural protein, which is not to be considered limiting, is set forth in the amino acid sequence of SEQ ID NO: 2 and SEQ ID NO: 36. Furthermore, the VP4 structural protein may comprise the sequence set forth in SEQ ID NO: 2, SEQ ID NO: 36 or a sequence having at least about 90-100% sequence similarity thereto, including any percent similarity within these ranges, such as 91, 92, 93, 94, 95, 96, 97, 98, 99% sequence similarity thereto. In addition, a VP4 structural protein may be encoded by a nucleotide sequence as set forth in SEQ ID NO: 15, 16, 47, 50 or 51 or a sequence having at least about 80-100% sequence similarity thereto, including any percent similarity within these ranges, such as 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99% sequence similarity thereto.

[00114] An example of a VP6 structural protein, which is not to be considered limiting, is set forth in the amino acid sequence of SEQ ID NO: 3 and SEQ ID NO: 31. Furthermore, the VP6 structural protein may comprise the sequence set forth in SEQ ID NO: 3, SEQ ID NO: 31 or a sequence having at least about 90-100% sequence similarity thereto, including any percent similarity within these ranges, such as 91, 92, 93, 94, 95, 96, 97, 98, 99% sequence similarity thereto. In addition, a VP6 structural protein may be encoded by a nucleotide sequence as set forth in SEQ ID NO:17, 18, or 46 or a sequence having at least about 80-100% sequence similarity

thereto, including any percent similarity within these ranges, such as 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99% sequence similarity thereto.

[00115] An example of a VP7 structural protein, which is not to be considered limiting, is set forth in the amino acid sequence of SEQ ID NO: 4, SEQ ID NO: 39, SEQ ID NO: 43, and SEQ ID NO: 47. Furthermore, the VP7 structural protein may comprise the sequence set forth in SEQ ID NO: 4, SEQ ID NO: 39 and SEQ ID NO: 43, or a sequence having at least about 90-100% similarity thereto, including any percent similarity within these ranges, such as 91, 92, 93, 94, 95, 96, 97, 98, 99% sequence similarity thereto. In addition, a VP7 structural protein may be encoded by a nucleotide sequence as set forth in SEQ ID NO:19, 20, 48, 49, 52, 53 or 54 or a sequence having at least about 80-100% sequence similarity thereto, including any percent similarity within these ranges, such as 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99% sequence similarity thereto.

[00116] Amino acid sequence similarity or identity may be computed by using the BLASTP and TBLASTN programs which employ the BLAST (basic local alignment search tool) 2.0 algorithm. Techniques for computing amino acid sequence similarity or identity are well known to those skilled in the art, and the use of the BLAST algorithm is described in ALTSCHUL et al. (1990, J Mol. Biol. 215: 403-410) and ALTSCHUL et al. (1997, Nucleic Acids Res. 25: 3389-3402).

[00117] The term "virus-like particle" (VLP), or "virus-like particles" or "VLPs" refers to structures that self-assemble and comprise one or more structural proteins such as for example rotavirus structural protein, for example but not limited to VP2, VP4, VP6 and/or VP7 structural protein. VLPs comprising rotavirus structural protein maybe also be referred to "rotavirus VLP", "rotavirus -like particle (RVLP)", "rotavirus -like particle (RLP)" , "rotavirus -like particle", "RVLP" or "RLP" . VLPs or RLPs are generally morphologically and antigenically similar to virions produced in an infection, but lack genetic information sufficient to replicate and thus are non-infectious. VLPs may be produced in suitable host cells including plant host cells. Following extraction from the host cell and upon isolation and further purification under suitable conditions, VLPs may be purified as intact structures. The RLP may be a single-, double or triple-layered RLP. Single-layered RLPs may be obtained by expressing one rotavirus structural protein, such as VP2 or VP6. Double-layered RLPs may be obtained by expressing two rotavirus structural proteins, such as

for example by co-expressing both VP2 and VP6, with or without VP4. Triple-layered RLPs may be obtained by the simultaneous expression of at least three rotavirus structural proteins for example the co-expression of VP2, VP6 and VP7, with or without VP4. Co-expression of VP4 results in a particle with spikes that resembles native rotavirus. VP4 may be processed or cleaved to produce VP5 and VP8. This processing may take place within the host using endogenous proteases, or by co-expressing a suitable protease, for example, trypsin, a trypsin-like protease, a serine protease, a chymotrypsin-like protease, subtilisin. Alternatively, VP4 may be processed to produce VP5 and VP8 by adding a suitable protease, for example, trypsin, a trypsin-like protease, a serine protease, a chymotrypsin-like protease, subtilisin during any step of the RLP extraction procedure, or after RLP purification.

[00118] Each of the rotavirus structural proteins has different characteristics and size, and is required in different amounts for assembly into RLP. The term "rotavirus VLP", "rotavirus virus-like particle (RVLP)", "rotavirus virus-like particle (RLP)", "rotavirus virus-like particle", "RVLP" or "RLP" refers to a virus-like particle (VLP) comprising one or more rotavirus structural proteins. Example of rotavirus structural proteins may include, but are not limited to VP2, VP4 (or VP5 and VP8) VP6 and VP7 structural protein.

[00119] The present invention also provides for a method of producing RLPs in a plant, wherein a first nucleic acid (a first nucleic acid) encoding a first rotavirus structural protein, for example a VP2 or VP6 protein, is co-expressed with a second nucleic acid encoding a second rotavirus structural protein, for example a VP6 or VP2 protein. Furthermore, a third nucleic acid encoding a third rotavirus structural protein, for example VP4 or VP7 may be co-expressed with the first and second nucleic acid so that the first, the second nucleic acids and third nucleic acids are co-expressed in the plant. The first nucleic acid, second nucleic acid and third nucleic acid, may be introduced into the plant in the same step, or may be introduced to the plant sequentially. The VP4 may be processed or cleaved to produce VP5 and VP8 within the host by co-expressing a nucleic acid encoding a suitable protease, for example, trypsin, a trypsin-like protease, a serine protease, a chymotrypsin-like protease, subtilisin. Alternatively, VP4 may be processed during any step of RLP extraction, or after RLP purification by adding a suitable protease, for example, trypsin, a trypsin-like protease, a serine protease, a chymotrypsin-like protease, subtilisin.

[00120] Furthermore, a plant that expresses a first nucleic acid encoding a first rotavirus structural protein, a second nucleic acid encoding a second rotavirus structural protein and a third nucleic acid encoding a third rotavirus structural protein may be further transformed with a fourth nucleic acid encoding a fourth rotavirus structural protein, for example a VP7 or VP4 protein, so that the first, the second nucleic acids, third and fourth nucleic acids are co-expressed in the plant. The VP4 may be processed or cleaved to produce VP5 and VP8 within the host by co-expressing a nucleic acid encoding a suitable protease, for example, trypsin, a trypsin-like protease, a serine protease, a chymotrypsin-like protease, subtilisin. Alternatively, VP4 may be processed during any step of RLP extraction, or after RLP purification by adding a suitable protease, for example, trypsin, a trypsin-like protease, a serine protease, a chymotrypsin-like protease, subtilisin.

[00121] Furthermore, a first plant expressing the first nucleic acid encoding one or more rotavirus structural protein, for example a VP2 or VP6 protein, may be crossed with a second plant expressing the second nucleic acid encoding one or more rotavirus structural protein for example but not limited to VP6 or VP2 protein, to produce a progeny plant (third plant) that co-expresses the first and second nucleic acids encoding VP2 and VP6 or VP6 and VP2, respectively. Furthermore, the third plant expressing the first and second nucleic acids encoding VP2 and VP6 or VP6 and VP2, respectively, may be crossed with a fourth plant expressing the third nucleic acid encoding one or more rotavirus structural protein for example but not limited to VP4 or VP7, to produce a further progeny plant (fifth plant) that co-expresses the first, second and third nucleic acids encoding VP2, VP6, and VP4 or VP7 respectively. The VP4 may be processed or cleaved to produce VP5 and VP8 within the plant using host a protease, or by co-expressing a nucleic acid encoding a suitable protease, for example, trypsin, a trypsin-like protease, a serine protease, a chymotrypsin-like protease, subtilisin within one of the first, second, third or fourth plants. Alternatively, VP4 may be processed during any step of RLP extraction, or after RLP purification by adding a suitable protease, for example, trypsin, a trypsin-like protease, a serine protease, a chymotrypsin-like protease, subtilisin.

[00122] As described in more detail below, RLPs may be produced in a plant by expressing a nucleic acid (a first nucleic acid) encoding one or more rotavirus structural protein, for example but not limited to VP2, VP6 or VP7. A second nucleic

acid encoding a second rotavirus structural protein, for example but not limited to VP7, VP6 or VP2 might be co-expressed in the plant. Furthermore, a third nucleic acid encoding a third rotavirus structural protein, for example but not limited to VP6, VP7 or VP2 might be co-expressed in the plant. The nucleic acid, second nucleic acid and third nucleic acid may be introduced to the plant in the same step, or they may be introduced to the plant sequentially. The nucleic acid, second nucleic acid and third nucleic acid may be introduced in the plant in a transient manner, or in a stable manner.

[00123] Furthermore, a plant that expresses a first nucleic acid encoding a first rotavirus structural protein, for example a VP2 protein, may be transformed with a second nucleic acid encoding a second rotavirus structural protein, for example but not limited to VP6 or VP7 so that both the first and the second nucleic acids are co-expressed in the plant. The plant might be further transformed with a third nucleic acid encoding a third rotavirus structural protein, for example but not limited to VP7 or VP6.

[00124] Alternatively, a plant that expresses a VP6 or VP7 protein, (second nucleic acid) may be transformed with the first nucleic acid encoding the VP2 protein, so that both the first and the second nucleic acids are co-expressed in the plant. The plant might be further transformed with a third nucleic acid encoding a third rotavirus structural protein, for example but not limited to VP7 or VP6.

[00125] In addition, a plant expressing a first and second nucleic acid encoding a first and second rotavirus structural protein for example a VP2 and VP6 protein, may be transformed with a third nucleic acid encoding a third rotavirus structural protein example VP4 or VP7. The VP4 may be processed or cleaved to produce VP5 and VP8 by co-expressing a nucleic acid encoding a suitable protease, for example, trypsin, a trypsin-like protease, a serine protease, a chymotrypsin-like protease, subtilisin. Alternatively, VP4 may be processed during any step of RLP extraction, or after RLP purification by adding a suitable protease, for example, trypsin, a trypsin-like protease, a serine protease, a chymotrypsin-like protease, subtilisin.

[00126] The present invention also provides a method of producing RLPs in a plant that involves introducing one or more nucleic acid encoding one or more rotavirus structural protein operatively linked to a regulatory region active in the

plant, and one or more than one compartment targeting sequence and/or an amplification elements, into the plant, portion of the plant or plant cell. The plant, portion of the plant or plant cell is then incubated under conditions that permit the expression of the one or more nucleic acid, thereby producing the RLPs. The one or more rotavirus structural protein may be VP2, VP4 (or VP5 and VP8), VP6, VP7 a fragment of the VP2, VP4 (or VP5 and VP8), VP6, VP7 or a combination thereof.

[00127] The present invention further provides for a RLP comprising one or more rotavirus structural protein for example but not limited to VP2, VP4 (or VP5 and VP8), VP6, VP7 or a combination thereof. The RLP may be produced by one or more of the methods as provided by the present invention.

[00128] The occurrence of RLPs may be detected using any suitable method for example density gradient centrifugation or size exclusion chromatography. RLPs may be assessed for structure and size by, for example electron microscopy, or by size exclusion chromatography.

[00129] For size exclusion chromatography, total soluble proteins may be extracted from plant tissue by homogenizing (Polytron) sample of frozen-crushed plant material in extraction buffer, and insoluble material removed by centrifugation. Precipitation with ice cold acetone or PEG may also be of benefit. The soluble protein is quantified, and the extract passed through a Sephacryl™ column, for example a Sephacryl™ S500 column. Blue Dextran 2000 may be used as a calibration standard. Following chromatography, fractions may be further analyzed by immunoblot to determine the protein complement of the fraction.

[00130] The separated fraction may be for example a supernatant (if centrifuged, sedimented, or precipitated), or a filtrate (if filtered), and is enriched for proteins, or suprastructure proteins, such as for example nanotubes, nanospheres or higher-order, higher molecular weight, particles such as single-layered (sl), double-layered (dl) or triple-layered (tl) RLPs.

[00131] The separated fraction may be further processed to isolate, purify, concentrate or a combination thereof, the proteins, suprastructure proteins or higher-order particles by, for example, additional centrifugation steps, precipitation, chromatographic steps (e.g. size exclusion, ion exchange, affinity chromatography), tangential flow filtration, or a combination thereof. The presence of purified proteins,

suprastructure proteins or higher-order particles such as RLPs, may be confirmed by, for example, native or SDS-PAGE, Western analysis using an appropriate detection antibody, capillary electrophoresis, electron microscopy, or any other method as would be evident to one of skill in the art.

5 [00132] The RLP's produced according to the present invention may be purified, partially purified from a plant, portion of a plant or plant matter, or may be administered as an oral vaccine, using methods as know to one of skill in the art.

[00133] RLP purification may involve gradient centrifugation, for example sucrose, iodixanol, OptiPrep™ or cesium chloride (CsCl) density gradients may be
10 used to purify or partially purify the RLPs from transformed plant biomass. As shown for example in Figure 45, an iodixanol step gradient or iodixanol continuous gradient might be used to purify the RLP and/or expressed rotavirus structural proteins.

[00134] Calcium (Ca²⁺) concentration has been shown to be important for the triple-layer particle (TLP) to double layer particle (DLP) transformation and is strain
15 dependent (see for example Martin et al. Journal of Virology, Jan 2002, which is incorporated herein by reference). Complete loss of the outer-capsid proteins from TLPs (TLP decapsidation) takes place in the nanomolar range of [Ca²⁺]. Therefore the purification and/or extraction of RLP may be performed in the presence of calcium, and the step of gradient centrifugation may be performed in the presence of
20 calcium, for example in the present of CaCl₂. The concentration of CaCl₂ maybe between for example, 1 mM and 1000 mM, or any amount there between, such as 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 40, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 50, 600, 650, 700, 750, 800, 850, 900, 950 mM or any amount therebetween.

25 [00135] The plants, or plant fragments may be minimally processed. By the term "minimal processing" it is meant plant matter, for example, a plant or portion thereof comprising a protein of interest and /or the RLP which is partially purified to yield a plant extract, homogenate, fraction of plant homogenate or the like (i.e. minimally processed). Partial purification may comprise, but is not limited to
30 disrupting plant cellular structures thereby creating a composition comprising soluble plant components, and insoluble plant components which may be separated for example, but not limited to, by centrifugation, filtration or a combination thereof. In

this regard, proteins secreted within the extracellular space of leaf or other tissues could be readily obtained using vacuum or centrifugal extraction, or tissues could be extracted under pressure by passage through rollers or grinding or the like to squeeze or liberate the protein free from within the extracellular space. Minimal processing could also involve preparation of crude extracts of soluble proteins, since these preparations would have negligible contamination from secondary plant products. Further, minimal processing may involve aqueous extraction of soluble protein from leaves, followed by precipitation with any suitable salt. Other methods may include large scale maceration and juice extraction in order to permit the direct use of the extract. The RLPs may be purified or extracted using any suitable method for example mechanical or biochemical extraction.

[00136] The one or more rotavirus structural protein may be synthesized at an amount up to 2 g per kilogram of plant fresh weight. For example, the amount of synthesized structural protein may be between 1 and 2 g per kilogram of fresh weight, or any amount there between, such as 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2 g per kilogram of fresh weight or any amount therebetween. For example, the structural protein may be synthesized at an amount up to 1.54 g per kilogram of plant fresh weight.

[00137] Furthermore, the RLP may be synthesized at an amount up to 1.5 g per kilogram of plant fresh weight. For example, the amount of synthesized RLP may be between 0.5 and 1.5 g per kilogram of fresh weight, or any amount there between, such as 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5 g per kilogram of fresh weight. For example, the RLP may be synthesized at an amount of up to 1.1g per kilogram of plant fresh weight.

[00138] The size (i.e. the diameter) of the above-defined RLPs, maybe measures for example by dynamic light scattering (DLS) or electron microscope (EM) techniques, is usually between 50 to 110 nm, or any size therebetween. For example, the size of the intact RLP structure may range from about 70 nm to about 110 nm, or any size therebetween, such as 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 100 nm, 105 nm or any size therebetween.

[00139] The present invention further provides a nucleic acid comprising a nucleotide sequence encoding one or more rotavirus structural protein operatively

linked to a regulatory region active in a plant. The nucleotide sequence may be optimized for example for human codon usage or plant codon usage. Furthermore one or more rotavirus structural protein may be operatively linked to one or more than one amplification elements. In addition one or more rotavirus structural protein may be operatively linked to one or more than one compartment targeting sequence. The one or more rotavirus structural protein encoded by the nucleotide sequence may be for example VP2, VP4, VP6 or VP7. Furthermore the one or more rotavirus structural protein encoded by the nucleotide sequence may be for example from any rotavirus group A to G, but more preferably from rotavirus group A. Furthermore, the one or more rotavirus structural protein encoded by the nucleotide sequence maybe from any rotavirus strain having a genotype of any combinations of G- and P- types from G1 to G27 and from P1 to P34, and more preferably from G1 to G19 and from P1 to P27, including, but not limited to G1P[8], G2P[4], G2P[8], G3P[8], G4P[8], G9P[6], G9P[8], rotavirus A WA strain, rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] strain or rotavirus SA11 strain.

[00140] A nucleic acid sequence referred to in the present invention, may be “substantially homologous” , “substantially similar” or “substantially identical” to a sequence, or a compliment of the sequence if the nucleic acid sequence hybridise to one or more than one nucleotide sequence or a compliment of the nucleic acid sequence as defined herein under stringent hybridisation conditions. Sequences are “substantially homologous” “substantially similar” “substantially identical” when at least about 70%, or between 70 to 100%, or any amount therebetween, for example 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100%, or any amount therebetween, of the nucleotides match over a defined length of the nucleotide sequence providing that such homologous sequences exhibit one or more than one of the properties of the sequence, or the encoded product as described herein.

[00141] For example the present invention provides an isolated polynucleotide comprising a nucleic acid which encodes one or more rotavirus structural protein that is at least 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% 100% or any amount therebetween identical to sequences as defines for example in SEQ ID NO: 13, 14, 15, 16, 17, 18, 19, 20, 45, 46, 47, 49, 50, 51, 52, 53, 54. The polynucleotide may be human codon optimized by any of the methods known in the art.

[00142] Furthermore, the present invention provides RLPS that comprise rotavirus structural proteins that are for example encoded by nucleic acids that are at least 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% 100% or any amount therebetween identical to sequences as defines for example in SEQ ID NO: 13, 14, 15, 16, 17, 18, 19, 20, 45, 46, 47, 49, 50, 51, 52, 53, 54.

[00143] Such a sequence similarity or identity may be determined using a nucleotide sequence comparison program, such as that provided within DNASTIS (using, for example but not limited to, the following parameters: GAP penalty 5, #of top diagonals 5, fixed GAP penalty 10, k tuple 2, floating gap 10, and window size 5). However, other methods of alignment of sequences for comparison are well-known in the art for example the algorithms of Smith & Waterman (1981, Adv. Appl. Math. 2:482), Needleman & Wunsch (J. Mol. Biol. 48:443, 1970), Pearson & Lipman (1988, Proc. Nat'l. Acad. Sci. USA 85:2444), and by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and BLAST, available through the NIH.), or by manual alignment and visual inspection (see, e.g., Current Protocols in Molecular Biology, Ausubel et al., eds. 1995 supplement), or using Southern or Northern hybridization under stringent conditions (see Maniatis et al., in Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory, 1982). Preferably, sequences that are substantially homologous exhibit at least about 80% and most preferably at least about 90% sequence similarity over a defined length of the molecule.

[00144] An example of one such stringent hybridization conditions may be overnight (from about 16-20 hours) hybridization in 4 X SSC at 65°C, followed by washing in 0.1 X SSC at 65°C for an hour, or 2 washes in 0.1 X SSC at 65°C each for 20 or 30 minutes. Alternatively an exemplary stringent hybridization condition could be overnight (16-20 hours) in 50% formamide, 4 X SSC at 42°C, followed by washing in 0.1 X SSC at 65°C for an hour, or 2 washes in 0.1 X SSC at 65°C each for 20 or 30 minutes, or overnight (16-20 hours), or hybridization in Church aqueous phosphate buffer (7% SDS; 0.5M NaPO₄ buffer pH 7.2; 10 mM EDTA) at 65°C, with 2 washes either at 50°C in 0.1 X SSC, 0.1% SDS for 20 or 30 minutes each, or 2 washes at 65°C in 2 X SSC, 0.1% SDS for 20 or 30 minutes each for unique sequence regions.

[00145] A nucleic acid encoding a rotavirus structural polypeptide may be described as a "rotavirus nucleic acid", a "rotavirus nucleotide sequence", a "rotavirus nucleic acid", or a "rotavirus nucleotide sequence". For example, which is not to be considered limiting, a virus-like particle comprising one or more rotavirus structural protein or rotavirus structural polypeptide, may be described as a "rotavirus VLP", "RVLP" or "RLP".

[00146] Many organisms display a bias for use of particular codons to code for insertion of a particular amino acid in a growing peptide chain. Codon preference or codon bias, differences in codon usage between organisms, is afforded by degeneracy of the genetic code, and is well documented among many organisms. Codon bias often correlates with the efficiency of translation of messenger RNA (mRNA), which is in turn believed to be dependent on, inter alia, the properties of the codons being translated and the availability of particular transfer RNA (tRNA) molecules. The predominance of selected tRNAs in a cell is generally a reflection of the codons used most frequently in peptide synthesis. Accordingly, genes can be tailored for optimal gene expression in a given organism based on codon optimization. The process of optimizing the nucleotide sequence coding for a heterologously expressed protein can be an important step for improving expression yields. The optimization requirements may include steps to improve the ability of the host to produce the foreign protein.

[00147] "Codon optimization" is defined as modifying a nucleic acid sequence for enhanced expression in cells of interest by replacing at least one, more than one, or a significant number, of codons of the native sequence with codons that may be more frequently or most frequently used in the genes of another organism or species. Various species exhibit particular bias for certain codons of a particular amino acid.

[00148] The present invention includes synthetic polynucleotide sequences that have been codon optimized for example the sequences have been optimized for human codon usage or plant codon usage. The codon optimized polynucleotide sequences may then be expressed in plants. More specifically the sequences optimized for human codon usage or plant codon usage may be expressed in plants. Without wishing to be bound by theory, it is believed that the sequences optimized for human codon increases the guanine-cytosine content (GC content) of the sequence and improves expression yields in plants.

[00149] There are different codon-optimisation techniques known in the art for improving, the translational kinetics of translationally inefficient protein coding regions. These techniques mainly rely on identifying the codon usage for a certain host organism. If a certain gene or sequence should be expressed in this organism, the coding sequence of such genes and sequences will then be modified such that one will replace codons of the sequence of interest by more frequently used codons of the host organism.

[00150] The rotavirus structural protein or polypeptide may be expressed in an expression system comprising a viral based, DNA or RNA, expression system, for example but not limited to, a comovirus-based expression cassette and geminivirus-based amplification element.

[00151] The expression system as described herein may comprise an expression cassette based on a bipartite virus, or a virus with a bipartite genome. For example, the bipartite viruses may be of the Comoviridae family. Genera of the Comoviridae family include Comovirus, Nepovirus, Fabavirus, Cheravirus and Sadwavirus. Comoviruses include Cowpea mosaic virus (CPMV), Cowpea severe mosaic virus (CPSMV), Squash mosaic virus (SqMV), Red clover mottle virus (RCMV), Bean pod mottle virus (BPMV), Turnip ringspot virus (TuRSV), Broad bean true mosaic virus (BBtMV), Broad bean stain virus (BBSV), Radish mosaic virus (RaMV). Examples of comovirus RNA-2 sequences comprising enhancer elements that may be useful for various aspects of the invention include, but are not limited to: CPMV RNA-2 (GenBank Accession No. NC_003550), RCMV RNA-2 (GenBank Accession No. NC_003738), BPMV RNA-2 (GenBank Accession No. NC_003495), CPSMV RNA-2 (GenBank Accession No. NC_003544), SqMV RNA-2 (GenBank Accession No. NC_003800), TuRSV RNA-2 (GenBank Accession No. NC_013219.1), BBtMV RNA-2 (GenBank Accession No. GU810904), BBSV RNA2 (GenBank Accession No. FJ028650), RaMV (GenBank Accession No. NC_003800).

[00152] Segments of the bipartite comoviral RNA genome are referred to as RNA-1 and RNA-2. RNA-1 encodes the proteins involved in replication while RNA-2 encodes the proteins necessary for cell-to-cell movement and the two capsid proteins. Any suitable comovirus-based cassette may be used including CPMV, CPSMV, SqMV, RCMV, or BPMV, for example, the expression cassette may be based on CPMV.

[00153] “Expression cassette” refers to a nucleotide sequence comprising a nucleic acid of interest under the control of, and operably (or operatively) linked to, an appropriate promoter or other regulatory elements for transcription of the nucleic acid of interest in a host cell.

5 [00154] The expression systems may also comprise amplification elements from a geminivirus for example, an amplification element from the bean yellow dwarf virus (BeYDV). BeYDV belongs to the Mastreviruses genus adapted to dicotyledonous plants. BeYDV is monopartite having a single-strand circular DNA genome and can replicate to very high copy numbers by a rolling circle mechanism.
10 BeYDV-derived DNA replicon vector systems have been used for rapid high-yield protein production in plants.

[00155] As used herein, the phrase “amplification elements” refers to a nucleic acid segment comprising at least a portion of one or more long intergenic regions or long intergenic repeat (LIR) of a geminivirus genome. As used herein, “long
15 intergenic region” or “long intergenic repeat” refers to a region of a long intergenic region that contains a rep binding site capable of mediating excision and replication by a geminivirus Rep protein. In some aspects, the nucleic acid segment comprising one or more LIRs, may further comprises a short intergenic region or small intergenic region (SIR) of a geminivirus genome. As used herein, “short intergenic region” or
20 “small intergenic region” refers to the complementary strand (the short IR (SIR) of a Mastreviruses). Any suitable geminivirus-derived amplification element may be used herein. See, for example, WO2000/20557; WO2010/025285; Zhang X. et al. (2005, Biotechnology and Bioengineering, Vol. 93, 271-279), Huang Z. et al. (2009, Biotechnology and Bioengineering, Vol. 103, 706-714), Huang Z. et al.(2009,
25 Biotechnology and Bioengineering, Vol. 106, 9-17); which are herein incorporated by reference). If more than one LIR is used in the construct, for example two LIRs, then the promoter, CMPV-HT regions and the nucleic acid sequence of interest and the terminator are bracketed by each of the two LIRs. Furthermore, the amplification element might for example originate from the sequence as disclosed in Halley-Stott
30 et al. (2007) Archives of Virology 152: 1237-1240, deposited under Gen Bank accession number DQ458791, which are herein incorporated by reference. The nucleic acid segment comprising LIRs are joined nucleotides 2401 to 2566 and 1 to 128. The nucleic acid segment comprising SIRs are nucleotides 1154 to 1212.

[00156] As described herein, co-delivery of bean yellow dwarf virus (BeYDV)-derived vector and a Rep/RepA-supplying vector, by agroinfiltration of *Nicotiana benthamiana* leaves results in efficient replicon amplification and robust protein production.

5 [00157] A comovirus-based expression cassette and a geminivirus-derived amplification element may be comprised on separate vectors, or the component parts may be included in one vector. If two vectors are used, the first and second vectors may be introduced into a plant cell simultaneously, or separately.

10 [00158] A viral replicase may also be included in the expression system as described herein to increase expression of the nucleic acid of interest. An non-limiting example of a replicase is a BeYDV replicase (pREP110) encoding BeYDV Rep and RepA (C2/C1; Huang et al., 2009, *Biotechnol. Bioeng.* 103, 706-714; which is incorporated herein by reference). Another non-limiting example of a replicase is disclosed in Halley-Stott et al. (2007) *Archives of Virology* 152: 1237-1240,
15 deposited under Gen Bank accession number DQ458791, which are herein incorporated by reference. The nucleic acid segment comprising C1:C2 gene are nucleotides 1310 to 2400.

[00159] By “co-expressed” it is meant that two or more than two nucleotide sequences are expressed at about the same time within the plant, and within the same
20 tissue of the plant. However, the nucleotide sequences need not be expressed at exactly the same time. Rather, the two or more nucleotide sequences are expressed in a manner such that the encoded products have a chance to interact. The two or more than two nucleotide sequences can be co-expressed using a transient expression system, where the two or more sequences are introduced within the plant at about the
25 same time under conditions that both sequences are expressed. Alternatively, a platform plant comprising one of the nucleotide sequences may be transformed in a stable manner, with an additional sequence encoding the protein of interest for example one or more rotavirus structural protein, introduced into the platform plant in a transient manner.

30 [00160] Correct folding of the protein may be important for stability of the protein, formation of multimers, formation of RLPs and function. Folding of a protein may be influenced by one or more factors, including, but not limited to, the

sequence of the protein, the relative abundance of the protein, the degree of intracellular crowding, the availability of cofactors that may bind or be transiently associated with the folded, partially folded or unfolded protein. Furthermore the compartment or sub-compartment within the plant where the protein is expressed may influence the expression levels and folding of the protein.

[00161] The expression of the one or more rotavirus structural protein may be targeted to specific plant cell compartment and/or sub-compartments by agroinfiltration in transgenic plants. The compartment or sub-compartments may be for example plastids, endoplasmic reticulum (ER), chloroplast or apoplast. Without wishing to be bound by theory, compartment or sub-compartments targeting may increase protein accumulation into the targeted compartment or sub-compartments over cytoplasmic accumulation. Compartment or sub-compartment accumulation may protect protein from degradation by proteases present in the cytoplasm and/or allow it to accumulate to higher concentration without affecting the function of the plant cell.

[00162] Therefore, the expression cassette or vector may be adapted to direct the vector or rotavirus structural protein or polypeptide expressed from the vector to the desired compartment or sub-compartment in the plant.

[00163] For example the expression cassette or vector may be adapted to target plastids by causing an expressed rotavirus structural protein or polypeptide to include a portion capable of interacting with the thylakoid membranes of the plastids, in particular the transfer mechanism of the thylakoid membranes. This interaction may cause the rotavirus structural protein or polypeptide to be imported into the plastid from the cytoplasm where it is expressed. Without wishing to be bound by theory, the mechanism of importation from the cytoplasm may be important for proper folding of the proteins. It will be appreciated that the expression cassette or vector may be adapted to target the plastids themselves to become transformed and expression of the rotavirus structural protein or polypeptide may occur wholly within the plastid.

[00164] By the term "targeting sequence" it is meant that the targeting sequences may be included in the vector or expression cassette. Such targeting sequences may be translated into a peptide which directs the vector or product thereof to the desired compartment or sub-compartment in the plant, such as a plastid. For example, plastid signal peptides (also referred to as "plastid transit peptides" in the

art) for targeting proteins into plastids are known in the art. A non limiting example of a plastid transit peptide that may be used is that of *rbcs1-cTP*. As suitable example of a chloroplast-transit peptide sequence is the Rubisco small-subunit gene (*rbcs1*), for example, from *Solanum tuberosum*.

5 [00165] Therefore, the rotavirus structural protein or polypeptide may include a signal peptide that is the same as, or heterologous with, the remainder of the polypeptide or protein. The term "signal peptide" is well known in the art and refers generally to a short (about 5-30 amino acids) sequence of amino acids, found generally at the N-terminus of a polypeptide that may direct translocation of the
10 newly-translated polypeptide to a particular organelle, or aid in positioning of specific domains of the polypeptide chain relative to others. As a non-limiting example, the signal peptide may target the translocation of the protein into the endoplasmic reticulum and/or aid in positioning of the N-terminus proximal domain relative to a membrane-anchor domain of the nascent polypeptide to aid in cleavage and folding of
15 the mature protein, for example which is not to be considered limiting, a rotavirus structural protein.

[00166] A signal peptide (SP) may be native to the protein or virus protein, or a signal peptide may be heterologous with respect to the primary sequence of the protein or virus protein being expressed. For example the native signal peptide of
20 rotavirus structural protein may be used to express the rotavirus structural protein in a plant system.

[00167] A signal peptide may also be non-native, for example, from a protein, viral protein or native structural protein of a virus other than rotavirus protein, or from a plant, animal or bacterial polypeptide. A non limiting example of a signal
25 peptide that may be used is that of alfalfa protein disulfide isomerase (PDISP) (nucleotides 32-103 of Accession No. Z11499). Furthermore, the signal peptide may be completely deleted or truncated. By truncation or truncated it is meant that 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100% or any amount therebetween of amino acid residues are
30 deleted from the signal peptide. Preferably, the truncated amino acid residues are continuous, and the truncation occurs from the second methionine onward

[00168] The present invention therefore provides for a rotavirus structural protein, such as for example VP2, VP4, VP6 and/or VP7, comprising a native, a non-native signal peptide or truncated signal peptide, and nucleic acids encoding such rotavirus structural proteins.

5 [00169] The one or more than one genetic constructs of the present invention may be expressed in any suitable plant host that is transformed by the nucleotide sequence, or constructs, or vectors of the present invention. Examples of suitable hosts include, but are not limited to, agricultural crops including alfalfa, canola, Brassica spp., maize, Nicotiana spp., potato, ginseng, pea, oat, rice, soybean, wheat,
10 barley, sunflower, cotton and the like.

[00170] The nucleotide sequences encoding for the rotavirus structural proteins may be transferred into the plant host using 1, 2, 3, 4 or 5 binary plasmid vectors. Each binary plasmid vector may therefore contain 1, 2, 3, 4 or 5 nucleotide sequences encoding for a rotavirus structural protein.

15 [00171] The one or more genetic constructs of the present invention can further comprise a 3' untranslated region. A 3' untranslated region refers to that portion of a gene comprising a DNA segment that contains a polyadenylation signal and any other regulatory signals capable of effecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by effecting the addition of
20 polyadenylic acid tracks to the 3' end of the mRNA precursor. Polyadenylation signals are commonly recognized by the presence of homology to the canonical form 5' AATAAA-3' although variations are not uncommon. Non-limiting examples of suitable 3' regions are the 3' transcribed nontranslated regions containing a polyadenylation signal of Agrobacterium tumor inducing (Ti) plasmid genes, such as
25 the nopaline synthase (NOS) gene, plant genes such as the soybean storage protein genes, the small subunit of the ribulose-I, 5-bisphosphate carboxylase gene (ssRUBISCO; US 4,962,028; which is incorporated herein by reference), the promoter used in regulating plastocyanin expression, described in US 7,125,978 (which is incorporated herein by reference).

30 [00172] One or more of the genetic constructs of the present invention may also include further enhancers, either translation or transcription enhancers, as may be required. Enhancers may be located 5' or 3' to the sequence being transcribed.

Enhancer regions are well known to persons skilled in the art, and may include an ATG initiation codon, adjacent sequences or the like. The initiation codon, if present, may be in phase with the reading frame ("in frame") of the coding sequence to provide for correct translation of the transcribed sequence.

5 [00173] The constructs of the present invention can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, micro-injection, electroporation, etc. For reviews of such techniques see for example Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academy Press, New York VIII, pp. 421-463 (1988); Geierman and Corey, *Plant Molecular Biology*, 10 2d Ed. (1988); and Miki and Iyer, *Fundamentals of Gene Transfer in Plants*. In *Plant Metabolism*, 2d Ed. DT. Dennis, DH Turpin, DD Lefebvre, DB Layzell (eds), Addison Wesley, Langmans Ltd. London, pp. 561-579 (1997). Other methods include direct DNA uptake, the use of liposomes, electroporation, for example using protoplasts, micro-injection, microprojectiles or whiskers, and vacuum infiltration. 15 See, for example, Bilang, et al. (*Gene* 100: 247-250 (1991), Scheid et al. (*Mol. Gen. Genet.* 228: 104-112, 1991), Guerche et al. (*Plant Science* 52: 111-116, 1987), Neuhauser et al. (*Theor. Appl. Genet.* 75: 30-36, 1987), Klein et al., *Nature* 327: 70-73 (1987); Howell et al. (*Science* 208: 1265, 1980), Horsch et al. (*Science* 227: 1229-1231, 1985), DeBlock et al., *Plant Physiology* 91: 694-701, 1989), *Methods for Plant* 20 *Molecular Biology* (Weissbach and Weissbach, eds., Academic Press Inc., 1988), *Methods in Plant Molecular Biology* (Schuler and Zielinski, eds., Academic Press Inc., 1989), Liu and Lomonossoff (*J. Virol. Meth.* 105:343-348, 2002.), U.S. Pat. Nos. 4,945,050; 5,036,006; and 5,100,792, U.S. patent application Ser. Nos. 08/438,666, filed May 10, 1995, and 07/951,715, filed Sep. 25, 1992, (all of which are hereby 25 incorporated by reference).

Transient expression

[00174] Without wishing to be bound by theory, the protein concentration and ratio of the different rotavirus structural proteins may be important for the assembly efficiency of RLPs. Therefore multiplicity and time of infection, may be important to 30 manipulate protein concentration and the overall assembly efficiency of RLPs in plants.

[00175] The construct of the present invention may be transiently expressed in a plants or portion of a plant. A transient expression system relying on the epichromosomal expression of recombinant *Agrobacterium tumefaciens* in a plant, portion of a plant or plant cell may be used to express the rotavirus structural protein, targeted to various cell compartments or sub-compartments. A transient expression system allows for a high production speed. Furthermore, large amounts of protein can be attained within a few days after infiltration of recombinant *Agrobacterium* in plants (Rybicki, 2010; Fischer et al., 1999). It is also possible to express long gene sequences and have more than one gene simultaneously expressed in the same cell, allowing for efficient assembly of multimeric proteins (Lombardi et al., 2009).

[00176] The nucleotide sequences encoding for the rotavirus structural proteins may be transferred into the plant host into 1, 2, 3, 4 or 5 transformed *Agrobacterium tumefaciens* strain.

[00177] However, during transient expression post-transcriptional gene silencing may limit the expression of the heterologous proteins in plants. The co-expression of a suppressor of silencing, for example, but not limited to Nss from Tomato spotted wilt virus may be used to counteract the specific degradation of transgene mRNAs (Brigneti et al., 1998). Alternate suppressors of silencing are well known in the art and may be used as described herein (Chiba et al., 2006, Virology 346:7-14; which is incorporated herein by reference), for example but not limited to HcPro, TEV -p1/HC-Pro (Tobacco etch virus-p1/HC-Pro), BYV -p21, p19 of Tomato bushy stunt virus (TBSV p19), capsid protein of Tomato crinkle virus (TCV -CP), 2b of Cucumber mosaic virus; CMV-2b), p25 of Potato virus X (PVX-p25), p11 of Potato virus M (PVM-p11), p11 of Potato virus S (PVS-p11), p16 of Blueberry scorch virus, (BScV -p16), p23 of Citrus tristexa virus (CTV-p23), p24 of Grapevine leafroll-associated virus-2, (GLRaV-2 p24), p10 of Grapevine virus A, (GVA-p10), p14 of Grapevine virus B (GVB-p14), p10 of Heracleum latent virus (HLV-p10), or p16 of Garlic common latent virus (GCLV-p16). Therefore, a suppressor of silencing, for example HcPro, TEV -p1/HC-Pro, BYV-p21, TBSV p19, TCV-CP, CMV-2b, PVX-p25, PVM-p11, PVS-p11, BScV-p16, CTV-p23, GLRaV-2 p24, GBV-p14, HLV-p10, GCLV-p16or GVA-p10, may be co-expressed along with one or more rotavirus structural protein for example VP2, VP4, VP6, or a combination

thereof, to further ensure high levels of protein production within a plant or portion of a plant.

[00178] The present invention also provides a methods as described above, wherein an additional (second, third, fourth, or fifth) nucleotide sequence is expressed within the plant, the additional (second, third, fourth, or fifth) nucleotide sequence encoding a suppressor of silencing is operatively linked with an additional (second, third, fourth, or fifth) regulatory region that is active in the plant. The nucleotide sequence encoding a suppressor of silencing may be, for example Nss, HcPro, TEV -p1/HC-Pro, BYV-p21, TBSV p19, TCV-CP, CMV-2b, PVX-p25, PVM-p11, PVS-p11, BScV-p16, CTV-p23, GLRaV-2 p24, GBV-p14, HLV-p10, GCLV-p16 or GVA-p10.

[00179] As described below, transient expression methods may be used to express the constructs of the present invention (see Liu and Lomonossoff, 2002, Journal of Virological Methods, 105:343-348; which is incorporated herein by reference). Alternatively, a vacuum-based transient expression method, as described by Kapila et al., 1997, which is incorporated herein by reference) may be used. These methods may include, for example, but are not limited to, a method of Agro-inoculation or Agro-infiltration, syringe infiltration, however, other transient methods may also be used as noted above. With Agro-inoculation, Agro-infiltration, or syringe infiltration, a mixture of Agrobacteria comprising the desired nucleic acid enter the intercellular spaces of a tissue, for example the leaves, aerial portion of the plant (including stem, leaves and flower), other portion of the plant (stem, root, flower), or the whole plant. After crossing the epidermis the Agrobacteria infect and transfer t-DNA copies into the cells. The t-DNA is episomally transcribed and the mRNA translated, leading to the production of the protein of interest in infected cells, however, the passage of t-DNA inside the nucleus is transient.

[00180] To aid in identification of transformed plant cells, the constructs of this invention may be further manipulated to include plant selectable markers. Useful selectable markers include enzymes that provide for resistance to chemicals such as an antibiotic for example, gentamycin, hygromycin, kanamycin, or herbicides such as phosphinothrycin, glyphosate, chlorosulfuron, and the like. Similarly, enzymes providing for production of a compound identifiable by colour change such as GUS (beta-glucuronidase), or luminescence, such as luciferase or GFP, may be used.

[00181] Also considered part of this invention are transgenic plants, plant cells or seeds containing the gene construct of the present invention. Methods of regenerating whole plants from plant cells are also known in the art. In general, transformed plant cells are cultured in an appropriate medium, which may contain selective agents such as antibiotics, where selectable markers are used to facilitate identification of transformed plant cells. Once callus forms, shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the shoots transferred to rooting medium for regeneration of plants. The plants may then be used to establish repetitive generations, either from seeds or using vegetative propagation techniques. Transgenic plants can also be generated without using tissue cultures.

[00182] The use of the terms "regulatory region", "regulatory element" or "promoter" in the present application is meant to reflect a portion of nucleic acid typically, but not always, upstream of the protein coding region of a gene, which may be comprised of either DNA or RNA, or both DNA and RNA. When a regulatory region is active, and in operative association, or operatively linked, with a gene of interest, this may result in expression of the gene of interest. A regulatory element may be capable of mediating organ specificity, or controlling developmental or temporal gene activation. A "regulatory region" may includes promoter elements, core promoter elements exhibiting a basal promoter activity, elements that are inducible in response to an external stimulus, elements that mediate promoter activity such as negative regulatory elements or transcriptional enhancers. "Regulatory region", as used herein, may also includes elements that are active following transcription, for example, regulatory elements that modulate gene expression such as translational and transcriptional enhancers, translational and transcriptional repressors, upstream activating sequences, and mRNA instability determinants. Several of these latter elements may be located proximal to the coding region.

[00183] In the context of this disclosure, the term "regulatory element" or "regulatory region" typically refers to a sequence of DNA, usually, but not always, upstream (5') to the coding sequence of a structural gene, which controls the expression of the coding region by providing the recognition for RNA polymerase and/or other factors required for transcription to start at a particular site. However, it is to be understood that other nucleotide sequences, located within introns, or 3' of the

sequence may also contribute to the regulation of expression of a coding region of interest. An example of a regulatory element that provides for the recognition for RNA polymerase or other transcriptional factors to ensure initiation at a particular site is a promoter element. Most, but not all, eukaryotic promoter elements contain a TATA box, a conserved nucleic acid sequence comprised of adenosine and thymidine nucleotide base pairs usually situated approximately 25 base pairs upstream of a transcriptional start site. A promoter element comprises a basal promoter element, responsible for the initiation of transcription, as well as other regulatory elements (as listed above) that modify gene expression.

[00184] There are several types of regulatory regions, including those that are developmentally regulated, inducible or constitutive. A regulatory region that is developmentally regulated, or controls the differential expression of a gene under its control, is activated within certain organs or tissues of an organ at specific times during the development of that organ or tissue. However, some regulatory regions that are developmentally regulated may preferentially be active within certain organs or tissues at specific developmental stages, they may also be active in a developmentally regulated manner, or at a basal level in other organs or tissues within the plant as well. Examples of tissue-specific regulatory regions, for example see-specific a regulatory region, include the napin promoter, and the cruciferin promoter (Rask et al., 1998, J. Plant Physiol. 152: 595-599; Bilodeau et al., 1994, Plant Cell 14: 125-130). An example of a leaf-specific promoter includes the plastocyanin promoter (see US 7,125,978, which is incorporated herein by reference).

[00185] An inducible regulatory region is one that is capable of directly or indirectly activating transcription of one or more DNA sequences or genes in response to an inducer. In the absence of an inducer the DNA sequences or genes will not be transcribed. Typically the protein factor that binds specifically to an inducible regulatory region to activate transcription may be present in an inactive form, which is then directly or indirectly converted to the active form by the inducer. However, the protein factor may also be absent. The inducer can be a chemical agent such as a protein, metabolite, growth regulator, herbicide or phenolic compound or a physiological stress imposed directly by heat, cold, salt, or toxic elements or indirectly through the action of a pathogen or disease agent such as a virus. A plant cell containing an inducible regulatory region may be exposed to an inducer by

externally applying the inducer to the cell or plant such as by spraying, watering, heating or similar methods. Inducible regulatory elements may be derived from either plant or non-plant genes (e.g. Gatz, C. and Lenk, L.R.P., 1998, Trends Plant Sci. 3, 352-358; which is incorporated by reference). Examples, of potential inducible promoters include, but not limited to, tetracycline-inducible promoter (Gatz, C., 1997, Ann. Rev. Plant Physiol. Plant Mol. Biol. 48, 89-108; which is incorporated by reference), steroid inducible promoter (Aoyama, T. and Chua, N.H., 1997, Plant 1. 2, 397-404; which is incorporated by reference) and ethanol-inducible promoter (Salter, M.G., et al, 1998, Plant Journal 16, 127-132; Caddick, M.X., et al, 1998, Nature Biotech. 16, 177-180, which are incorporated by reference) cytokinin inducible IB6 and CKI 1 genes (Brandstatter, I. and Kieber, J.J., 1998, Plant Cell 10, 1009-1019; Kakimoto, T., 1996, Science 274, 982-985; which are incorporated by reference) and the auxin inducible element, DR5 (Ulmasov, T., et al., 1997, Plant Cell 9, 1963-1971; which is incorporated by reference).

[00186] A constitutive regulatory region directs the expression of a gene throughout the various parts of a plant and continuously throughout plant development. Examples of known constitutive regulatory elements include promoters associated with the CaMV 35S transcript (Odell et al., 1985, Nature, 313: 810-812), the rice actin 1 (Zhang et al, 1991, Plant Cell, 3: 1155-1165), actin 2 (An et al., 1996, Plant J., 10: 107-121), or tms 2 (U.S. 5,428,147, which is incorporated herein by reference), and triosephosphate isomerase 1 (Xu et al., 1994, Plant Physiol. 106: 459-467) genes, the maize ubiquitin 1 gene (Cornejo et al, 1993, Plant Mol. Biol. 29: 637-646), the Arabidopsis ubiquitin 1 and 6 genes (Holtorf et al, 1995, Plant Mol. Biol. 29: 637-646), and the tobacco translational initiation factor 4A gene (Mandel et al, 1995, Plant Mol. Biol. 29: 995-1004).

[00187] The term "constitutive" as used herein does not necessarily indicate that a gene under control of the constitutive regulatory region is expressed at the same level in all cell types, but that the gene is expressed in a wide range of cell types even though variation in abundance is often observed. Constitutive regulatory elements may be coupled with other sequences to further enhance the transcription and/or translation of the nucleotide sequence to which they are operatively linked. For example, the CPMV-HT system is derived from the untranslated regions of the Cowpea mosaic virus (CPMV) and demonstrates enhanced translation of the

associated coding sequence. By "native" it is meant that the nucleic acid or amino acid sequence is naturally occurring, or "wild type". By "operatively linked" it is meant that the particular sequences, for example a regulatory element and a coding region of interest, interact either directly or indirectly to carry out an intended function, such as mediation or modulation of gene expression. The interaction of operatively linked sequences may, for example, be mediated by proteins that interact with the operatively linked sequences.

[00188] The RLP produced within a plant may induce a rotavirus VP7 structural protein comprising plant-specific N-glycans. Therefore, this invention also provides for a RLP comprising VP7 having plant specific N-glycans.

[00189] Furthermore, modification of N-glycan in plants is known (see for example U.S. 60/944,344; which is incorporated herein by reference) and VP7 having modified N-glycans may be produced. VP7 comprising a modified glycosylation pattern, for example with reduced fucosylated, xylosylated, or both, fucosylated and xylosylated, N-glycans may be obtained, or VP7 having a modified glycosylation pattern may be obtained, wherein the protein lacks fucosylation, xylosylation, or both, and comprises increased galactosylation. Furthermore, modulation of post-translational modifications, for example, the addition of terminal galactose may result in a reduction of fucosylation and xylosylation of the expressed VP7 when compared to a wild-type plant expressing VP7.

[00190] For example, which is not to be considered limiting, the synthesis of VP7 having a modified glycosylation pattern may be achieved by co-expressing VP7 along with a nucleotide sequence encoding beta-1.4 galactosyltransferase (GalT), for example, but not limited to mammalian GalT, or human GalT however GalT from another sources may also be used. The catalytic domain of GalT may also be fused to a CTS domain (i.e. the cytoplasmic tail, transmembrane domain, stem region) of N-acetylglucosaminyl transferase (GNT1), to produce a GNT1-GalT hybrid enzyme, and the hybrid enzyme may be co-expressed with VP7. The VP7 may also be co-expressed along with a nucleotide sequence encoding N-acetylglucosaminyl transferase III (GnT-III), for example but not limited to mammalian GnT-III or human GnT-III, GnT-III from other sources may also be used. Additionally, a GNT1-GnT-III hybrid enzyme, comprising the CTS of GNT1 fused to GnT-III may also be used.

[00191] Therefore the present invention also provides RLPs comprising VP7 having modified N-glycans.

[00192] Without wishing to be bound by theory, the presence of plant N-glycans on VP7 may stimulate the immune response by promoting the binding of VP7 by antigen presenting cells. Stimulation of the immune response using plant N-glycan has been proposed by Saint-Jore-Dupas et al. (2007).

[00193] The present invention will be further illustrated in the following examples.

Examples

Example 1

Expression of rotavirus proteins and production of VLPs in *N. benthamiana* plant leaves

[00194] The following analysis utilized rotavirus capsid proteins from the G9P[6] rotavirus strain, and assessed whether rotavirus-like particles were formed in the various compartments of tobacco *N. benthamiana* leaf cells. Co-expression of VP2 and VP6 as well as various combinations of VP2, VP6, VP7 and VP4 in tobacco plant leaves were investigated.

Materials and Methods

Plasmid construction

[00195] Plant codon optimized rotavirus cDNAs for VP2, VP4, VP6 and VP7 were supplied by Geneart, Germany. The plasmid DNA was transformed into DH5- α chemically competent *E. coli* cells (*E. coli*TM, Lucigen) as per the manufacturer's instructions. Novel binary *Agrobacterium* vectors pTRAc (cytoplasm), pTRAc-rbcs1-cTP (chloroplast targeting) and pTRAc-ERH (endoplasmic reticulum targeting) supplied by Rainer Fischer (Fraunhofer Institute for Molecular Biology and Applied Ecology, IME, Germany) were used in this study. An additional vector, pTRAc-A (apoplast), was derived from the modification of pTRAc-ERH by restriction enzyme (RE) digestion at sites NcoI and XhoI in the multiple cloning site (Figure 3). This removes the histidine tag and KDEL sequence which retain proteins in the ER. The proteins are instead targeted to the apoplast.

[00196] VP2, VP4 and VP6 cDNA was restriction enzyme (RE) digested with NcoI/XhoI while VP7 was cut with AflIII/XhoI. Restriction enzymes AflIII, NcoI and MluI have compatible sticky ends. For direct cloning of the DNA into pTRAc, pTRAc-rbcs-cTP and pTRAc-A, the vectors were each RE digested at sites AflIII/XhoI, MluI/XhoI and NcoI/XhoI, respectively. Cloning of DNA in the vectors was carried out as per standard protocol followed by transformation into chemically competent *E. coli* DH5- α cells (*E. cloni*TM, Lucigen). Selected recombinant colonies were verified by colony PCR. For cloning in pTRAc-ERH, a NotI restriction enzyme site was added to replace the stop codon of each the four rotavirus cDNA by PCR amplification. The cDNA was amplified with primers as detailed in Table 1. The PCR reaction conditions included denaturation at 95°C for 5 min, followed by five cycles of denaturation at 95°C for 30 sec, annealing at 52°C for 1 min, and elongation at 72°C for 1.5 min. A further 20 cycles were done as follows; 95°C for 30 sec, 57°C for 1 min, 72°C for 1.5 min and 72°C for 5 min. The amplified fragments were then cloned into pGEM-T-Easy (Promega) as per the manufacturer's instructions. Transformation was carried out in chemically competent *E. coli* DH5- α (*E. cloni*TM, Lucigen). Colony PCR was then carried out on selected colonies as done for the other three constructs.

Table 1: Rotavirus cDNA primers for ER vector cloning

Primer	Sequence	R.E site added	Orientation
VP2F	5'-TTCCATGGCTTACCGTAAAGG-3'	-	SEQ ID NO: 5 Forward
VP2R	5'-ATGCGGCCGCAAGCTCGTTCATAATCCTCATG-3'	NotI	SEQ ID NO: 6 Reverse
VP4F	5'-TTCCATGGCTTCCCTCATCTAC-3'	-	SEQ ID NO: 7 Forward
VP4R	5'-ATGCGGCCGCAAGACGGCACTGGAGAATGAG-3'	NotI	SEQ ID NO: 8 Reverse
VP6F	5'-TTCCATGGATGTGCTCTACTC-3'	-	SEQ ID NO: 9 Forward
VP6R	5'-ATGCGGCCGCCCTTCACGAGCATGGAACG-3'	NotI	SEQ ID NO: 10 Reverse
VP7F	5'-GTACATGTACGGAATCGAGTAC-3'	-	SEQ ID NO: 11 Forward
VP7R	5'-ATGCGGCCGCCACACGGTAGTAGAAAGCAGC-3'	NotI	SEQ ID NO: 12 Reverse

[00197] The pGEM-VP DNAs from positive colonies were sequenced to verify fidelity of the PCR. DNA was digested with NcoI/NotI and the appropriate DNA fragment cloned into pTRAc-ERH at sites NcoI and NotI to form pTRAc-ERH-VP. Transformation was then carried out into *E. coli* DH5- α cells as previously done. Colony PCR was also performed to check for rotavirus DNA in selected colonies.

Agrobacterium transformation

[00198] Agrobacterium tumefaciens GV3101 strain was provided by Professor Rainer Fischer (Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Aachen, Germany) and made electrocompetent as previously described (Shen and Forde, 1989). Three hundred nanograms of isolated rotavirus pTRA-VP constructs were mixed with 100 µl of electrocompetent GV3101 cells in a 0.1 cm electrogap cuvette (BioRad™) then electroporated in a GenePulser (BioRad) under the following settings: 1.8 kV, 25 µF and 200 Ω. Incubation was allowed for 1 hr at 27°C in 900 µl of LB before plating on LA plates containing 50 µg/ml carbenicillin (carb), 30 µg/ml kanamycin (kan) and 50 µg/ml rifampicin (rif). The plates were incubated at 27°C for 3 days. To check for positive transformants, plasmid DNA was isolated from the recombinant Agrobacterium colonies and back-transformed into E. coli competent DH5-α cells. These were then selected on 100 µg/ml ampicillin (amp) LA. Colony PCR and restriction enzyme digests on cDNA were done to verify successful transformants. Glycerol stocks of relevant recombinant Agrobacterium were made and stored at -70 °C.

Recombinant Agrobacterium infiltration

[00199] A. tumefaciens LBA 4404 (pBIN-NSs) used in this study was obtained from Marcel Prins (Laboratory of Virology, Wageningen University, Binnenhaven, Netherlands). It contains the NSs silencing suppressor found in Tomato spotted wilt virus (TSWV). Recombinant Agrobacterium (pTRA-VPs) from glycerol stocks were grown at 27 °C overnight in LB with 50 µg/ml carb, 30 µg/ml kan and 50 µg/ml rif. The recombinant Agrobacterium and LBA4404 (pBIN-NSs) were then each inoculated in induction medium (LB, 10 mM 2-(N-morpholino) ethanesulphonic acid MES, 2 mM MgSO₄, 20 µM acetosyringone, 50 µg/ml carb, 30 µg/ml kan and 50 µg/ml rif, and pH 5.6).

[00200] Cultures were incubated at 27°C overnight. Agrobacterium cells were collected by centrifugation at 4000 rpm for 5 min at 4°C and then resuspended in 2 ml infiltration medium (10 mM MES, 10 mM MgCl₂, 3 % sucrose, pH 5.6, 200 µM acetosyringone and sterile water). Optical density (OD₆₀₀) of the cells was verified and diluted with infiltration medium to obtain an OD₆₀₀ of 0.25. For each pTRA-VP construct, LBA4404 was mixed with recombinant Agrobacterium to final OD₆₀₀ of

0.5. For co-expression studies, each construct was added to total an OD600 of 0.5, for example; VP2 – 0.25 and VP6 – 0.25, until the OD600 for mixture equalled 0.5. Acetosyringone used in the induction and infiltration medium helps in activation of vir genes in *Agrobacterium*.

5 [00201] Wounded plant cells release phenolic compounds which are detected by Vir A and Vir G genes in *Agrobacterium* subsequently leading to induction of protein expression in the host cells (Zupan, J. et al., 2000). The cells were then incubated at room temperature for 1 h to allow acetosyringone to induce vir genes. Three week old wild type *N. benthamiana* plants were infiltrated with recombinant
10 *Agrobacterium* expressing the VP proteins. This involved either vacuum infiltration of whole plants or injection of recombinant *Agrobacterium* (pTRA-VP) into the abaxial air spaces on the ventral side of plant leaves. Recombinant *agrobacterium* was infiltrated either with or without the silencing suppressor LBA 4404 (pBIN-NSs).

15 [00202] Initially, 2 ml of *Agrobacterium* infiltration medium suspension was injected into each plant using a syringe per construct. One plant was used per construct over a seven day time trial. Co-expression of rotavirus proteins was also carried out in which VP2, VP6 and VP4 were simultaneously expressed in the cytoplasm of *N. benthamiana* plant leaves. Combinations investigated were VP2/6 and VP2/6/4. VP4 “spike” protein may bind to VP6, and thus there is a possibility that
20 they could add to RLP structures. VP7 cloning was attempted but toxicity issues to host cells proved to be a problem. Recombinant VP7 *Agrobacterium* killed leaf cells within a day after infiltration. Several methods were tried to evade this such as infiltrating plants at a low temperature of 17° C and infiltrating after day 3 and/or day 5 of the time trials. As such, VP7 was omitted in the co-expression studies due to its
25 toxic nature in the tobacco plants.

Protein Extraction

30 [00203] Whole leaves or two leaf discs per construct were harvested and ground in liquid nitrogen. Ground leaf matter was resuspended in sterile PBS containing Complete Protease Inhibitor (EDTA-free; Roche). This was then centrifuged for 5 min at 13000 rpm and the pellets (plant leaf matter) discarded. 100 µl of each construct were then mixed with 5X SDS-PAGE loading buffer and boiled for 2 min at 95 °C, ready for further analysis on SDS-PAGE gels and western blots.

The rest of the samples were stored at -20 °C for future use. Figure 4 shows an overview of the cloning and infiltration procedure for the rotavirus cDNA.

Apoplast protein extraction

[00204] An additional extraction procedure was carried out on the pTRAc-A, apoplast constructs. The apoplast is the free diffusional space between the plasma membrane and cell walls of plant cells (Figure 5a). Proteins expressed in the cytoplasm have an export sequence that targets them to the apoplast hence they accumulate here. In the extraction procedure that followed, whole leaves from each extraction day were either vacuum or injection infiltrated with sterile PBS containing Complete Protease Inhibitor. For vacuum infiltration, individual plant leaves were suspended in PBS and put under a vacuum at 100 mbar for 10 min in a vacuum tank. The leaves were then rolled and gently placed in spin columns (similar to Qiagen spin columns) with a hole at the bottom (Figure 5b2). The holes allow easy passage of fluid from the leaves without allowing solid leaf matter to pass through. The spin columns were placed in 2 ml Eppendorf tubes and centrifugation carried out at 4000 rpm for 15 min (Figure 5 b3). The filtrate was collected and protein loading dye for SDS-PAGE gels and western blot analysis was added to 100 µl of each filtrate sample.

Western Blots and Coomassie stains

[00205] Western blots and Coomassie blue stained SDS-PAGE gels were used as previously described. Mouse anti-rotavirus VP6 antibody (US Biologicals) (1:5000), anti-mouse histidine tag antibody (Sigma®) (1:2000), chicken anti-VP2 and chicken anti-VP4 serum (1:2000) were used to probe each of the respective proteins in western blots. Coomassie blue stained SDS-PAGE gels were used to quantify proteins by density scanning of bands using a Syngene Gel Documentation System.

Electron Microscopy

[00206] To determine whether expressed proteins assembled into RLPs, transmission electron microscopy (TEM) of immuno-trapped particles was performed on day 3 of expression of cytoplasm expressed VP6, VP2/6 and VP2/6/4, all in the presence of a silencing suppressor Nss. Glow discharged carbon/copper grids were placed on 20 µl of mouse anti-rotavirus VP6 antibody (1:5000) for 5 min and then washed 3 times with sterile distilled water. The grids were then placed on 10 µl of the

protein extracts and left for 2 min before being washed 3 times again with sterile distilled water. Finally, the grids were floated on 20 µl of 2 % uranyl acetate for 1 min before viewing under a TEM (Zeiss 912 OMEGA Energy Filter Transmission Electron Microscope, University of Cape Town).

5 [00207] For samples isolated from sucrose gradients, the sucrose first had to be removed by dialysis before immune-trapping on the copper grids. If not removed, sucrose crystals inhibit definitive viewing of samples under the TEM as it forms crystals on the grids, disrupting the structure of bound carbon and material. The sucrose fractions were placed in 10 000 MW dialysis cassettes and dialyzed in sterile
10 PBS containing 0.4 M NaCl for 4 hr before exchanging the buffer and leaving it overnight at 4 °C with stirring. Since volume increases with dialysis, the protein samples required concentrating. The samples were vacuum freeze dried for 3 hours and resuspended in 2 ml of sterile PBS, ready for further analysis.

Sucrose gradient purification of RLPs

15 [00208] Plant protein extracts were initially filtered through miracloth to remove solid plant matter. Sucrose gradients from 10 to 60 % sucrose were set up in 40ml tubes each by creating six layers of 5 ml of sucrose dissolved in sterile PBS (pH 7.4). Clarified protein samples in 5 to 10 ml volumes were then loaded on top of each gradient column.

20 [00209] Ultracentrifugation at 150 000 g (SWTi28 swinging bucket rotor, Beckman Coulter) was carried out at 4 °C for 1 h 30 min. At the end of the centrifugation, 2 ml fractions were collected from the bottom of each column by tube puncture. Dot blots were then performed to determine fractions with proteins of interest. For each fraction, 1 µl of sample was loaded in a grid on a nitrocellulose
25 membrane, which was then blocked with BSA blocking buffer. Western blot analysis was then performed as usual. Proteins were probed with mouse anti-VP6 antibody (1:5000) for VP6 or chicken anti-VP2 and VP4 serum (1:5000) for the other two proteins.

Total Soluble Protein Assay

30 [00210] Total soluble protein (TSP) was determined by Bradford assays. This was carried out to compare the levels of accumulated proteins in cytoplasm co-expressed VP2/6. The protein IgG (1.43mg/ml stock) was used in a dilution series as a

standard. 5 µl of standard and samples were each added to a clean dry microtitre plate. Total Soluble Protein Reagents A and B were added as per manufacturer's instructions (Bio-Rad Dc Protein Assay). All experiments were done in triplicate. Absorbance readings were recorded at 750 nm using a microplate reader (Bio-tek PowerWave XS).

Results

Expression of VP6 in plant leaf cell compartments

[00211] VP6 was expressed and targeted to all cellular compartments (Figure 6; (the line marks VP6, at ~42 kDa)), with and without the silencing suppressor. In the cytoplasm, protein was expressed from day one of the time trial, with increasing protein accumulation in the cytoplasm during the week-long trial (Figure 6a). In the ER, protein accumulation was clearly seen at day 3 only (Figure 6b). The protein ran at a higher band size (approximately 11 kDa more) than the other proteins. This may be a result of the 6 histidine-tag added to the C-terminal end of the protein, as well as the cleavage site (refer to pProEx vector sequence)

[00212] Protein accumulation in the chloroplasts occurred between days 1 and 3 (Figure 6 "chloroplasts"). The silencing suppressor had an effect on the proteins as no proteins were detected in its absence. There was no protein expression at days 5 and 7. The apoplast, just as in the ER, had the best protein accumulation between days 3 and 5 of the time trial (Figure 6 "apoplast") and none at all at day 1 and day 7. The silencing suppressor had a positive effect especially on day 3, resulting in higher protein detection levels compared to when it was left out. It can also be seen that two bands are visible at the ~40 kDa mark probably as a result of cleavage of the signaling tag on the VP6 protein.

[00213] The ER, chloroplasts and apoplast all exhibited the highest protein expression on day 3, with the most protein accumulating in the presence of the silencing suppressor. The cytoplasm was the best in terms of protein accumulation as it exhibited high and increasing protein expression throughout the time-trial.

Expression of histidine-tagged rotavirus proteins in the cytoplasm

[00214] The four rotavirus VPs were cloned into an additional vector, pTRAc-HT. This vector includes a 6-histidine tag to proteins targeted to the cytoplasm and makes detection easy by use of an anti-histidine tag antibody if the antibodies for the

proteins of interest are unavailable. In our case, only VP6 has a commercially available antibody and hence we tried this procedure for early detection of all proteins while awaiting serum. The cytoplasm also worked well for VP6 expression and motivated us to try the other proteins.

5 [00215] Western blot results of day 3 extracts from a 7 day time trial showed successful expression of VP2, VP4 and VP6 (Figure 7a). In order to obtain expression of VP7 in plants, various techniques were tried. However, plants infiltrated with VP7 exhibited yellowing leaves from day 1 and proceeded to wilt during the course of the time trial (Figure 7b). No expression of protein was detected under these conditions,
10 even after day 1 of infiltration when the plant still looked reasonably good.

Expression of VP2 and VP4 in plants

[00216] VP2 and VP4 were infiltrated in *N. benthamiana* plant leaves and targeted to the ER, chloroplast, cytoplasm and apoplast. We were unable to express VP2 targeted to the apoplast vector as we could not get any positive clones in *E. coli*.
15 However, the protein was successfully expressed and targeted to all other 3 compartments (Figure 8a). Chicken anti-VP2 and anti-VP4 serum (1:2000) was used in western blot analysis of extracts. VP2 and VP4 bands were visible just below the 100 kDa mark (protein band indicated by arrow) as seen in Figure 8a and 8b respectively. Expression appeared to be best in the cytoplasm and ER for VP2 while
20 for VP4, in the cytoplasm and apoplast. The silencing suppressor did not have a significant effect on the expression of the proteins. It only slightly increased expression in the VP2 ER construct and not so much in the rest, as can be seen from the western blot. VP4 constructs were all expressed in the presence of the silencing suppressor.

Co-expression of VP2/6 and VP2/6/4 in the cytoplasm

[00217] The cytoplasm appeared to be best for rotavirus capsid protein expression and exhibited the highest extraction efficiencies. All further expression work was therefore done with proteins targeted to the cytoplasm.

[00218] VP2 and VP6 have been shown to form RLPs with protective
30 immunogenic responses in mice and therefore co-expression of VP2/6 and VP2/6/4 in the cytoplasm was investigated. Day 3 extracts of co-expressed VP2/6/4 were detected by western blot with anti-VP2 and VP4 serum (1/5000) and mouse anti-VP6

antibody (1:5000) (Figure 9). VP6 expression was very high as previously determined, but expression of VP2 and VP4 was very low as can be seen from the very faint band at the 100 kDa mark. This may have been attributed by the co-expression which resulted in more host cell resources being utilized in the over-expression of VP6, leaving less for VP2 and/or VP4. It was also not easy to determine if the detected band was both VP2 and VP4 or either of the 2 proteins. The very visible band running above 130 kDa may be dimerized VP6 proteins. The band visible at the 55 kDa mark is most likely the abundant plant enzyme Rubisco.

[00219] Transmission electron microscopic analysis on the cytoplasm-expressed VP6 as well as on co-expressed VP2/6 and VP2/6/4, were carried out to check for protein particles and assembled RLPs (Figure 10). This also determined if VP2 and/or VP4 were indeed co-expressed successfully. VP6 when expressed alone assembled to form sheaths of protein as indicated by the arrow in Figure 10b. On addition of VP2, the particles assembled to form RLPs (Figure 10c). VP2 acts as scaffolding protein that enables other proteins to assemble and ultimately form a complete rotavirus structure. VP6 as such bound to VP2 but it was still not easy to determine VP4 structures in co-expressed VP2/6/4. The electron micrograph in Figure 10d may be purely assembled VP2/6 particles. It has been shown however that VP4 binds to VP6 during protein assembly, and this occurs before VP7 binds. It is likely that these VP4 structures are not stable and may fall off the RLP structure during preparation procedures for electronic microscopy.

Sucrose gradient purification of VP2/6 and VP2/6/4

[00220] VP2/6 and VP2/6/4 were purified on a sucrose gradient ranging from 10 to 60 % sucrose (Figure 11a). 2 ml fractions were collected from the bottom of each of the tubes and probed with mouse anti-VP6 antibody and/or chicken anti-VP2 and VP4 serum to determine which fractions contained the proteins. For VP2/6, proteins were found in fractions 16 and 17 as these were positive for VP6 protein on the blot (Figure 11b). VP2/6/4 blot analysis with chicken anti-VP2 and VP4 serum showed positive results throughout all the fractions. This may have been as a result of the high levels of background protein detection by the chicken serum. However, intensity of dots was highest in fractions 17 and 18 as seen in Figure 11c, possibly due to higher concentration of the proteins of interest in these fractions.

[00221] Putting these results together (Figure 11b and 11c), the rotavirus proteins were in fractions ranging from 16 to 20.

Western Blot and Coomassie stains of fractions

[00222] A western blot and SDS-PAGE of co-expressed VP2/6 were done to verify presence of VP2 and VP6 proteins in fractions 13 to 20. Western blot analysis for VP6 protein probed with mouse anti-VP6 antibody was positive in fractions 16 right through to 20 (Figure 12a, bottom arrow and Figure 12c). VP2 protein probed with chicken anti-VP2 serum was detected in fractions 17 to 20 (Figure 12a, top arrow). VP2 has been shown to express less than VP6 in past co-expression studies and this was also shown here Figure 12a in which VP2 protein bands have a lower intensity in comparison to VP6.

[00223] Fractions 16 and 17 of co-expressed VP2/6 previously determined to contain VP6 protein by dot blots (Figure 11b) were electrophoresed on an SDS-PAGE gel. A protein of known concentration, SF9 insect cell expressed VP6 (0.91 µg/µl) was included so as to determine the concentration of VP2/6 crude proteins (Figure 11b and c). This was done by density scanning of the crude protein band (lane labeled crude) using a Syngene Gel Documentation System and consequently allowed us to determine the amount of VP2/6 per kilogram of leaf matter. Protein yield was found to be approximately 1.54 g/kg fresh weight (FW). 1.1 mg of purified RLPs were obtained from 1 gram of plant material (1.1g/kg).

Total Soluble Protein Assay of VP2/6

[00224] Total soluble protein (TSP) was determined on co-expressed VP2/6 fractions to determine the relative amounts of VP2/6 protein (Figure 13). Protein concentrations were calculated as 0.538 mg/ml and 1.012 mg/ml for fractions 17 and 18 respectively with the use of an IgG standard (Figure 13a). The protein bands corresponding to VP2/6 in these fractions were calculated by density scanning on a Syngene Gel Documentation System and found to be approximately 0.108 mg/ml and 0.202 mg/ml, respectively.

[00225] Thus, the TSP for VP2/6 in fractions 17 and 18 were both approximately 20 % TSP. Most of the RLPs in the sucrose column were found to be between 15 and 25 % sucrose, corresponding with fractions 15 to approximately 20, where the graph is noted to suddenly peak and then subside. The differences in

density of the various materials in the extract allowed me to separate and thereby purify the proteins of interest. SDS-PAGE gels stained with Coomassie blue showed only one prominent band indicating that the proteins are relatively pure (Figure 12b).

TEM of purified VP2/6

[00226] Purified VP2/6 fractions were pooled together and dialyzed in high salt PBS to remove sucrose before viewing on a transmission electron microscope. The TEM was done to determine purity and check if RLPs remained intact after the purification procedure. As can be seen in Figure 15, most of the background material which mainly consisted of the host cell's products (Figure 10b, c and d) was removed, leaving behind RLPs. Most of the RLPs remained intact but some appeared to have lost shape probably as a result of deformation due to the conditions on the EM grid.

Preliminary analysis of expression of rotavirus structural proteins in *N. benthamiana* leaves

[00227] This preliminary analysis focused on the expression of rotavirus structural proteins VP2 (SEQ ID NO:1), VP4 (SEQ ID NO:2), VP6 (SEQ ID NO:3) and VP7 (SEQ ID NO:4) in *N. benthamiana* leaves as an example host expression system. The strain of rotavirus selected here was a G9 P[6] strain which circulates predominantly in South Africa and other sub-Saharan regions. A RLP vaccine targeting this strain would help in alleviating the burden of disease in sub-Saharan Africa.

[00228] A transient expression system mediated by *Agrobacterium* was used in this analysis. Transient expression, as opposed to transgenic expression, permits rapid expression of proteins in a relatively short time, without integration of the rotavirus capsid protein genes in the host's chromosome. Most proteins were expressed and accumulated to detectable amounts by day 3 of recombinant *Agrobacterium* infiltration in *N. benthamiana* leaves. As shown below successful expression of several rotavirus structural proteins was observed including VP2, VP4 and VP6 in plant leaf cell compartments as detailed in table 2:

Table 2: Expression of rotavirus VP proteins in various leaf cell compartments

Capisd Protein	Leaf Cell Compartment			
	Apoplast	Chloroplast	Cytoplasm	ER
VP2	0	1	1	1
VP4	1	0	1	1
VP6	1	1	1	1
VP7	0	0	0	0

0 = no expression
1 = expressed

[00229] Expression of the glycoprotein VP7 was not observed possibly due to its toxic effects on plant cells. It is worth noting that for this preliminary study, a VP7 containing its native signal peptide had been used. Infiltrating at day 3 during co-expression trials was also tried. This was attempted to see if the protein was expressed and soon after assembled with VP2 and VP6 to form RLPs. The toxic nature of recombinant VP7 as observed in this study has been previously described (Williams et al., 1995; McCorquodale, 1987; Arias et al., 1986).

[00230] Past VP7 expression studies in transgenic potatoes has been reported (Li et al., 2006; Choi et al., 2005; Wu et al., 2003). Choi et. al. (2005) used a simian rotavirus VP7, and Li et. al. and Wu et. al. (Li et al., 2006; Wu et al., 2003) used human group A G1 VP7. The result described herein used human rotavirus G9 VP7.

[00231] VP2 was expressed and targeted to all compartments except the apoplast as we were unable to clone the appropriate cDNA, and time constraints only allowed us a few attempts before proceeding with the other constructs. Expression levels of VP2 were noted to be significantly low in all the compartments. In a past study quoted by Saldana et al. 2006, it was concluded that a VP2 having its sequence optimized for expression in the plant was impossible to express, despite mRNA being detected in the plant cells. They however managed to express it in tomato plant cells using synthetic DNA. The reason for the difficulty in VP2 expression is most likely as result of improper mRNA translation or that the mRNA contains some sequence motifs that destabilize the plant cells (Kawaguchi and Bailey-Serres, 2002). Evidence of low expression levels of VP2 in comparison to VP6 have been seen in both plant

and insect-cell expression studies by Mena et al. (2006), Saldana et al. (2006), Vieira et al. (2005), and Labbé et al. (1991).

[00232] The outer capsid protein, VP4, which forms spikes on the surface of the virion structure, was expressed and targeted for accumulation in the cytoplasm, ER and apoplast. No protein accumulation was detected in chloroplasts. As observed for VP2, protein expression levels for VP4 were lower than seen for VP6 on western blots. The protein has a trypsin cleavage site which results in two proteins, VP5 and VP8. It may be possible that local trypsin in *N. benthamiana* leaves cleaves some of the proteins as they are produced resulting in lower concentration levels of accumulated, intact VP4 in the designated compartments. The protein has been shown to be a major neutralizing antigen but there have been a few attempts to clone the whole protein for vaccine development (Khodabandehloo et al., 2009; Mahajan et al., 1995; Nishikawa et al., 1989). There have been, however, several studies in the insect-cell and yeast expression system showing expression of either the VP5 or VP8 subunits of VP4 (Andrés et al., 2006; Favacho et al., 2006; Kovacs-Nolan et al., 2001). To date, the present study represents the first study showing expression of the whole protein in a plant expression system.

[00233] VP6 was expressed in all compartments with over-expression being observed in the cytoplasm with protein accumulation observed from day 1 to day 7 in this compartment. This is contrary to some literature which suggests that protease activity and gene silencing reduce or hinder the accumulation of foreign protein in the cytoplasm (Fischer et al., 2004). In addition, given the right pH conditions, VP6 is known to self-assemble into tubular or helical particles, much like the particles seen in our study (Figure 9b) (Estes et al., 1987). VP6 makes up about 50 % of the viral core and therefore is a major antigen in the development of a rotavirus vaccine. The result attained above enabled us to further investigate the co-expression of VP2, VP6 and VP4 in the cytoplasm.

[00234] When co-expressed in the cytoplasm, VP2 and VP6 assembled to form RLPs. Very high protein yields from transient expression system of between 1.27 – 1.54 g/kg FW were observed. When purified on a sucrose column, the amount of VPs retained was 1.1 g/kg FW. This yield is comparable to that obtained for the production of an antibody, IgG, using a transient expression system in *N. benthamiana*, with a yield of up to 1.5 g/kg FW (Vézina et al., 2009). Saldana et al. (2006) were until now

the only group known to have successfully co-expressed rotavirus VP2 and VP6 in transgenic tomato plants and to levels of approximately 1 % total soluble protein. The assembly of VP2/6 in the insect-cell expression system has been well documented (Vieira et al., 2005;; O'Brien et al., 2000). These VP2/6 RLPs have also been shown to provide protective immunity against rotavirus infection (Zhou et al., 2011, Saldana et al., 2006). Thus the VP2/6 RLPs we produced in the plant expression system are suitable candidates for the development a subunit rotavirus vaccine.

[00235] VP2/6/4 were also co-expressed and detected. The first peak visible (Figure 14, fraction 16) in the total protein absorbance reading of the co-expressed proteins might be assembled VP2/6/4, but on examination of this fraction under a TEM, no RLPs were detected. The protein peak observed may result from an accumulation of VP4 monomers or its respective VP5 and VP8 subunits. The second peak (fraction 18), when examined under a TEM showed RLP structures very much similar to the ones seen in the VP2/6 sample. However, Crawford et al have previously reported that VP4 could not be seen under TEM, and that VP2/6/4 and VP2/6/4/7 particles had similar structure and diameter under TEM (Crawford 1994). We made the same observation for VP2/6/7 RLPs, VP 2/6/4/7 RLPs and VP2/6 RLPs, which all look similar under regular TEM.

Example 2

Constructs

A-2X35S/CPMV-HT/ RVA(WA) VP2(opt)/ NOS (Construct number 1710)

[00236] An optimized sequence encoding VP2 from Rotavirus A WA strain was cloned into 2X35S-CPMV-HT-NOS expression system in a plasmid containing Plasto_pro/P19/Plasto_ter expression cassette using the following PCR-based method. A fragment containing the VP2 coding sequence was amplified using primers IF-WA_VP2(opt).s1+3c (Figure 17A, SEQ ID NO: 21) and IF-WA_VP2(opt).s1-4r (Figure 17B, SEQ ID NO: 22), using optimized VP2 gene sequence (Figure 19, SEQ ID NO: 45) as template. For sequence optimization, VP2 protein sequence (Genbank accession number CAA33074) was backtranslated and optimized for human codon usage, GC content and mRNA structure. The PCR product was cloned in 2X35S/CPMV-HT/NOS expression system using In-Fusion cloning system (Clontech, Mountain View, CA). Construct number 1191 (Figure 17C) was digested

with SacII and StuI restriction enzyme and the linearized plasmid was used for the In-Fusion assembly reaction. Construct number 1191 is an acceptor plasmid intended for “In Fusion” cloning of genes of interest in a CPMV-HT-based expression cassette. It also incorporates a gene construct for the co-expression of the TBSV P19 suppressor of silencing under the alfalfa Plastocyanin gene promoter and terminator. The backbone is a pCAMBIA binary plasmid and the sequence from left to right t-DNA borders is presented in Figure 18 (SEQ ID NO: 23). The resulting construct was given number 1710 (Figure 23, SEQ ID NO: 27). The amino acid sequence of VP2 from Rotavirus A strain WA is presented in Figure 20 (SEQ ID NO: 25). A representation of plasmid 1710 is presented in Figure 21.

B-2X35S/CPMV-HT/RVA(WA) VP2(opt)/NOS into BeYDV(m)+Replicase amplification system (Construct number 1711)

[00237] An optimized sequence encoding VP2 from Rotavirus A WA strain was cloned into 2X35S/CPMV-HT/NOS comprising the BeYDV(m)+replicase amplification system in a plasmid containing Plasto_pro/P19/Plasto_ter expression cassette using the following PCR-based method. A fragment containing the VP2 coding sequence was amplified using primers IF-WA_VP2(opt).s1+3c (Figure 17A, SEQ ID NO: 21) and IF-WA_VP2(opt).s1-4r (Figure 17B, SEQ ID NO: 22), using optimized VP2 gene sequence (SEQ ID NO: 45) as template. For sequence optimization, VP2 protein sequence (Genbank accession number CAA33074) was backtranslated and optimized for human codon usage, GC content and mRNA structure. The PCR product was cloned in 2X35S/CPMV-HT/NOS expression cassette into the BeYDV(m) amplification system using In-Fusion cloning system (Clontech, Mountain View, CA). Construct 193 (Figure 22A) was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for the In-Fusion assembly reaction. Construct number 193 is an acceptor plasmid intended for “In Fusion” cloning of genes of interest in a CPMV-HT-based expression cassette into the BeYDV(m) amplification system. It also incorporates a gene construct for the co-expression of the TBSV P19 suppressor of silencing under the alfalfa Plastocyanin gene promoter and terminator. The backbone is a pCAMBIA binary plasmid and the sequence from left to right t-DNA borders is presented in Figure 22B (SEQ ID NO: 26). The resulting construct was given number 1711 (Figure 23, SEQ ID NO: 27).

The amino acid sequence of VP2 from Rotavirus A strain WA is presented in Figure 20 (SEQ ID NO: 25). A representation of plasmid 1711 is presented in Figure 24.

C-2X35S/CPMV-HT/RVA(WA) VP6(opt)/NOS (Construct number 1713)

[00238] An optimized sequence encoding VP6 from Rotavirus A WA strain was cloned into 2X35S-CPMV-HT-NOS expression system in a plasmid containing Plasto_pro/P19/Plasto_ter expression cassette using the following PCR-based method. A fragment containing the VP6 coding sequence was amplified using primers IF-WA_VP6(opt).s1+3c (Figure 25a, SEQ ID NO: 28) and IF-WA_VP6(opt).s1-4r (Figure 25b, SEQ ID NO: 29), using optimized VP6 gene sequence (SEQ ID NO: 46) as template. For sequence optimization, VP6 protein sequence (Genbank accession number AAA47311) was backtranslated and optimized for human codon usage, GC content and mRNA structure. The PCR product was cloned in 2X35S/CPMV-HT/NOS expression system using In-Fusion cloning system (Clontech, Mountain View, CA). Construct number 1191 (Figure 17c) was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for the In-Fusion assembly reaction. Construct number 1191 is an acceptor plasmid intended for "In Fusion" cloning of genes of interest in a CPMV-HT-based expression cassette. It also incorporates a gene construct for the co-expression of the TBSV P19 suppressor of silencing under the alfalfa Plastocyanin gene promoter and terminator. The backbone is a pCAMBIA binary plasmid and the sequence from left to right t-DNA borders is presented in Figure 18 (SEQ ID NO: 23). The resulting construct was given number 1713 (Figure 25c, SEQ ID NO: 30). The amino acid sequence of VP6 from Rotavirus A strain WA is presented in Figure 26 (SEQ ID NO: 31). A representation of plasmid 1713 is presented in Figure 27.

D-2X35S/CPMV-HT/RVA(WA) VP6(opt)/NOS into BeYDV(m)+Replicase amplification system (Construct number 1714)

[00239] An optimized sequence encoding VP6 from Rotavirus A WA strain was cloned into 2X35S/CPMV-HT/NOS comprising the BeYDV(m)+replicase amplification system in a plasmid containing Plasto_pro/P19/Plasto_ter expression cassette using the following PCR-based method. A fragment containing the VP6 coding sequence was amplified using primers IF-WA_VP6(opt).s1+3c (Figure 25a, SEQ ID NO: 28) and IF-WA_VP6(opt).s1-4r (Figure 25b, SEQ ID NO: 29), using

optimized VP6 gene sequence (SEQ ID NO: 46) as template. For sequence optimization, VP6 protein sequence (Genbank accession number AAA47311) was backtranslated and optimized for human codon usage, GC content and mRNA structure. The PCR product was cloned in 2X35S/CPMV-HT/NOS expression cassette into the BeYDV(m) amplification system using In-Fusion cloning system (Clontech, Mountain View, CA). Construct 193 (Figure 22A) was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for the In-Fusion assembly reaction. Construct number 193 is an acceptor plasmid intended for “In Fusion” cloning of genes of interest in a CPMV-HT-based expression cassette into the BeYDV(m) amplification system. It also incorporates a gene construct for the co-expression of the TBSV P19 suppressor of silencing under the alfalfa Plastocyanin gene promoter and terminator. The backbone is a pCAMBIA binary plasmid and the sequence from left to right t-DNA borders is presented in Figure 22B (SEQ ID NO: 26). The resulting construct was given number 1714 (Figure 28, SEQ ID NO: 32). The amino acid sequence of VP6 from Rotavirus A strain WA is presented in Figure 26 (SEQ ID NO: 31). A representation of plasmid 1714 is presented in Figure 29.

C-2X35S/CPMV-HT/RVA(Rtx) VP4(opt)/NOS (Construct number 1730)

[00240] An optimized sequence encoding VP4 from Rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] strain was cloned into 2X35S/CPMV-HT/NOS in a plasmid containing Plasto_pro/P19/Plasto_ter expression cassette using the following PCR-based method. A fragment containing the VP4 coding sequence was amplified using primers IF-Rtx_VP4(opt).s1+3c (Figure 30A, SEQ ID NO: 33) and IF-Rtx_VP4(opt).s1-4r (Figure 30B, SEQ ID NO: 34), using optimized VP4 gene sequence (Figure 31B, (SEQ ID NO: 47) as template. For sequence optimization, VP4 protein sequence (Genbank accession number AEX30660) was backtranslated and optimized for human codon usage, GC content and mRNA structure. The PCR product was cloned in 2X35S/CPMV-HT/NOS expression cassette using In-Fusion cloning system (Clontech, Mountain View, CA). Construct number 1191 (Figure 18, SEQ ID NO: 23) was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for the In-Fusion assembly reaction. Construct number 1191 is an acceptor plasmid intended for “In Fusion” cloning of genes of interest in a CPMV-HT-based expression cassette. It also incorporates a gene construct for the co-expression of the TBSV P19 suppressor of silencing under the alfalfa Plastocyanin

gene promoter and terminator. The backbone is a pCAMBIA binary plasmid and the sequence from left to right t-DNA borders is presented in (Figure 18, SEQ ID NO: 23). The resulting construct was given number 1730 (Figure 31C, SEQ ID NO: 50). The amino acid sequence of VP4 from Rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] is presented in Figure 32 (SEQ ID NO: 36). A representation of plasmid 1730 is presented in Figure 33A.

E-2X35S/CPMV-HT/RVA(Rtx) VP4(opt)/NOS into BeYDV(m)+Replicase amplification system (Construct number 1731)

[00241] An optimized sequence encoding VP4 from Rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] strain was cloned into 2X35S/CPMV-HT/NOS comprising the BeYDV(m)+replicase amplification system in a plasmid containing Plasto_pro/P19/Plasto_ter expression cassette using the following PCR-based method. A fragment containing the VP4 coding sequence was amplified using primers IF-Rtx_VP4(opt).s1+3c (Figure 30A, SEQ ID NO: 33) and IF-Rtx_VP4(opt).s1-4r (Figure 30B, SEQ ID NO: 34), using optimized VP4 gene sequence (SEQ ID NO: 47) as template. For sequence optimization, VP4 protein sequence (Genbank accession number AEX30660) was backtranslated and optimized for human codon usage, GC content and mRNA structure. The PCR product was cloned in 2X35S/CPMV-HT/NOS expression cassette into the BeYDV(m) amplification system using In-Fusion cloning system (Clontech, Mountain View, CA). Construct 193 (Figure 22A) was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for the In-Fusion assembly reaction. Construct number 193 is an acceptor plasmid intended for "In Fusion" cloning of genes of interest in a CPMV-HT-based expression cassette into the BeYDV(m) amplification system. It also incorporates a gene construct for the co-expression of the TBSV P19 suppressor of silencing under the alfalfa Plastocyanin gene promoter and terminator. The backbone is a pCAMBIA binary plasmid and the sequence from left to right t-DNA borders is presented in Figure 22B (SEQ ID NO: 26). The resulting construct was given number 1731 (Figure 31, SEQ ID NO: 35). The amino acid sequence of VP4 from Rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] is presented in Figure 32 (SEQ ID NO: 36). A representation of plasmid 1731 is presented in Figure 33B.

F-2X35S/CPMV-HT/RVA(Rtx) VP7(opt)/NOS (Construct number 1733)

[00242] An optimized sequence encoding VP7 with is native signal peptide from Rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] strain was cloned into 2X35S-CPMV-HT-NOS expression system in a plasmid containing Plasto_pro/P19/Plasto_ter expression cassette using the following PCR-based method.

5 A fragment containing the VP7 coding sequence was amplified using primers IF-Rtx_VP7(opt).s1+3c (Figure 34A, SEQ ID NO: 37) and IF-Rtx_VP7(opt).s1-4r (Figure 34B, SEQ ID NO: 38), using optimized VP7 gene sequence (SEQ ID NO: 54) as template. For sequence optimization, VP7 protein sequence (Genbank accession number AEX30682) was backtranslated and optimized for human codon usage, GC

10 content and mRNA structure. The PCR product was cloned in 2X35S/CPMV-HT/NOS expression system using In-Fusion cloning system (Clontech, Mountain View, CA). Construct number 1191 (Figure 17C) was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for the In-Fusion assembly reaction. Construct number 1191 is an acceptor plasmid intended for “In Fusion”

15 cloning of genes of interest in a CPMV-HT-based expression cassette. It also incorporates a gene construct for the co-expression of the TBSV P19 suppressor of silencing under the alfalfa Plastocyanin gene promoter and terminator. The backbone is a pCAMBIA binary plasmid and the sequence from left to right t-DNA borders is presented in Figure 18 (SEQ ID NO: 23). The resulting construct was given number

20 1733 (Figure 34C, SEQ ID NO: 24). The amino acid sequence of VP7 with is native signal peptide from Rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] strain is presented in Figure 35 (SEQ ID NO: 39). A representation of plasmid 1733 is presented in Figure 36.

D-2X35S/CPMV-HT/TrSp-RVA(Rtx) VP7(opt)/NOS (Construct number 1734)

[00243] An optimized sequence encoding VP7 with a truncated version of the native signal peptide from Rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] strain was cloned into 2X35S-CPMV-HT-NOS expression system in a plasmid containing Plasto_pro/P19/Plasto_ter expression cassette using the following PCR-based method. A fragment containing the VP7

30 coding sequence was amplified using primers IF-TrSP+Rtx_VP7(opt).s1+3c (Figure 44A, SEQ ID NO: 55) and IF-Rtx_VP7(opt).s1-4r (Figure 44B, SEQ ID NO: 56), using optimized VP7 gene sequence (corresponding to nt 88-891 from Figure 44C, SEQ ID NO: 57) as template. For sequence optimization, VP7 protein sequence

(Genbank accession number AEX30682) was backtranslated and optimized for human codon usage, GC content and mRNA structure. The PCR product was cloned in 2X35S/CPMV-HT/NOS expression system using In-Fusion cloning system (Clontech, Mountain View, CA). Construct number 1191 (Figure 17C) was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for the In-Fusion assembly reaction. Construct number 1191 is an acceptor plasmid intended for “In Fusion” cloning of genes of interest in a CPMV-HT-based expression cassette. It also incorporates a gene construct for the co-expression of the TBSV P19 suppressor of silencing under the alfalfa Plastocyanin gene promoter and terminator. The backbone is a pCAMBIA binary plasmid and the sequence from left to right t-DNA borders is presented in Figure 18 (SEQ ID NO: 23). The resulting construct was given number 1734 (Figure 44D, SEQ ID NO: 58). The amino acid sequence of VP7 with truncated signal peptide from Rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] strain is presented in Figure 44E (SEQ ID NO: 59). A representation of plasmid 1734 is presented in Figure 44F.

G-2X35S/CPMV-HT/PDISP/RVA(WA) VP7(opt)/NOS into BeYDV(m)+Replicase amplification system (Construct number 1735)

[00244] A sequence encoding VP7 from Rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] strain was cloned into 2X35S-CPMV-HT-PDISP-NOS expression system in a plasmid containing Plasto_pro/P19/Plasto_ter expression cassette using the following PCR-based method. A fragment containing the VP7 coding sequence without his wild type signal peptide was amplified using primers IF-Rtx_VP7(opt).s2+4c (Figure 37A, SEQ ID NO: 40) and IF-Rtx_VP7(opt).s1-4r (Figure 34B, SEQ ID NO: 38), using optimized VP7 gene sequence (SEQ ID NO: 54). For sequence optimization, VP7 protein sequence (Genbank accession number AEX30682) was backtranslated and optimized for human codon usage, GC content and mRNA structure. The PCR product was cloned in-frame with alfalfa PDI signal peptide in 2X35S/CPMV-HT/NOS expression system using In-Fusion cloning system (Clontech, Mountain View, CA). Construct 1192 (Figure 38) was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for the In-Fusion assembly reaction. Construct number 1192 is an acceptor plasmid intended for “In Fusion” cloning of genes of interest in frame with an alfalfa PDI signal peptide in a CPMV-HT-based expression cassette. It also incorporates a gene construct for the co-

expression of the TBSV P19 suppressor of silencing under the alfalfa Plastocyanin gene promoter and terminator. The backbone is a pCAMBIA binary plasmid and the sequence from left to right t-DNA borders is presented in Figure 39 (SEQ ID NO: 41). The resulting construct was given number 1735 (Figure 40, SEQ ID NO: 42). The amino acid sequence of PDISP/VP7 from Rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] strain is presented in Figure 41 (SEQ ID NO: 43). A representation of plasmid 1735 is presented in Figure 42.

Table 3. Description of synthesized genes for the production of RLPS.

SEQ ID No	Antigen	Strain of origin	Sequence type*	Figure in disclosure
45	VP2	WA	Optimized	19B
46	VP6	WA	Optimized	25D
47	VP4	Rotarix	Optimized	31B
50	VP4	SA11	Wild-type	43A
51	VP4	SA11	Optimized	43B
54	VP7	Rotarix	Optimized	34E
53	VP7	SA11	Wild-type	43D
52	VP7	SA11	Optimized	43C

* Optimized sequences were modified to favor the use of preferred human codons and increase GC content

Table 4. Description of assembled and tested construct for the production of RLPS.

Expression system	Amplification system	Signal peptide [†]	Antigen (strain) ^{**}	Gene SEQ ID used for PCR	Construct number
CPMV HT	-	-	RVA(WA) VP2 [optimized]	SEQ ID NO: 45	1710
CPMV HT	BeYDV(m)+rep	-	RVA(WA) VP2 [optimized]	SEQ ID NO: 45	1711
CPMV HT	-	-	RVA(WA) VP6 [optimized]	SEQ ID NO: 46	1713
CPMV HT	BeYDV(m)+rep	-	RVA(WA) VP6 [optimized]	SEQ ID NO: 46	1714
CPMV HT	-	-	RVA(Rtx) VP4 [optimized]	SEQ ID NO: 47	1730
CPMV HT	BeYDV(m)+rep	-	RVA(Rtx) VP4 [optimized]	SEQ ID NO: 47	1731

CPMV HT	-	WtSp	RVA(Rtx) VP7 [optimized]	SEQ ID NO: 54	1733
CPMV HT	-	TrSp	RVA(Rtx) VP7 [optimized]	SEQ ID NO: 54	1734
CPMV HT	-	SpPDI	RVA(Rtx) VP7 [optimized]	SEQ ID NO: 54	1735
CPMV HT	BeYDV(m)+rep	WtSp	RVA(Rtx) VP7 [optimized]	SEQ ID NO: 54	1736
CPMV HT	BeYDV(m)+rep	TrSp	RVA(Rtx) VP7 [optimized]	SEQ ID NO: 54	1737
CPMV HT	BeYDV(m)+rep	SpPDI	RVA(Rtx) VP7 [optimized]	SEQ ID NO: 54	1738
CPMV HT	-	-	RVA(SA11) VP4	SEQ ID NO: 50	1760
CPMV HT	BeYDV(m)+rep	-	RVA(SA11) VP4	SEQ ID NO: 50	1761
CPMV HT	-	-	RVA(SA11) VP4 [optimized]	SEQ ID NO: 51	1770
CPMV HT	BeYDV(m)+rep	-	RVA(SA11) VP4 [optimized]	SEQ ID NO: 51	1771
CPMV HT	-	WtSp	RVA(SA11) VP7	SEQ ID NO: 53	1763
CPMV HT	-	TrSp	RVA(SA11) VP7	SEQ ID NO: 53	1764
CPMV HT	-	SpPDI	RVA(SA11) VP7	SEQ ID NO: 53	1765
CPMV HT	BeYDV(m)+rep	WtSp	RVA(SA11) VP7	SEQ ID NO: 53	1766
CPMV HT	BeYDV(m)+rep	TrSp	RVA(SA11) VP7	SEQ ID NO: 53	1767
CPMV HT	BeYDV(m)+rep	SpPDI	RVA(SA11) VP7	SEQ ID NO: 53	1768
CPMV HT	-	WtSp	RVA(SA11) VP7 [optimized]	SEQ ID NO: 52	1773
CPMV HT	-	TrSp	RVA(SA11) VP7 [optimized]	SEQ ID NO: 52	1774
CPMV HT	-	SpPDI	RVA(SA11) VP7 [optimized]	SEQ ID NO: 52	1775
CPMV HT	BeYDV(m)+rep	WtSp	RVA(SA11) VP7 [optimized]	SEQ ID NO: 52	1776
CPMV HT	BeYDV(m)+rep	TrSp	RVA(SA11) VP7[optimized]	SEQ ID NO: 52	1777
CPMV HT	BeYDV(m)+rep	SpPDI	RVA(SA11) VP7 [optimized]	SEQ ID NO: 52	1778

† WtSp: Wild type signal peptide, SpPDI: Signal peptide of plant origin, cloned from alfalfa protein disulfide isomerase gene, TrSp: truncated wild type signal peptide, TrSp start at 2nd Met in WtSp (M30).

* [optimized] means that the sequence has been optimized based on codon usage, GC content and RNA structure.

Example 3

Assembly of gene constructs and Agrobacterium transformation

5 [00245] All plasmids, including plasmids 1710, 1713, 1730 and 1734, were used to transform *Agrobacterium tumefaciens* (AGL1; ATCC, Manassas, VA 20108, USA) by electroporation (Mattanovich et al., 1989, Nucleic Acid Res. 17:6747) alternatively, heat shock using CaCl₂-prepared competent cells (XU et al., 2008, Plant Methods 4) may be used. The integrity of the plasmids in the *A. tumefaciens* strains
10 created was confirmed by restriction mapping. The *A. tumefaciens* strain transformed with a given binary plasmid is named AGL1/"plasmid number". For example, the *A. tumefaciens* strain transformed with construct number 1710 is termed "AGL1/1710".

Preparation of plant biomass, inoculum, agroinfiltration, and harvesting

15 [00246] *Nicotiana benthamiana* plants were grown from seeds in flats filled with a commercial peat moss substrate. The plants were allowed to grow in the greenhouse under a 16/8 photoperiod and a temperature regime of 25°C day/20°C night. Three weeks after seeding, individual plantlets were picked out, transplanted in pots and left to grow in the greenhouse for three additional weeks under the same environmental conditions.

20 [00247] *Agrobacteria* transfected with each construct were grown in a LB medium from vegetal origin and supplemented with 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) and 50 µg/ml kanamycin pH5.6 until they reached an OD₆₀₀ between 0.6 and 2.5. *Agrobacterium* suspensions were mixed to reach appropriate ratio for each construct and brought to 2.5X OD₆₀₀ with infiltration
25 medium (10 mM MgCl₂ and 10 mM MES pH 5.6). *A. tumefaciens* suspensions were stored overnight at 4°C. On the day of infiltration, culture batches were diluted with infiltration medium in 2.5 suspension volumes and allowed to warm before use. Whole plants of *N. benthamiana* were placed upside down in the bacterial suspension in an air-tight stainless steel tank under a vacuum of 20-40 Torr for 2-min. Following
30 infiltration, plants were returned to the greenhouse for a 3-12 day incubation period until harvest. Harvested biomass was kept frozen (-80°C) until use for purification of particles.

Extraction and purification of rotavirus-like particles

[00248] Proteins were extracted from frozen biomass by mechanical extraction in a blender with 3 volumes of extraction buffer (TNC: 10 mM Tris pH 7.4, 140 mM NaCl, 10 mM CaCl₂). The slurry was filtered through a large pore nylon filter to remove large debris and centrifuged 5000 g for 5 min at 4°C. The supernatant was collected and centrifuged again at 5000 g for 30 min (4°C) to remove additional debris. The supernatant was depth-filtered and ultra-filtered and the filtrate was centrifuged at 75 000 g for 20 min (4°C) to concentrate the rotavirus-like particles. The pellet containing the particles was resuspended in 1/12 volume of TNC and the insoluble were remove with a centrifugation at 5000 g for 5 minutes. The supernatant was filtered on Miracloth before being loaded on iodixanol density gradients.

[00249] Density gradient centrifugation was performed as follows. Tubes containing step gradients from 5% to 45% of iodixanol were prepared and overlaid with the filtered extracts containing the rotavirus-like particles. The gradients were centrifuged at 120 000 g for 4 hours (4°C). After centrifugation, 1 ml fractions were collected from the bottom to the top and analysed by Coomassie-stained SDS-PAGE and Western blot. To remove iodixanol for the fractions selected for further analysis, selected fractions were centrifuged 75 000 g for 20 min (4°C) and the pelleted particles were resuspended in fresh TNC buffer.

SDS-PAGE and immunoblotting

[00250] Protein concentrations were determined by the BCA protein assay (Pierce Biochemicals, Rockport IL). Proteins were separated by SDS-PAGE under reducing or non-reducing conditions and stained with Coomassie Blue. Stained gels were scanned and densitometry analysis performed using ImageJ Software (NIH).

[00251] For immunoblotting, electrophoresed proteins were electrotransferred onto polyvinylene difluoride (PVDF) membranes (Roche Diagnostics Corporation, Indianapolis, IN). Prior to immunoblotting, the membranes were blocked with 5% skim milk and 0.1% Tween-20 in Tris-buffered saline (TBS-T) for 16-18h at 4°C.

[00252] Immunoblotting was performed by incubation with a suitable antibody (Table 5), in 2 µg/ml in 2% skim milk in TBS-Tween 20 0.1%. Secondary antibodies used for chemiluminescence detection were as indicated in Table 5, diluted as indicated in 2% skim milk in TBS-Tween 20 0.1%. Immunoreactive complexes were

detected by chemiluminescence using luminol as the substrate (Roche Diagnostics Corporation). Horseradish peroxidase–enzyme conjugation of human IgG antibody was carried out by using the EZ-Link Plus® Activated Peroxidase conjugation kit (Pierce, Rockford, IL).

- 5 Table 5: Electrophoresis conditions, antibodies, and dilutions for immunoblotting of rotavirus antigens.

Rotavirus antigen	Electrophoresis condition	Primary antibody	Dilution	Secondary antibody	Dilution
VP2	Reducing	Rabbit polyclonal anti-VP2 (kindly provided by professor Koki Taniguchi)	1 µg/ml	Goat anti-rabbit (JIR 111-035-144)	1:10 000
VP6	Reducing	ABIN 308233	1:20000	Goat anti-rabbit (JIR 111-035-144)	1:10 000
VP7	Non-Reducing	Rabbit polyclonal anti-VP7 (kindly provided by professor Koki Taniguchi)	1:2000	Goat anti-rabbit (JIR 111-035-144)	1:10 000

Anti-VP4 Enzyme-linked immuno sorbent assay (ELISA)

- U-bottom 96-well microtiter plates were coated with a mouse monoclonal anti-VP4 (kindly provided by Professor Koki Taniguchi) diluted 1:100000 in 10 mM PBS pH7.4 (phosphate-buffered saline), 150 mM NaCl for 16-18 hours at 4°C. After incubation, plates were washed three times with 10 mM PBS pH7.4, 1 M NaCl containing 0.1% Tween-20 and blocked with 5% BSA in 10 mM PBS pH7.4, 150 mM NaCl containing 0.1% Tween-20 for 1 hour at 37°C. After the blocking step, plates were washed three times with 10 mM PBS pH7.4, 1 M NaCl containing 0.1% Tween-20. Samples were added and plates were incubated for 1 hour at 37°C. Plate were then washed 3 times with 10 mM PBS pH7.4, 1 M NaCl, 1 mM CaCl₂, 0.5 mM MgCl₂ containing 0.1% Tween-20. For all remaining wash steps, washing buffer remain the same and during the third wash, plates were incubated 10 minutes at room temperature before completely removing washing solution. Rabbit polyclonal

antibody raised against Rotavirus (kindly provided by Professor Koki Taniguchi) diluted 1:10000 with 3% BSA in 10 mM PBS pH7.4, 150 mM NaCl, 1 mM CaCl₂, 0.5 mM MgCl₂ containing 0.1% Tween-20 was added and plates were incubated for 1 hour at 37°C. Plates were then washed 3 times and horseradish peroxidase-conjugated goat anti-rabbit antibody (111-035-144, Jackson ImmunoResearch, West Grove, PA) diluted 1:5000 with 3% BSA in 10 mM PBS pH7.4, 150 mM NaCl, 1 mM CaCl₂, 0.5 mM MgCl₂ containing 0.1% Tween-20 was added and plates were incubated for 1 hour at 37°C. Plates were washed 3 times. After final washes, plates were incubated with SureBlue TMB peroxidase substrate (KPL, Gaithersburg, MD) for 20 minutes at room temperature. Reaction was stopped by the addition of 1N HCl and A450 values were measured using a Multiskan Ascent plate reader (Thermo Scientific, Waltham, MA).

Production of rotavirus-like particles comprising VP2 and VP6.

[00253] Rotavirus-like particles comprising VP2 and VP6 were produced by transient expression in *Nicotiana benthamiana*. Plants were agro-infiltrated with an inoculum of *Agrobacteria* containing a mixture of AGL1/1710 and AGL1/1713 in a 1:1 proportion and incubated for 7 days before harvest. Rotavirus-like particles were purified from the biomass using the methodology described in the materials and methods section. After centrifugation of the clarified extracts on iodixanol density gradient, the first ten fractions from the bottom of the tube were analyzed by Coomassie-stained SDS-PAGE. As shown in Figure 45A, the rotavirus antigens (VP2 and VP6) were mainly found in fractions 2 and 3 of the density gradient where the concentration of iodixanol is approximately 35%, a concentration where rotavirus-like particles are expected to be found. Very little contamination by plant proteins was found in these fractions. Western blot analysis of the fractions with anti-rotavirus hyperimmune rabbit serum and polyclonal rabbit anti-VP2 antibodies confirmed the identity of VP2 and VP6 in the density gradient fractions (Figures 45B and 45C). Fractions 2 and 3 were pooled and iodixanol was removed by high speed centrifugation and resuspension, and the purified particles were sent for cryo-electron microscopy analysis (NanoImaging Services Inc., La Jolla, CA) to confirm the assembly of the VP2 and VP6 into particles resembling the rotavirus particle. As shown in Figure 49 (left panel) the cryoEM images of VP2/VP6 particles confirmed the correct assembly of the antigens into rotavirus-like particles.

Production of rotavirus-like particles comprising VP2, VP6 and VP7.

[00254] Rotavirus-like particles comprising VP2, VP6 and VP7 were produced by transient expression in *Nicotiana benthamiana*. Plants were agro-infiltrated with an inoculum of *Agrobacteria* containing a mixture of AGL1/1710 , AGL1/1713, AGL1/1734 in a 1:1:1 proportion and incubated for 7 days before harvest. Rotavirus-like particles were purified from the biomass using the methodology described in the materials and methods section. After centrifugation of the clarified extracts on iodixanol density gradient, the first ten fractions from the bottom of the tube were analyzed by Coomassie-stained SDS-PAGE. As shown in Figure 46A, the rotavirus antigens (VP2, VP6 and VP7) were mainly found in fractions 2 and 3 of the density gradient where the concentration of iodixanol is approximately 35%, a concentration where rotavirus-like particles are expected to be found. Very little contamination by plant proteins was found in these fractions. Western blot analysis of the fractions with anti-rotavirus hyperimmune rabbit serum and polyclonal rabbit anti-VP7 antibodies confirmed the identity of VP6 and VP7 in the density gradient fractions (Figures 46B and 46C).

Production of rotavirus-like particles comprising VP2, VP4, VP6 and VP7.

[00255] Rotavirus-like particles comprising VP2, VP4, VP6 and VP7 were produced by transient expression in *Nicotiana benthamiana*. Plants were agro-infiltrated with an inoculum of *Agrobacteria* containing a mixture of AGL1/1710, AGL1/1730 , AGL1/1713, AGL1/1734 in a 1:1:1:1 proportion and incubated for 7 days before harvest. Rotavirus-like particles were purified from the biomass using the methodology described in the materials and methods section. After centrifugation of the clarified extracts on iodixanol density gradient, the first ten fractions from the bottom of the tube were analyzed by Coomassie-stained SDS-PAGE. As shown in figure 47A, 3 of the 4 rotavirus antigens (VP2, VP6 and VP7) were visible and mainly found in fractions 3 of the density gradient where the concentration of iodixanol is approximately 35%, a concentration where rotavirus-like particles are expected to be found. Very little contamination by plant proteins was found in these fractions. The absence of detectable level of VP4 in the Coomassie-stained gel was expected since VP4 cannot be observed when the same analysis is performed on purified human rotavirus virion. Western blot analysis of the fractions with anti-rotavirus hyperimmune rabbit serum and polyclonal rabbit anti-VP7 antibodies confirmed the

identity of VP6 and VP7 in the density gradient fractions (Figures 47B and 47C). Iodixanol was removed from fraction 3 by high speed centrifugation and resuspension and the purified particles were analyzed by ELISA to confirm the presence of VP4. The results presented in Figure 48 clearly show that the ELISA specifically recognizes VP4 as the negative control particles comprising VP2/VP6 and VP7 only resulted in background signal level. In contrast, the analysis of 3 different lots of purified particles comprising VP2, VP4, VP6 and VP7 antigens showed strong and uniform signals when assayed under the same conditions. Purified VP2/VP4/VP6/VP7 RLPs were sent for cryo-electron microscopy analysis (NanoImaging Services Inc., La Jolla, CA) to confirm the assembly of the four antigens into particles resembling the rotavirus particle. As shown in Figure 49 (right panel) the cryoEM images of VP2/VP4/VP6/VP7 particles confirmed the correct assembly of the antigens into rotavirus-like particles.

[00256] Table 6 lists sequences provided in various embodiments of the invention.

Table 6: Sequence description for sequence identifiers.

SEQ ID NO	Description	Page/Figure
1	Amino acid sequence of optimized G9P6_VP2	Figure 16A
2	Amino acid sequence of optimized G9P6_VP4	Figure 16B
3	Amino acid sequence of optimized G9P6_VP6	Figure 16C
4	Amino acid of optimized G9P6_VP7	Figure 16D
5	Primer VP2F	Table 1
6	Primer VP2R	Table 1
7	Primer VP4F	Table 1
8	Primer VP4R	Table 1
9	Primer VP6F	Table 1
10	Primer VP6R	Table 1
11	Primer VP7F	Table 1
12	Primer VP7R	Table 1
13	Nucleotide sequence of G9P6_VP2	Figure 16A
14	Nucleotide sequence of optimized G9P6_VP2	Figure 16A
15	Nucleotide sequence of G9P6_VP4	Figure 16B
16	Nucleotide sequence of G9P6_VP4	Figure 16B
17	Nucleotide sequence of G9P6_VP6	Figure 16C
18	Nucleotide sequence of optimized G9P6_VP6	Figure 16C
19	Nucleotide sequence of G9P6_VP7	Figure 16D
20	Nucleotide sequence of G9P6_VP7	Figure 16D

21	Primer IF-WA_VP2(opt).s1+3c	Figure 17A
22	Primer IF-WA_VP2(opt).s1-4r	Figure 17B
23	Construct 1191 from left to right t-DNA borders (underlined). 2X35S/CPMV-HT/NOS with Plastocyanine-P19-Plastocyanine silencing inhibitor expression cassette	Figure 18
24	Expression cassette number 1733 from 2X35S promoter to NOS terminator. VP7 from Rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] strain is underlined.	Figure 34C
25	Amino acid sequence of VP2 from Rotavirus A WA strain	Figure 20
26	Construct 193 from left to right t-DNA borders (underlined). 2X35S/CPMV-HT/NOS into BeYDV(m)+Replicase amplification system with Plastocyanine-P19-Plastocyanine silencing inhibitor expression cassette	Figure 22B
27	Expression cassette number 1710 from 2X35S promoter to NOS terminator. VP2(opt) from Rotavirus A WA strain is underlined.	Figure 23
28	Primer IF-WA_VP6(opt).s1+3c	Figure 25a
29	Primer IF-WA_VP6(opt).s1-4r	Figure 25b
30	Expression cassette number 1713 from 2X35S promoter to NOS terminator. VP6(opt) from Rotavirus A WA strain is underlined.	Figure 25c
31	Amino acid sequence of VP6 from Rotavirus A WA strain	Figure 26
32	Expression cassette number 1714 from 2X35S promoter to NOS terminator. VP6(opt) from Rotavirus A WA strain is underlined.	Figure 28
33	Primer IF-Rtx_VP4(opt).s1+3c	Figure 30A
34	Primer IF-Rtx_VP4(opt).s1-4r	Figure 30B
35	Expression cassette number 1731 from 2X35S promoter to NOS terminator. VP4(opt) from Rotavirus A Rotarix strain is underlined.	Figure 31A
36	Amino acid sequence of VP4 from rotavirus A Rotarix strain	Figure 32
37	Primer IF-Rtx_VP7(opt).s1+3c	Figure 34A
38	Primer IF-Rtx_VP7(opt).s1-4r	Figure 34B
39	Amino acid sequence of VP7 from Rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] strain	Fig 35
40	Primer IF-Rtx_VP7(opt).s2+4c	Figure 37A
41	Construct 1192 from left to right t-DNA borders (underlined). 2X35S/CPMV-HT/PDISP/NOS with Plastocyanine-P19-Plastocyanine silencing inhibitor expression cassette	Figure 39
42	Expression cassette number 1735 from 2X35S promoter to NOS terminator. PDISP/VP7(opt) from Rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] strain is underlined.	Figure 40A
43	Amino acid sequence of PDISP/VP7 from Rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] strain	41

44	Expression cassette number 1730 from 2X35S promoter to NOS terminator. VP4(opt) from Rotavirus A Rotarix strain is underlined.	Figure 31C
45	Nucleotide sequence encoding VP2(opt) from Rotavirus A WA strain	Figure 19
46	Nucleotide sequence encoding VP6(opt) from Rotavirus A WA strain	Figure 25d
47	Optimized coding sequence of Rotavirus A VP4 from strain RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P1A[8]	Figure 31B
48	Nucleotide sequence encoding VP7 from Rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] strain	Figure 34D
49	Nucleotide sequence encoding PDISP/VP7(opt) from Rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] strain	Figure 40B
50	Coding sequence of Rotavirus A VP4 from strain RVA/Simian-tc/ZAF/SA11-H96/1958/G3P5B[2]	Figure 43A
51	Optimized coding sequence of Rotavirus A VP4 from strain RVA/Simian-tc/ZAF/SA11-H96/1958/G3P5B[2]	Figure 43B
52	Optimized coding sequence of Rotavirus A VP7 from strain RVA/Simian-tc/ZAF/SA11-H96/1958/G3P5B[2]	Figure 43C
53	Coding sequence of Rotavirus A VP7 from strain RVA/Simian-tc/ZAF/SA11-H96/1958/G3P5B[2]	Figure 43D
54	Optimized coding sequence of Rotavirus A VP7 from strain RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P1A[8]	Figure 34E
55	Primer IF-TrSP+Rtx_VP7(opt).s1+3c	Figure 44A
56	Primer IF-Rtx_VP7(opt).s1-4r	Figure 44B
57	Nucleotide sequence of Optimized coding sequence of Rotavirus A VP7 from strain RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P1A[8]	Figure 44C
58	Expression cassette number 1734 from 2X35S promoter to NOS terminator. VP7 from Rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] strain is underlined.	Figure 44D
59	Amino acid sequence of TrSp-VP7 from Rotavirus A vaccine USA/Rotarix-A41CB052A/1988/G1P1A[8] strain	Figure 44E

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[00257] All citations are hereby incorporated by reference.

[00258] The present invention has been described with regard to one or more embodiments. However, it will be apparent to persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention as defined in the claims.

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WHAT IS CLAIMED IS:

1. A method of producing a rotavirus like particle (RLP) in a plant, portion of a plant or plant cell, comprising:
 - a) introducing a first nucleic acid comprising a first regulatory region active in the plant operatively linked to a first nucleotide sequence encoding a first rotavirus structural protein selected from one of VP2, VP6 and VP7, a second nucleic acid comprising a second regulatory region active in the plant operatively linked to a second nucleotide sequence encoding a second rotavirus structural protein selected from one of VP2, VP6 and VP7 and a third nucleic acid comprising a third regulatory region active in the plant operatively linked to a third nucleotide sequence encoding a third rotavirus structural protein selected from one of VP2, VP6 and VP7, wherein each of VP2, VP6 and VP7 are introduced into the plant, portion of the plant or plant cell, wherein the first, second or third nucleotide sequence encoding the VP7 comprises a truncated signal peptide or non-native signal peptide;
 - b) incubating the plant, portion of the plant or plant cell under conditions that permit the transient expression of the first, second and third nucleic acid, thereby producing the RLP,
 - c) harvesting the plant, portion of the plant or plant cell, and
 - d) extracting and purifying the RLP from the plant, portion of the plant or plant cell, in the presence of calcium.
2. The method of claim 1, wherein a fourth nucleic acid comprising a fourth regulatory region active in the plant and operatively linked to a fourth nucleotide sequence encoding a fourth rotavirus structural protein is introduced in step a), and is expressed when incubating the plant, portion of the plant or plant cell in step b), wherein the fourth rotavirus structural protein is VP4.
3. The method of claim 1, wherein the first, second and third nucleic acid are introduced into the plant, portion of the plant or plant cell by agro-infiltration with an inoculum comprising an *Agrobacteria* mixture, the mixture comprising a first, second and third *Agrobacterium* in a ratio of 1:1:1, each *Agrobacterium* comprising one of the first, second, and third nucleic acid.
4. The method of claim 2, wherein the first, second, third and fourth nucleic acid are introduced into the plant, portion of the plant or plant cell by agro-infiltration with an inoculum comprising an *Agrobacteria* mixture, the mixture comprising a first, second, third and fourth *Agrobacterium* in a ratio of 1:1:1:1, each *Agrobacterium* comprising one of the first, second, third and fourth nucleic acid.
5. The method of claim 1 or 2, wherein the codon usage of the nucleotide sequence is adjusted to preferred human codon usage, increased GC content, or a combination thereof.
6. The method of claim 1, wherein the first, second and third nucleotide sequence is operatively linked to a translation enhancer element.
7. The method of claim 6, wherein the translation enhancer element comprises a Cowpea Mosaic Virus (CPMV)

regulatory region.

8. The method of claim 1, wherein the first, second or third nucleotide sequence or a combination thereof is operatively linked to a Cowpea Mosaic Virus (CPMV) regulatory region.

9. The method of claim 2, wherein the first, second, third or fourth nucleotide sequence or a combination thereof is operatively linked to a Cowpea Mosaic Virus (CPMV) regulatory region.

10. The method of claim 1, wherein the non-native signal peptide is a signal peptide obtained from a plant.

11. The method of claim 10, wherein the non-native signal peptide is a protein disulfide isomerase signal (PDI) peptide.

12. The method of claim 1 wherein the nucleotide sequence encoding VP2 comprises from 80% to 100% identity with a nucleotide sequence as defined by SEQ ID NO:13, SEQ ID NO:14, or SEQ ID NO: 45, the nucleotide sequence encoding VP6 comprises from 80% to 100% identity with a nucleotide sequence as defined by SEQ ID NO:17, SEQ ID NO:18 or SEQ ID NO:46, the nucleotide sequence encoding VP7 comprises from 80% to 100% identity with a nucleotide sequence as defined by SEQ ID NO: 19, 20, 48, 49, 52, 53, 54 or 57.

13. The method of claim 2 wherein the nucleotide sequence encoding VP2 comprises from 80% to 100% identity with a nucleotide sequence as defined by SEQ ID NO:13, SEQ ID NO:14, or SEQ ID NO: 45, the nucleotide sequence encoding VP6 comprises from 80% to 100% identity with a nucleotide sequence as defined by SEQ ID NO:17, SEQ ID NO:18 or SEQ ID NO:46, the nucleotide sequence encoding VP7 comprises from 80% to 100% identity with a nucleotide sequence as defined by SEQ ID NO: 19, 20, 48, 49, 52, 53, 54 or 57, the nucleotide sequence encoding VP4 comprises from 80% to 100% identity with a nucleotide sequence as defined by SEQ ID NO: 15, 16, 47, 50, or 51.

14. A method of producing a rotavirus like particle (RLP) in a plant, portion of a plant or plant cell comprising:

a) providing a plant, portion of a plant or plant cell comprising a first nucleic acid comprising a first regulatory region active in the plant operatively linked to a first nucleotide sequence encoding a first rotavirus structural protein selected from one of VP2, VP6 and VP7, a second nucleic acid comprising a second regulatory region active in the plant operatively linked to a second nucleotide sequence encoding a second rotavirus structural protein selected from one of VP2, VP6 and VP7 and a third nucleic acid comprising a third regulatory region active in the plant operatively linked to a third nucleotide sequence encoding a third rotavirus structural protein selected from one of VP2, VP6 and VP7, wherein the plant, portion of the plant or plant cell comprises each of VP2, VP6 and VP7; and

wherein the first, second or third nucleotide sequence encoding the VP7 comprises a truncated signal peptide or non-native signal peptide;

b) incubating the plant, portion of a plant or plant cell under conditions that permit the transient expression of

the first, second and third nucleic acid, thereby producing the RLP;

c) harvesting the plant, portion of the plant or plant cell, and

d) extracting and purifying the RLP from the plant, portion of the plant or plant cell, in the presence of calcium.

15. The method of claim 14, wherein the plant, portion of the plant or plant cell is provided with a fourth nucleic acid comprising a fourth regulatory region active in the plant and operatively linked to a fourth nucleotide sequence encoding a fourth rotavirus structural protein in step a), and the fourth rotavirus structural protein is expressed when incubating the plant, portion of the plant or plant cell in step b), wherein the fourth rotavirus structural protein is VP4.

16. The method of claim 14 or 15, wherein the codon usage of the nucleotide sequence is adjusted to preferred human codon usage, increased GC content, or a combination thereof.

17. The method of claim 14, wherein the first, second and third nucleotide sequence is operatively linked to a translation enhancer element.

18. The method of claim 17, wherein the translation enhancer element comprises a Cowpea Mosaic Virus (CPMV) regulatory region.

19. The method of claim 15, wherein the first, second, third or fourth nucleotide sequence or a combination thereof is operatively linked to a Cowpea Mosaic Virus (CPMV) regulatory region.

20. The method of claim 14, wherein the non-native signal peptide is a signal peptide obtained from a plant.

21. The method of claim 14, wherein the non-native signal peptide is a protein disulfide isomerase signal (PDI) peptide.

22. A RLP produced by the method of claim 15, wherein the RLP is of a size from 70-100 nm.

23. A composition comprising an effective dose of the RLP of claim 22 for inducing an immune response in a subject, and a pharmaceutically acceptable carrier.

24. Composition of claim 23 for use in the treatment of a rotavirus infection in a subject.

25. A method of producing a rotavirus like particle (RLP) in a plant, portion of a plant or plant cell, comprising:

a) introducing a first nucleic acid comprising a first regulatory region active in the plant operatively linked to a first nucleotide sequence encoding a first rotavirus structural protein selected from one of VP2, VP6 and VP7, a second nucleic acid comprising a second regulatory region active in the plant operatively linked to a second nucleotide sequence encoding a second rotavirus structural protein selected from one of VP2, VP6 and VP7, and a third nucleic acid comprising a third regulatory region active in the plant operatively linked to a third nucleotide sequence encoding a third rotavirus structural protein selected from one of VP2, VP6 and VP7, into the plant, portion of the plant or plant cell, wherein the first, second and third rotavirus structural proteins are

not identical to each other, and the first, second or third nucleotide sequence encoding VP7 comprises a nucleotide sequence encoding a truncated signal peptide obtained from a Rotavirus structural protein, or comprises a nucleotide sequence encoding a signal peptide obtained from a plant, or

a) providing the plant, portion of the plant or plant cell comprising a first nucleic acid comprising a first regulatory region active in the plant operatively linked to a first nucleotide sequence encoding a first rotavirus structural protein, a second nucleic acid comprising a second regulatory region active in the plant operatively linked to a second nucleotide sequence encoding a second rotavirus structural protein and a third nucleic acid comprising a third regulatory region active in the plant operatively linked to a third nucleotide sequence encoding a third rotavirus structural protein, wherein the first rotavirus structural protein is VP2, the second rotavirus structural protein is VP6 and the third rotavirus structural protein is VP7, and wherein the third nucleotide sequence encoding VP7 comprises a nucleotide sequence encoding a truncated signal peptide obtained from a Rotavirus structural protein, or comprises a nucleotide sequence encoding a signal peptide obtained from a plant,

b) incubating the plant, portion of the plant or plant cell under conditions that permit the transient expression of the first, second and third nucleic acid, thereby producing the RLP, the RLP comprising VP2, VP6 and VP7,

c) harvesting the plant, portion of the plant or plant cell.

26. The method of claims 1, 14 or 25, wherein the plant is *Nicotiana benthamiana*.