

PICORNAVIRUS-LIKE PARTICLE PRODUCTION IN PLANTS

FIELD OF INVENTION

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

BACKGROUND OF THE INVENTION

[0002] Picornaviruses are small non-enveloped positive strand RNA viruses that can cause a wide range of clinical manifestations in humans and animals. Based on a number of properties including sequence homologies and acid sensitivity,

Picornaviruses are separated into a number of genera among them are many important pathogens of humans and animals.

[0003] Picornaviruses have naked nucleocapsid. The capsid is an arrangement of 60 protomers in a tightly packed icosahedral structure. Each protomer consists of 4 polypeptides known as VP (viral protein)1, 2, 3 and 4. VP2 and VP4 polypeptides originate from one precursor known as VP0, which is cleaved after the internalization of the viral genomic RNA into the cell. VP4 is located on the internal side of the capsid. Depending on the type and degree of dehydration the viral particle is around 27-30 nm in diameter.

[0004] Picornaviruses have a monopartite, linear, polyadenylated ssRNA(+) genome of 7.1-8.9 kb, that is composed of a single ORF encoding a polyprotein. Viral genomic RNA has a viral protein (VPg) at its 5' end instead of a methylated nucleotide cap structure. The long UTR at the 5' end contains an internal ribosome entry site (IRES). The P1 region encodes the structural polypeptides. The P2 and P3 regions encode the nonstructural proteins associated with replication. The shorter 3' UTR is important in (-)strand synthesis. L is an additional N-terminal leader protein present in some genera that can either be a protease (aphthoviruses, erboviruses) or have other function (kobuvirus, cardiovirus).

[0005] The virion RNA is infectious and serves as both the genome and viral messenger RNA. The IRES allows direct translation of the polyprotein. The

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polyprotein is initially processed by the viral protease(s) into various precursor and mature proteins to yield the structural proteins, replicase, VPg, and a number of proteins that modify the host cell, ultimately leading to cell lysis.

[0006] Enterovirus 71 (EV71) is a member of the *Picornaviridae* family of single stranded RNA viruses. It is a non-enveloped virus and its capsid is constituted of multiple coat proteins produced as fragments of a single viral translation product. The processing of viral polyprotein into structural and non-structural components is presented in Figure 1 (prior art). The P1 region of the polyprotein gene encodes the structural proteins while P2 and P3 regions encode non-structural components of the virus. After cleavage of the structural protein precursor P1 (1ABCD in Figure 1) from the polyprotein by the viral protease 2A, the P1 precursor is processed into the capsid proteins VP0, VP1 (1D fragment in Figure 1) and VP3 (1C fragment in Figure 1). The 3C component and its precursor 3CD – encoded by the P3 region – are the viral proteases responsible for processing the P1 precursor into capsid proteins. The VP0, VP1 and VP3 protomers spontaneously assemble into empty capsids and it is believed that viral RNA is packaged into the particles after the assembly of empty particles. Association of the empty capsid with genomic RNA results in a structural shift, internalization of the RNA, autocatalytic cleavage of VP0 into VP2 (1B fragment in Figure 1) and VP4 (1A fragment in Figure 1), and maturation into a stable 150S virion. Empty capsids, containing the uncleaved VP0 precursor, are commonly found during picornavirus infections.

[0007] Production of EV71 VLPs in insect cells has been obtained from the co-expression of the P1 precursor protein with the 3CD protease (Hu et al., 2003, *Biotechnology Letters* 25: 919-925). Use of a single baculovirus vector for the production of P1 and 3CD is described by Chung et al. (2008, *Vaccine* 26: 1855-1862). Immunogenicity studies in mice showed that purified EV71 VLPs conferred protection to a challenge with lethal doses of the virus.

[0008] The VP1 protein from EV71 has been produced in fruits of transgenic tomatoes, and feeding mice with transgenic fruit containing VP1 resulted in the development of VP1-specific fecal IgA and serum IgG (Chen et al., 2006, *Vaccine* 24: 2944-2951).

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[0009] The P1 precursor protein and protease 3C of the foot and mouth disease virus (FMDV) was co-expressed in transgenic alfalfa (Dus Santos et al. 2005, Vaccine 23: 1838-1843). The alfalfa was stably transformed with a single vector comprising the genomic region of FMDV P1 (1A, 1B, 1C, 1D), 2A, the first 16 amino acid residues of the N terminus of 2B, the complete sequence of 3B1, 3B2, 3B3, 3C and the first 16 amino acid residues of the N terminus of 3D. Immunogenicity of crude protein extracts from the transgenic plants was demonstrated by intraperitoneal administration in Balb/c mice. Immunized mice were also protected against a lethal FMDV challenge. The levels of antigen expression were low for practical purposes.

[0010] Argentinean Application AR078257 discloses a transgenic plant expressing an empty capsid virus, wherein the transgenic plant comprises in its genome a DNA construct encoding a P1 precursor polypeptide linked to autocatalytic 2A protease. The DNA construct may further contain protein fragment 2B attached to the sequence encoding the 3C protease linked to a fragment of the sequence encoding a protein fragment 3D.

SUMMARY OF THE INVENTION

[0011] The present invention relates to producing picornavirus structural proteins in plants. More specifically, the present invention also relates to producing virus-like particles comprising picornavirus structural protein in plants.

[0012] According to the present invention there is provided a method (A) of producing a Picornavirus-like particle (PVLV) in a plant comprising:

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

b) introducing 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 protease;

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c) incubating the plant, portion of the plant under conditions that permit the expression of the first and second nucleic acid, thereby producing thePVLP.

[0013] The present invention also provides a method (B) of producing a
5 Picornavirus-like particle (PVLP) 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 one or more picornavirus polyprotein and a second nucleic acid comprising a second regulatory region
10 active in the plant operatively linked to a second nucleotide sequence encoding one or more protease;

b) incubating the plant, portion of the plant, or plant cell under conditions that permit the expression of the nucleic acids, thereby producing the PVLP.

[0014] The first regulatory region active in the plant, and the second regulatory region active in the plant may be the same or different.

[0015] Furthermore, in method (A) or (B) the percent ratio of the first nucleic acid to the second nucleic acid introduced into the plant, portion of the plant, or plant cell may be between 95%:5% to 50%:50%, or from between about 20:1 to about 0.5:1.

[0016] The present invention also includes the methods (A) or (B) as described above, wherein the first nucleic acid sequence comprises the first regulatory region operatively linked with a one or more than one comovirus enhancer, the nucleotide sequence encoding the polyprotein, and one or more than one geminivirus amplification element, and a third nucleic acid encoding a geminivirus replicase is
20 introduced into the plant or portion of the plant. The one or more than one comovirus enhancer may be a comovirus UTR, for example, a Cowpea Mosaic Virus hypertranslatable (CPMV-HT) UTR such as the CPMV-HT 5', 3'UTR, or a combination thereof. The one or more than one geminivirus amplification element
25 may be selected from a Bean Yellow Dwarf Virus long intergenic region (BeYDV LIR), and a BeYDV short intergenic region (BeYDV SIR).
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[0017] The methods as described above (Method A) may also involving introducing another nucleic acid sequence encoding a suppressor of silencing, for example HcPro or p19.

[0018] The methods as described above (Method B) may also involving further providing the plant, portion of plant, or plant cell comprising another nucleic acid sequence encoding a suppressor of silencing, for example HcPro or p19.

[0019] The present invention also includes the method (A) as described above, wherein in the step of introducing (step a), the nucleic acid is transiently expressed in the plant. Alternatively, in the step of introducing (step a), the nucleic acid is stably expressed in the plant.

[0020] The methods (A) and (B) as described above may further comprising a step of harvesting the plant and purifying the PVLPs.

[0021] The present invention includes a composition comprising an effective dose of the PVLP as just described for inducing an immune response, and a pharmaceutically acceptable carrier.

[0022] The present invention also includes a method of inducing immunity to picornavirus infection in a subject, comprising administering the PVLP as just described to the subject. The PVLP may be administered to a subject orally, intradermally, intranasally, intramuscularly, intraperitoneally, intravenously, or subcutaneously.

[0023] The present invention also provides plant matter comprising a PVLP produced by the method (A) and/or (B) described above. The plant matter may be used in inducing immunity to a picornavirus infection in a subject. The plant matter may also be admixed as a food supplement.

[0024] This summary of the invention does not necessarily describe all features of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] 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:

[0026] **Figure 1** shows a prior art representation of a picornavirus genome (enterovirus 71) and polyprotein processing intermediates. (from ViralZone webpage)

[0027] **Figure 2** shows a Western blot analysis of P1 expression and processing by 3CD. Ten micrograms of protein extracts from plants transformed with the expression vectors identified above were loaded and electrophoresed under non-reducing conditions. Mouse anti-VP1 monoclonal antibodies were used for immunodetection. The ratios indicate the proportion of P1 (construct 1300 or 1301 see table1) to 3CD (construct 1310, 1311 or 1315 see table 1) *Agrobacterium* strains in the bacterial suspension used for transformation. The expected position of P1 and VP1 are indicated.

[0028] **Figure 3** shows the Screening of 3CD expression strategies for maximal accumulation of VP1. Five micrograms of protein extracts from plants transformed with the expression vectors identified above were loaded and electrophoresed under non-reducing conditions. Mouse anti-VP1 monoclonal antibodies were used for immunodetection. The ratios indicate the proportion of P1 (1301) to 3CD (1310, 1311, 1312 and 1313) *Agrobacterium* strains in the bacterial suspension used for transformation.

[0029] **Figure 4** shows the assessment of EV71 capsid assembly. (A) Coomassie-stained SDS-PAGE and western blot analysis of elution fractions from size exclusion chromatography (SEC) separation of protein extracts from plants co-expressing P1 and 3CD (constructs 1301+1310 (4:0.5)). The band putatively corresponding to VP1 in the Coomassie-stained gel is indicated. (B) Negative staining transmission electron microscopy examination of SEC elution fraction 12. The bar represents 100 nm.

[0030] **Figure 5** shows the characterization of purified EV71 PVLPS. (A) Coomassie-stained SDS-PAGE and western blot analysis of purified EV71 PVLPS. The band corresponding to VP1 in the Coomassie-stained gel is indicated. Other bands, corresponding in molecular weight to other EV71 capsid proteins are also identified.

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(B) Negative staining transmission electron microscopic examination of purified EV71 PVLPS. The sample was diluted 1/100 prior to examination. The bar represents 100 nm.

[0031] **Figure 6** shows characterization of lot 479-23-018 by electron microscopy.

5 [0032] **Figure 7** shows cryo-electron microscopy analysis of EV71 PVLPS extracted by enzyme-assisted method, processes and selected by HIC (lot no. 479-31-020).

[0033] **Figure 8** shows cryo-electron microscopy analysis of EV71 PVLPS extracted by mechanical extraction method (pH 8.0) with heat treatment (lot no. 479-32-020).

10 **Figure 9A** shows the 3CD from EV71 strain HK08 comprising amino acids 1549-2193 (SEQ ID NO: 1), as set forth under GenBank ID ADG57603. **Figure 9B** shows 3CD from EV71 strain HK08 comprising nucleotide 5387 -7321 (SEQ ID NO: 2) set forth under GenBank ID GQ279369. **Figure 9C** shows 3CD from EV71 strain GDFS08 comprising amino acids 1549-2193 (SEQ ID NO: 3) as set forth under GenBank ID ACI25378. **Figure 9D** shows 3CD from EV71 strain GDFS08
15 comprising nucleotide 5387 -7321 (SEQ ID NO: 4) set forth under GenBank ID FJ194964. **Figure 9E** shows P1 amino acids sequence GenBank ID ADG57603 (amino acids 1-862) (SEQ ID NO: 5). **Figure 9F** shows P1 nucleotide sequence GenBank ID GQ279369 (nucleotides 743-3328) (SEQ ID NO: 6). **Figure 9G** shows PVgp1 polyprotein nucleotide sequence from Human enterovirus C serotype PV-1
20 (GenBank ID NC_002058 for genome and NP_041277 for polyprotein: nt 5438-7369) (SEQ ID NO: 7). **Figure 9H** shows amino acid sequence of polyprotein from Poliovirus (aa 1566-2209 from GenBank ID NP_041277) (SEQ ID NO: 8). **Figure 9I** shows nucleotide sequence of PVgp1 polyprotein [*Human enterovirus C*] (nt 743-3385 from GenBank ID NC_002058) (SEQ ID NO: 9). **Figure 9J** shows amino acid
25 sequence of polyprotein [*Human enterovirus C*] GenBank ID NP_041277 (aa 1-881 from GenBank ID NP_041277) (SEQ ID NO: 10).

DETAILED DESCRIPTION

[0034] The following description is of a preferred embodiment.

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[0035] The present invention relates to virus-like particles (VLPs) comprising one or more picornavirus structural protein (i.e. a picornavirus like protein, or PVLP), and methods of producing PVLPs in plants or in portions of the plant. The PVLP may therefore comprise one or more than one picornavirus structural protein. For example, the PVLP may comprise one or more than one enterovirus structural protein.

[0036] The picornavirus may be selected from the group of Aphthovirus, Avihepatovirus, Cardiovirus, Enterovirus, Erbovirus, Hepatovirus, Kobuvirus, Parechovirus, Sapelovirus, Senecavirus, Teschovirus and Tremovirus. In a non-limiting example the picornavirus may be an Enterovirus, for example Enterovirus 71 (EV71) or Human enterovirus C (also known as poliovirus).

[0037] The present invention in part provides a method of producing a VLP, for example a PVLP or an enterovirus like particle 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 one or more picornavirus polyprotein into the plant, or portion of the plant and introducing a second nucleic acid comprising a second regulatory region active in the plant operatively linked to a second nucleotide sequence encoding a protease. Followed by incubating the plant or portion of the plant under conditions that permit the expression of the nucleic acids, thereby producing the PVLP.

[0038] The term "virus-like particle" (VLP), or "virus-like particles" or "VLPs" refers to structures that self-assemble and comprise one or more than one structural protein, for example one or more than one picornavirus structural protein, or one or more than one enterovirus structural protein, or a combination thereof, for example but not limited to VP0, VP1, VP2, VP3, VP4 structural protein, or a combination thereof. VLPs 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.

[0039] The term "Picornavirus-like particle" (PVLP), refers to a VLP or VLPs comprising one or more than one picornavirus structural protein. The term or

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"enterovirus-like particle" refers to a VLP or VLPs comprising one or more than one enterovirus structural protein. Example of picornavirus structural proteins may include, but not limited to VP0, VP1, VP2, VP3, VP4, or a combination thereof structural protein. Example of enterovirus structural proteins may include, but not limited to VP0, VP1, VP2, VP3, VP4, or a combination thereof, structural protein.

[0040] By polyprotein is meant a protein that comprises one or more than one protein or protein precursor, which when proteolytic processed provide one or more protein. For example the polyprotein may comprise one or more than one structural protein. The one or more proteins for example structural protein, in the polypeptide may for example be separated by cleavage sites, such for example protease cleavage sites. A non-limiting example for a "polyprotein" is the structural protein precursor P1 also referred to as "P1 region". The P1 region is defined as that part of the picornavirus polyprotein which generates "structural proteins" or "coat proteins" for example VP0, VP1, VP2, VP3, VP4 or a combination thereof. Non-limiting examples of picornavirus P1, or fragments of P1 that may be used according to the present invention include those P1 from enterovirus for example enterovirus 71.

[0041] An example of a P1 region, which is not to be considered limiting, is the amino acid sequence set forth under GenBank ID ADG57603 comprising amino acids 1-862 (SEQ ID NO: 5) or a sequence having at least about 90-100% sequence similarity thereto, including any percent similarity within these ranges, such as 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100% sequence similarity thereto. Furthermore, a non-limiting example of a nucleotide sequence encoding a P1 region is set forth under GenBank ID GQ279369 comprising nucleotides 743-3328 (SEQ ID NO: 6) or a sequence having at least about 90-100% sequence similarity thereto, including any percent similarity within these ranges, such as 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100% sequence similarity thereto.

[0042] Another example of a P1 region, which is not to be considered limiting, is the amino acid sequence set forth under GenBank ID NP_041277 comprising amino acids 1566-2209 (SEQ ID NO: 8) or a sequence having at least about 90-100% sequence similarity thereto, including any percent similarity within these ranges, such as 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100% sequence similarity thereto.

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Furthermore, a non-limiting example of a nucleotide sequence encoding a P1 region is set forth under GenBank ID NC_002058 comprising nucleotides 5438-7369 (SEQ ID NO: 7) or a sequence having at least about 90-100% sequence similarity thereto, including any percent similarity within these ranges, such as 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100% sequence similarity thereto. In another example which is not to be considered limiting the P1 region has the amino acid sequence set forth under GenBank ID NP_041277 comprising amino acids 1-881 (SEQ ID NO: 10) or a sequence having at least about 90-100% sequence similarity thereto, including any percent similarity within these ranges, such as 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100% sequence similarity thereto. Furthermore, a non-limiting example of a nucleotide sequence encoding a P1 region is set forth under GenBank ID NC_002058 comprising nucleotides 743-3385 (SEQ ID NO: 9) or a sequence having at least about 90-100% sequence similarity thereto, including any percent similarity within these ranges, such as 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100% sequence similarity thereto.

[0043] A “picornavirus polyprotein” refers to all or a portion of a picornavirus polyprotein isolated from picornavirus, present in any naturally occurring or variant picornavirus strain or isolate, for example an enterovirus polyprotein. Similarly, the “picornavirus structural protein” may refer to all or a portion of a picornavirus structural protein isolated from picornavirus, present in any naturally occurring or variant picornavirus strain or isolate, for example an enterovirus structural protein, for example obtained from a poliovirus or enterovirus 71. Thus, the term “picornavirus polyprotein” and “picornavirus structural protein” and the like include naturally occurring variants of picornavirus polyprotein, picornavirus structural protein, or a combination thereof, 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.). The term “picornavirus polyprotein” further includes “enterovirus polyprotein” and “enterovirus structural protein” and the like include naturally occurring variants of enterovirus polyprotein, enterovirus structural protein, or a combination thereof, 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.). The term “picornavirus polyprotein” may also include “poliovirus

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polyprotein” and “poliovirus structural protein” and the like include naturally occurring variants of poliovirus polyprotein, poliovirus structural protein, or a combination thereof, 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, native and variants of picornavirus, enterovirus or poliovirus polyprotein, or picornavirus, enterovirus or poliovirus structural protein may be also produced using recombinant techniques.

[0044] The polyprotein may comprise one or more structural proteins for example capsid proteins. Non-limiting examples of picornavirus structural protein or capsid proteins are picornavirus protein VP0, VP1, VP2, VP3 and VP4 and a fragment of VP0, VP1, VP2, VP3 and VP4. Non-limiting examples of VP0, VP1, VP2, VP3 and VP4, or fragments of VP0, VP1, VP2, VP3 and VP4 protein that may be used according to the present invention include those VP0, VP1, VP2, VP3 and VP4 protein from enterovirus, for example poliovirus or enterovirus 71. Furthermore, the polyprotein structural protein, or a combination thereof may be for example from enterovirus 71 strain HK08 or strain GDFS08. In another non limiting example the polyprotein structural protein or a combination thereof may be from human enterovirus C, also known as poliovirus.

[0045] 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).

[0046] In the present invention picornavirus, enterovirus (including poliovirus) VLPs are produced in a plant, portion of a plant or plant cell, by co-expressing a nucleic acid (a first nucleic acid) encoding a picornavirus, enterovirus or poliovirus polyprotein, for example but not limited to P1, with a second nucleic acid encoding a protease, for example a picornavirus, enterovirus or poliovirus protease such as for example but not limited to 3CD, and thereby producing VLP.

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[0047] An example of a protease, which is not to be considered limiting, is an amino acid sequence from the EV71 strain HK08 comprising amino acids 1549-2193, as set forth under GenBank ID ADG57603 (SEQ ID NO:1). The nucleotide sequence set forth under GenBank ID GQ279369 (SEQ ID NO:2) from nucleotide 5387 to nucleotide 7321 or a sequence having at least about 90-100% sequence similarity to SEQ ID NO:2, including any percent similarity within this range, such as 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100% sequence similarity thereto. Another non-limiting example is the amino acid sequence from the EV71 strain GDFS08 comprising amino acids 1549-2193 as set forth under GenBank ID ACI25378 (SEQ ID NO:3). The nucleotide sequence set forth under GenBank ID FJ194964 (SEQ ID NO:4), from nucleotide 5387 to nucleotide 7321 may be used to produce the amino acid sequence. Furthermore, a sequence having between about 90-100% sequence similarity to SEQ ID NO:4, including any percent similarity within this range, such as 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100% sequence similarity thereto.

[0048] The first nucleic acid, and second nucleic acid, may be introduced to the plant in the same step, or may be introduced to the plant sequentially.

Sequences

[0049] Non-limiting example of sequences that may be used with the present invention include:

[0050] The P1 sequence from Aphthovirus, Avihepatovirus, Cardiovirus, Enterovirus, Erbovirus, Hepatovirus, Kobuvirus, Parechovirus, Sapelovirus, Senecavirus, Teschovirus and Tremovirus may be used to produce a P1 polypeptide. In a non-limiting example sequence encoding the the P1 polypeptide may be from Enterovirus, for example Enterovirus 71 or human enterovirus C (also known as poliovirus).

[0051] Furthermore non-limiting examples of sequences that may be used to encode a protease for use as described herein include the sequence of protease 3CD, for example, obtained from Aphthovirus, Avihepatovirus, Cardiovirus, Enterovirus, Erbovirus, Hepatovirus, Kobuvirus, Parechovirus, Sapelovirus, Senecavirus, Teschovirus and Tremovirus. In a non-limiting example the sequence encoding the

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3CD protease may be from Enterovirus, for example Enterovirus 71 or Poliovirus (also known as human enterovirus C).

[0052] It has been found that by introducing and co-expressing the polyprotein and the protease in the plant or portion of the plant that the yield of the VLP produced may be modulated. The polyprotein and the protease may be provided on separate nucleic acid constructs and co-expressed, or they may be provided on the same construct but each sequence differentially expressed, as required, to optimize VLP production as described below.

[0053] By “co-expressed” it is meant that two, or more than two, nucleotide sequences are expressed at about the same time within the plant, within the same tissue of the plant and within the same cells in the plant. Moreover, the two, or more than two, nucleotide sequences may need to be expressed within the same cellular compartment such as, for example, the endoplasmic reticulum, Golgi apparatus, apoplast, cytosol, mitochondria, chloroplast, peroxysome. 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. For example, the protease may be expressed either before or during the period when the polyprotein is expressed so that cleavage of the polyprotein into structural proteins may take place. 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 same time under conditions that both sequences are expressed. The two or more than two sequences may be present on different constructs, and co-expression requires introduction of each of the constructs into the plant, portion of plant or plant cell, or the two or more than two sequences may be present on one construct and the construct introduced into the plant, portion of plant or plant cell.

[0054] Alternatively, a plant comprising one of the nucleotide sequences, for example the sequence encoding the protease may be transformed, either transiently or in a stable manner, with an additional sequence encoding the polyprotein. In this case, the sequence encoding the protease may be expressed within a desired tissue, during a desired stage of development, or its expression may be induced using an inducible

promoter, and the additional sequence encoding polypeptide may be expressed under similar conditions and in the same tissue, to ensure that the nucleotide sequences are co-expressed. Additionally, the sequence encoding the polypeptide may be transformed, either transiently or in a stable manner, with an additional sequence encoding the protease. In this case, the sequence encoding the polypeptide may be expressed within a desired tissue, during a desired stage of development, or its expression may be induced using an inducible promoter, and the additional sequence encoding the protease may be expressed under similar conditions and in the same tissue, to ensure that the nucleotide sequences are co-expressed.

[0055] As may be seen in Figures 2 and 3, the level of VLP accumulation in the plant, portion of the plant or plant cell, is influenced by the ratio of the polypeptide-containing *Agrobacterium*, to protease-containing *Agrobacterium* infiltrated into the plant, portion of the plant or plant cell. The ratio of the polypeptide-containing to protease-containing *Agrobacterium* may range for example from about 20:1 to about 0.5:1 (polypeptide:protease), or any amount therebetween, for example from about 20:1, 18:1, 16:1, 14:1, 12:1, 10:1, 9:1, 8:1, 7:1, 6:1, 5:1, 4:1, 3:1, 2:1, 1:1, 0.5:1 (polypeptide:protease), or any amount therebetween.

[0056] The ratio of polypeptide to protease may be varied for example by introducing different ratios of *Agrobacterium* containing the first nucleic acid to *Agrobacterium* containing the second nucleic acid into the plant, portion of the plant or plant cell. Alternatively, if the polypeptide and protease are present on the same construct, and therefore are introduced into the same *Agrobacterium*, they may be differentially expressed within the plant, portion of the plant or plant cell using suitable promoters so that the desired ratio of polypeptide to protease is obtained.

[0057] Therefore the present invention also provides a method for increased PVLP production yield by modulating the ratio between the first and second nucleic acid.

[0058] In one embodiment the percentage of the *Agrobacterium* containing protease may be between 0.5% to 50% of total *Agrobacterium* infiltrated or any amount therebetween. For example the percent ratio of *Agrobacterium* containing protease may be 0, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%,

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30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48% , 49% or 50% or any amount therebetween.

[0059] The percentage ratio of *Agrobacterium* containing polyprotein to *Agrobacterium* containing protease may be 95%:5% to 40%:60% of total *Agrobacterium* infiltrated, or any amount therebetween. For example the percentage of *Agrobacterium* containing polyprotein within the total amount of *Agrobacterium* infiltrated may be 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81%, 80%, 79%, 78%, 77%, 76%, 75%, 74%, 73%, 72%, 71%, 70%, 69%, 68%, 67%, 66%, 65%, 64%, 63%, 62%, 61%, 60%, 59%, 58%, 57%, 56%, 55%, 54%, 53%, 52% or 51%. For example, the percentage ratio of *Agrobacterium* containing polyprotein to *Agrobacterium* containing protease may be between 50%:50% and 95%:5%, or any percent ratio in between, or the percentage ratio between *Agrobacterium* containing polyprotein and *Agrobacterium* containing protease may be 50%:50%, 55%:45%, 60%:40%, 65%:35%, 70%:30%, 75%:25%, 80%:20%, 85%:15%, 90%:10%, 95%:5%, or any percentage ratio in between.

[0060] Expression of the first and second nucleotide sequence within a plant cell forms a VLP, and the VLP may be used for example to produce an antibody that is capable of binding a virus protein such for example picornavirus structural protein, including but not limited to VP0, VP1, VP2, VP3 and/or VP4. The VLP, when administered to a subject, induces an immune response.

[0061] As described further below the ratio of polyprotein to protease may further be varied for example by differentially expressing the polyprotein and the protease. Expression may be varied by modulating for example replication, transcription, translation, or a combination thereof, of the polyprotein, the protease, or both the protein and the protease. For example different regulatory elements, including promoters, amplification elements, enhancers or a combination thereof, may be used in addition to varying the ratio of the polyprotein-containing *Agrobacterium* to protease-containing *Agrobacterium* infiltrated as described above. A first set or combination of regulatory elements may be used to regulate the replication, transcription or a combination thereof, of the first nucleic acid and a second set or

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combination of regulatory elements may be used to regulate the replication, transcription or a combination thereof, of the second nucleic acid. The first set or combination of regulatory elements is different from the second set or combination of regulatory elements and permits differential expression of the first and second nucleic acids to permit modulating the ratio of polyprotein:protease *in vivo*. For example, which is not to be considered limiting, one set or combination of regulatory elements, for example the first set, may include an amplification element for example elements obtained from BeYDV, while the amplification element, for example those obtained from BeYDV, may be absent in the other set or combination of regulatory elements, for example the second set. Alternatively, the second set may include an amplification element (for example elements obtained from BeYDV), while the amplification element (for example elements obtained from BeYDV) may be absent in the first set or combination of regulatory elements. In a similar manner, the strength of a promoters may differ between the first and second set or combination of regulatory elements, or one of the promoters may be inducible, and the other constitutive, so that differential expression between the polyprotein relative to the protease is achieved *in vivo*.

Size

[0062] The occurrence of VLPs may be detected using any suitable method for example, sucrose gradients, or size exclusion chromatography. VLPs may be assessed for structure and size by, for example electron microscopy, or by size exclusion chromatography.

[0063] 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. Concentration by PEG-assisted precipitation may also be of benefit. The VLP may also be produced by preparing protoplasts or a protoplast fraction using the methods described in WO 2011/035422 (which is incorporated herein by reference). The soluble protein is quantified, and the extract passed through a SephacrylTM column, for example a SephacrylTM S500 column. Blue Dextran 2000 may be used as a calibration standard.

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[0064] Cellular debris might be eliminated by centrifugation. The centrifuged extract may then be filtered. Without wishing to be bound by theory it is believed that such filter step or steps may remove solids in suspension, reduce bioburden and stabilize and condition the extract prior to further purification. Due to their size, PVLP may be further purified using tangential flow filtration (TFF). Without wishing to be bound by theory, TFF efficiently and selectively eliminates soluble proteins of lower molecular weight found in the clarified extract, including enzymes used for cell wall depolymerisation. Furthermore, the TFF step also concentrates VLPs and enables a buffer exchange in preparation for chromatography. The TFF step might be followed by several chromatographic steps, for example anion exchange, cation exchange, hydrophobic interaction chromatography (HIC) and/or pseudo-affinity. Additional TFF steps may be added following the chromatograph steps. Following chromatography and/or TFF, fractions may be further analyzed by immunoblot to determine the protein complement of the fraction.

[0065] 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 higher-order, higher molecular weight, particles, or complete VLPs. 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 VLPs, 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.

[0066] Figure 4A, show an example of an elution profile of a size exclusion chromatography analysis of a plant extract comprising PVLPs. In this case, VLPs comprising enterovirus EV71 capsid, elute in fractions 9 to approx. 14, peaking in fraction 12.

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[0067] The VLPs may be purified or extracted using any suitable method for example chemical or biochemical extraction. VLPs can be relatively sensitive to desiccation, heat, pH, surfactants and detergents. Therefore it may be useful to use methods that maximize yields, minimize contamination of the VLP fraction with cellular proteins, maintain the integrity of the proteins, or VLPs, and, methods of loosening the cell wall to release the proteins, or VLP. For example, methods that produce protoplasts and/or spheroplasts may be used (see for example WO 2011/035422, which is incorporated herein by reference) to obtain VLPs as described herein. Minimizing or eliminating the use of detergents or surfactants such for example SDS or Triton X-100 may be beneficial for improving the yield of VLP extraction. VLPs may be then assessed for structure and size by, for example, electron microscopy, or by size exclusion chromatography as mentioned above, and submitted to analytical ultracentrifugation.

[0068] The size (i.e. the diameter) of the above-defined PVLPs, maybe measured for example by dynamic light scattering (DLS) or electron microscope (EM) techniques, is usually between 20 to 50 nm, or any size therebetween. For example, the size of the intact PVLP structure may range from about 25 nm to about 35 nm, or any size therebetween, or from 20nm, 21nm, 22nm, 23nm, 24nm, 25nm, 26nm, 27 nm, 28nm, 29nm, 30nm, 31nm, 32nm, 33nm, 34nm, 35nm, 36nm, 37nm, 38nm, 39nm, 40nm, 41nm, 42nm, 43nm, 44nm, 45nm, 46nm, 47nm, 48nm, 49nm, 50nm, or any size therebetween.

[0069] PVLP may be synthesized at an amount of up to 2g per kilogram of plant fresh weight, corresponding to about 40% of the total protein content of the plant. For example, as described herein the amount of synthesized VLP maybe between 10mg and 2.0 g per kilogram of fresh weight, or any amount there between, such as 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625, 650, 675, 700, 725, 750, 775, 800, 825, 850, 875, 900, 925, 950, 975, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900 or 2000 mg per kilogram of fresh weight or any amount therebetween.

Host

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[0070] The one or more than one genetic constructs of the present invention may be expressed in any suitable plant host or portion of the plant, for example, one or more leaves, the stem plus one or more leaves, or the roots, 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, barley, sunflower, cotton and the like.

[0071] 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 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 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).

[0072] 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.

[0073] The constructs of the present invention can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, micro-

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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*, 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. 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 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 Lomonosoff (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 incorporated by reference).

Transient expression

[0074] Without wishing to be bound by theory, the protein concentration and ratio of the different picornavirus structural proteins, the picornavirus polyprotein and/or the protease may be important for the assembly efficiency of PVLPs. Therefore multiplicity and time of infection, may be important to manipulate protein concentration and the overall assembly efficiency of VLPs in plants.

[0075] The construct of the present invention may be transiently expressed in a plant, portion of a plant, or a plant cell. A transient expression system relying on the epichromosomal expression of recombinant polyprotein introduced, via *Agrobacterium tumefaciens* infiltration, into a plant, portion of a plant, or a plant cell may be used to express the picornavirus structural protein, picornavirus polyprotein and/or protease, targeted to various cell compartments or sub-compartments. A

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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).

[0076] 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-p16 or GVA-p10, may be co-expressed along with one or more picornavirus structural protein, picornavirus polyprotein and/or protease to further ensure high levels of protein production within a plant, portion of a plant or plant cell.

[0077] The present invention also provides a method as described above, wherein an additional (third) nucleotide sequence is expressed within the plant, the additional (third) nucleotide sequence encoding a suppressor of silencing is operatively linked with an additional (third) 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-

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p11, BScV-p16, CTV-p23, GLRaV-2 p24, GBV-p14, HLV-p10, GCLV-p16 or GVA-p10.

[0078] As described below, transient expression methods may be used to express the constructs of the present invention (see Liu and Lomonosoff, 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.

[0079] Also considered part of this invention are transgenic plants, plant cells or seeds containing the nucleic acids or one or more than one 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 stably transformed plant cells. To aid in identification of stably 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. 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.

Amplification Elements

5 [0080] The ratio of polyprotein to protease may be varied for example by using different regulatory elements, or combination of regulatory elements, in the nucleic acid sequences used to drive expression of the polyprotein and protease. For example, a first set or combination of regulatory elements may be used to regulate the replication, transcription or a combination thereof, of the first nucleic acid and a
10 second set or combination of regulatory elements may be used to regulate the replication, transcription or a combination thereof, of the second nucleic acid so that a difference in the expression of the first and second nucleic acids is achieved thereby modulating the ratio of polyprotein:protease *in vivo*. For example, which is not to be considered limiting the first set or combination of regulatory elements may include an
15 amplification element, for example, elements obtained from BeYDV, while the amplification element may be absent in the second set or combination of regulatory elements. Alternatively, the second set may include an amplification element, for example, elements obtained from BeYDV, while the amplification element may be absent in the first set or combination of regulatory elements.

20 [0081] “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.

[0082] The expression system as described herein may comprise an expression
25 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
30 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)

[0083] 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.

[0084] 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. BeYDV-derived DNA replicon vector systems have been used for rapid high-yield protein production in plants.

[0085] 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 (LIR) of a geminivirus genome. As used herein, “long intergenic region” 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 (SIR) of a geminivirus genome. As used herein, “short 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, *Biotechnology and Bioengineering*, Vol. 106, 9-17); which are herein incorporated by reference).

Regulatory Element

[0086] The present invention is further directed to a gene construct comprising a nucleic acid encoding a polyprotein, such as one or more picornavirus protein, or a protease, for example but not limited to picornavirus protease, as described above, operatively linked to a regulatory element that is operative in a plant.

[0087] 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 include 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 include 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.

[0088] Examples of regulatory elements operative in a plant cell and that may be used in accordance with the present invention include but are not limited to a plastocyanin regulatory region (US 7,125,978; which is incorporated herein by reference), or a regulatory region of Ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO; US 4,962,028; which is incorporated herein by reference), chlorophyll a/b binding protein (CAB; Leutwiler et al; 1986; which is incorporated herein by reference), ST-LS1 (associated with the oxygen-evolving

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complex of photosystem II and described by Stockhaus et al. 1987, 1989; which is incorporated herein by reference).

[0089] 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.

[0090] 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).

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[0091] 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).

[0092] 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).

[0093] 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.

[0094] The ratio of polyprotein to protease may further be varied for example by using regulatory elements, amplification element and/or enhancers. For example the first nucleic acid may comprise a regulatory elements, amplification element and/or enhancers. The second nucleic acid may or may not comprise the same combination of a regulatory elements, amplification element and/or enhancers.

[0095] For example, different promoters may be used to drive differential expression between the polyprotein relative to the protease *in vivo*. For example, the first set or combination of regulatory elements may include an inducible promoter, while the promoter in the second set or combination of regulatory elements may be constitutive, or the second set or combination of regulatory elements may comprise an inducible promoter, while the promoter in the first set or combination of regulatory elements may be constitutive. The strength of the promoter may also differ between the first

and second set or combination of regulatory elements, so that differential expression between the polypeptide relative to the protease is achieved *in vivo*.

[0096] The present invention will be further illustrated in the following examples.

Example 1 Expression EV71

Gene synthesis

[0097] DNA segments encoding EV71 structural protein P1 and protease 3CD were used. The candidate sequences for P1 and 3CD are available in GenBank. Non limiting examples of these sequences are:

- *For P1* aa sequence: amino acids sequence GenBank ID ADG57603 (amino acids 1-862) (SEQ ID NO:5); nucleotide sequence: GenBank ID GQ279369 (nucleotides 743-3328) (SEQ ID NO:6);
- *For 3CD (strain HK08)*: amino acid sequence: GenBank ID ADG57603 (amino acids 1549-2193) (SEQ ID NO: 1); nucleotide sequence: GenBank ID GQ279369 (nucleotides 5386-7321) (SEQ ID NO:2);
- *For 3CD (strain GDFS08)*: amino acid sequence GenBank ID ACI25378 (amino acids 1549-2193)(SEQ ID NO: 3); nucleotide sequence: GenBank ID FJ194964 (nucleotides 5387-7321) (SEQ ID NO: 4).

[0098] Two P1 genes were synthesized. The first was produced using the wild-type sequence while the second was based on an optimized sequence (human codon usage) determined using standard methods as known in the art. The two 3CD genes were synthesized based on their wild-type sequences. The 3 wild-type genes were synthesized by Invitrogen™ (formerly GeneArt®) and the optimized P1 gene was optimized and synthesized by DNA2.0.

Molecular cloning

[0099] The synthesized genes were cloned into plant expression vectors. Selected vector components include transcription and translation regulatory elements from a

cowpea mosaic virus (CPMV)-based cassette or an alfalfa plastocyanin gene. Both regulatory elements have been used with success in our platform for high expression of recombinant proteins. DNA amplification elements from the Bean yellow dwarf geminivirus (BeYDV) are another feature that can be integrated into our plant expression vectors. It has led to a great increase in protein expression for some candidates. We have therefore cloned each gene construct in expression vectors with or without DNA amplification elements. Table 1 presents the plant expression cassettes assembled for the project.

Table 1. Plant expression cassettes assembled for the expression of EV71 structural polyprotein P1 and protease 3CD in *N. benthamiana*.

Coding region	Regulatory element	DNA amplification elements	Vector number
P1 (Wt HK08)	CPMV HT	-	1300
P1 (Wt HK08)	CPMV HT	BeYDV+rep	1301
P1 (Wt HK08)	Plastocyanin	-	1302
P1 (Wt HK08)	Plastocyanin	BeYDV+rep	1303
P1 (Opt HK08)	CPMV HT	-	1305
P1 (Opt HK08)	CPMV HT	BeYDV+rep	1306
P1 (Opt HK08)	Plastocyanin	-	1307
P1 (Opt HK08)	Plastocyanin	BeYDV+rep	1308
3CD (Wt HK08)	CPMV HT	-	1310
3CD (Wt HK08)	CPMV HT	BeYDV+rep	1311
3CD (Wt HK08)	Plastocyanin	-	1312
3CD (Wt HK08)	Plastocyanin	BeYDV+rep	1313
3CD (Wt GDFS08)	CPMV HT	-	1315
3CD (Wt GDFS08)	CPMV HT	BeYDV+rep	1316
3CD (Wt GDFS08)	Plastocyanin	-	1317
3CD (Wt GDFS08)	Plastocyanin	BeYDV+rep	1318

Analysis of expression - Selecting the best recombinant gene constructs

[00100] Each expression cassettes was cloned into a plasmid vector that was then transferred to *Agrobacterium tumefaciens*. Transient expression was initiated by vacuum infiltration of the transgenic *Agrobacterium* inoculum that leads to transfer of mobile DNA copies of the DNA constructs into plant cells. Transient expression of multiple components (co-expression) was performed by infiltration of mixes of *Agrobacterium* inoculums (co-infiltration). As one component being introduced into the plant was structural (P1), and the substrate of the second component, the 3CD protease, the level of expression of the two components was modulated. This was performed by the use of different promoters, DNA amplification systems of variable

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strength, by varying the relative abundance of each inoculum (P1 and 3CD) at the time of infiltration, or a combination thereof.

[00101] Expression vectors 1300 to 1308 were screened for their ability to express P1 alone, and when combined with vectors 1310 to 1318, for their ability to produce high levels of the proteolytic fragments VP1-4. As only an anti-VP1 antibody was available (Abnova, MAB1255-M05), accumulation of proteolytic fragments was monitored through accumulation of VP1 and disappearance of unprocessed P1. As shown in figure 2, the expression of P1 alone (vector no. 1300) led to the accumulation of a VP1-containing product having an apparent molecular weight corresponding to that of the unprocessed structural protein (98 kDa), indicating that plant proteases cannot cleave P1 to generate the viral capsid proteins. However, when P1 is co-expressed with 3CD (vectors no. 1300 + 1310 and 1300 + 1315), the 98 kDa signal completely disappears and a new product is detected that corresponds in molecular weight to VP1 (33.5 kDa). This result shows that the viral protease is produced and highly active in the plant and that it recognizes and cleaves its co-produced substrate in the plant cells to generate EV71 capsid proteins.

[00102] The results obtained indicated that the level of VP1 accumulation in the plant is influenced by the ratio of *Agrobacterium* containing the P1 protein to *Agrobacterium* containing 3CD protease, with higher accumulation being obtained with a lower proportion of *Agrobacterium* containing 3CD protease (Figure 2: compare 1300+1315 (4:2), 1300+1315 (4:1) and 1300+1315 (4:0.5)). The origin of 3CD, either HK08 vs GDFS08, also impacts on the accumulation level of VP1 in the plant (Figure 2: 1300+1310 (4:0.5) vs 1300+1315 (4:0.5)). Finally, it was observed that the highest VP1 accumulation level was obtained from expression vectors comprising DNA amplification elements (Figure 2: 1301+1311 (4:2)).

[00103] In the following experiment, P1 was maintained under the control of CPMV-HT+BeYDV (1301) while different 3CD expression cassettes were co-transformed at different dilutions at the time of infiltration. Western blot analysis using the anti-VP1 monoclonal antibody on crude protein extracts from the transformed plants indicated that VP1 accumulation was observed over a range of P1 to protease ratios and construct components. The vector combination resulting in the

highest level of VP1 accumulation was 1301 + 1310, with a ratio of *Agrobacterium* strain concentration of 4:0.5 (structural protein: protease) in the bacterial suspension (Figure 3).

Analysis of VLP formation

[00104] The incorporation of VP1 into VLPs was evaluated with the use of size exclusion chromatography (SEC) of concentrated extracts. Colloidal particles were concentrated from crude clarified extracts by high-speed centrifugation (75 000 xg for 20 min.). The pellet was washed and resuspended in 1/6 volume of resuspension buffer (50 mM PBS pH 7.4, 150 mM NaCl) and loaded onto a Sephacryl S-500 gel filtration column. The column was eluted with resuspension buffer and the elution fractions were characterized by SDS-PAGE and western blotting.

[00105] A protein extract from plants transiently transformed with 1301+1310 (4:0.5) was subjected to SEC separation and elution fractions were analyzed by Coomassie-stained SDS-PAGE and anti-VP1 western blot. The results presented in figure 4A show that most of the host proteins eluted from the column in the late fractions, peaking at fraction 16 while the VP1-specific signal was found in earlier fractions, peaking at fraction 12, where very little host protein is found. VP1 being a relatively small protein, it would be expected to elute from the column with the majority of the host proteins if not incorporated into high molecular weight structures. Hence, the elution profile observed for VP1 was strongly indicative that VP1 had been integrated into a high molecular weight structure. A combination of the western blot and the Coomassie-stained gel also suggested that the abundant protein identified by an arrow in the Coomassie-stained SDS-PAGE in figure 4A could be VP1.

[00106] A sample of elution fraction 12 from this experiment was sent to Institut Armand-Frappier (IAF, Laval, Québec) for analysis by transmission electron microscopy (TEM). The sample was examined after negative staining with 3% phosphotungstic acid. Figure 4B shows that spherical particles of 30 nm identical in size and appearance to empty EV71 particles found in EV71-infected Vero cell cultures (Liu et al., PLoS ONE 6, e20005) are observed in elution fraction 12. This result indicates that the high molecular weight structures in which VP1 is incorporated are genuine EV71 VLPs.

Partial purification

[00107] The VLP purification method of the *VLPExpress* screening platform was developed for the purification of enveloped VLPs (140 nm diameter) from transformed plant biomass. The method uses an enzymatic digestion of cell walls for the release of extracellular and cytosolic content and the extract obtained is subjected to deep filtration and to microfiltration before being centrifuged at 16 000 g for 6h to pellet VLPs. The pellet is resuspended in 1/60 volume of resuspension solution (100 mM Na/KPO₄ pH 7.4, 150 mM NaCl, 0.01% Tween-80) and sterile filtered (0.2 µm).

[00108] The *VLPExpress* purification method was tested for its capacity to concentrate the 30 nm non- enveloped EV71 VLPs. The purification method was applied to plants transformed with expression vectors 1301 + 1310 (4:0.5). The resulting product was analyzed by Coomassie-stained SDS- PAGE and anti-VP1 western blot (Figure 5A). Coomassie-stained SDS-PAGE analysis of the purification product showed the presence of proteins corresponding in molecular weight to EV71 coat proteins (indicated by arrows, Figure 5A, right panel). The identity of VP1 was confirmed by western blot (Figure 5A, left panel). For other capsid proteins, the identification was based on the estimated molecular weight; 37.5 kDa for VP0 and 26.5 kDa for VP3. VP4 and VP2 were expected to be found in the form of uncleaved VP0 since in the formation of viral particles the cleavage between VP4 and VP2 only occurs after the internalization of viral RNA. Transmission electron microscopic analysis of the purified product revealed abundant spherical structures of 30 nm, corresponding in size and shape to EV71 VLPs (Figure 5B).

Conclusions on the expression

[00109] The work performed to demonstrate the capacity of the plant-based transient expression platform to produce EV71 VLPs has led to the following conclusions:

- EV71 P1 and 3CD proteins are efficiently produced in the system
- 3CD is active *in planta* and correctly processes P1 into capsid proteins
- EV71 capsid proteins assemble into VLPs

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- EV71 VLPs are extractable and can be purified intact from plant biomass.

Example 2 – Expression Poliovirus Expression

5 *Gene synthesis*

[00110] DNA segments encoding poliovirus (PV) structural protein P1 and protease 3CD from Human enterovirus C serotype PV-1 may be used. The candidate sequences for P1 and 3CD are available in GenBank. Non limiting examples of these sequences are:

10 For P1 : amino acids sequence GenBank ID NP_041277 (amino acids 1-881) (SEQ ID NO:10); nucleotide sequence: GenBank ID NC_002058 (nucleotides 743-3385) (SEQ ID NO: 9);

15 For 3CD: amino acid sequence GenBank ID NP_041277 (amino acids 1566-2209) (SEQ ID NO:8); nucleotide sequence: GenBank ID NC_002058 (nucleotides 5438-7369) (SEQ ID NO:7).

[00111] Two P1 genes may be synthesized. The first may be produced using the wild-type sequence while the second may be based on an optimized sequence (human codon usage) determined using standard methods as known in the art. The 3CD gene may be synthesized based on its wild-type sequence. Both wild-type genes (P1 and 3CD) may be synthesized by Invitrogen™ (formerly GeneArt®) and the optimized P1 gene is optimized and synthesized by DNA2.0.

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Molecular cloning

[00112] The synthesized genes may be cloned into plant expression vectors. Selected vector components include transcription and translation regulatory elements from a cowpea mosaic virus (CPMV)-based cassette or an alfalfa plastocyanin gene, as both regulatory elements have previously been used with success for high expression of recombinant proteins. DNA amplification elements from the Bean yellow dwarf geminivirus (BeYDV) may also be integrated into the plant expression

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vectors. Each gene construct may therefore be cloned in expression vectors with or without DNA amplification elements. Table 2 presents the plant expression cassettes that may be assembled.

[00113] Table 2. Plant expression cassettes for the expression of PV structural polyprotein P1 and protease 3CD in *N. benthamiana*.

Coding region	Regulatory element	DNA amplification elements
P1 (Wt)	CPMV HT	-
P1 (Wt)	CPMV HT	BeYDV+rep
P1 (Wt)	Plastocyanin	-
P1 (Wt)	Plastocyanin	BeYDV+rep
P1 (Opt)	CPMV HT	-
P1 (Opt)	CPMV HT	BeYDV+rep
P1 (Opt)	Plastocyanin	-
P1 (Opt)	Plastocyanin	BeYDV+rep
3CD	CPMV HT	-
3CD	CPMV HT	BeYDV+rep
3CD	Plastocyanin	-
3CD	Plastocyanin	BeYDV+rep

Analysis of expression - Selecting the best recombinant gene constructs

[00114] Each expression cassettes may be cloned into a plasmid vector that may then be transferred to *Agrobacterium tumefaciens*. Transient expression may be initiated by vacuum infiltration of the transgenic *Agrobacterium* inoculum that leads to transfer of mobile DNA copies of the DNA constructs into plant cells. Transient expression of multiple components (co-expression) may be performed by infiltration of mixes of *Agrobacterium* inoculums (co-infiltration). As one component being introduced into the plant is structural (P1), and the substrate of the second component, the 3CD protease, the level of expression of the two components may be modulated. This may be performed by using different promoters, DNA amplification systems of variable strength, by varying the relative abundance of each inoculum (P1 and 3CD) at the time of infiltration, or a combination thereof.

[00115] Expression vectors with P1 may be first screened for their ability to express P1 alone, and when combined with 3CD vectors, for their ability to produce high levels of proteolytic fragments. Accumulation of proteolytic fragments may be monitored through disappearance of unprocessed P1. Viral protease is shown to be

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produced and highly active in the plant, as well as being able to recognize and cleave its co-produced substrate in the plant cells to generate PV capsid proteins.

[00116] The level of proteolytic fragments accumulation in the plant may be influenced by the ratio of Agrobacterium containing the P1 protein to Agrobacterium containing 3CD protease, with higher accumulation being obtained with a lower proportion of Agrobacterium containing 3CD protease. Observation is made with respect to the presence of DNA amplification elements and the use of the different regulatory elements on the processing of P1 and the accumulation of proteolytic fragments.

Analysis of VLP formation

[00117] The incorporation of VP1 into VLPs may be evaluated with the use of size exclusion chromatography (SEC) of concentrated extracts. Colloidal particles may be concentrated from crude clarified extracts by high-speed centrifugation. The pellet may be washed and resuspended in 1/6 volume of resuspension buffer and loaded onto a gel filtration column. The column may be eluted with resuspension buffer and the elution fractions are characterized by SDS-PAGE and western blotting.

[00118] A protein extract from plants transiently transformed may be subjected to SEC separation and elution fractions are analyzed by Coomassie-stained SDS-PAGE. The results may show that most of the host proteins eluted from the column in the late fractions, while the VP1-specific signal may be found in earlier fractions. VP1 being a relatively small protein, it would be expected to elute from the column with the majority of the host proteins if not incorporated into high molecular weight structures. Hence, the elution profile observed for VP1 may be strongly indicative that VP1 had been integrated into a high molecular weight structure. A combination of the western blot and the Coomassie-stained gel may also suggested that the abundant protein observed in the Coomassie-stained SDS-PAGE could be VP1.

[00119] A sample from this experiment may be sent to Institut Armand-Frappier (IAF, Laval, Québec) for analysis by transmission electron microscopy (TEM). The result indicates that the high molecular weight structures in which VP1 is incorporated are genuine PV VLPs.

Partial purification

[00120] The VLP purification method of the VLPEXpress screening platform was developed for the purification of enveloped VLPs (140 nm diameter) from transformed plant biomass. The method uses an enzymatic digestion of cell walls for the release of extracellular and cytosolic content and the extract obtained is subjected to deep filtration and to microfiltration before being centrifuged to pellet VLPs. The pellet is resuspended in resuspension solution and sterile filtered.

[00121] The VLPEXpress purification method may be tested for its capacity to concentrate the non- enveloped PV VLPs. Coomassie-stained SDS-PAGE analysis of the purification product may show the presence of proteins corresponding in molecular weight to PV coat proteins. Identification of the capsid proteins may be based on their estimated molecular weight.

Example 3- Purification

[00122] Protein extraction was performed using either mechanical extraction technique, or enzymatic degradation of the cell wall as described in WO 2011/035422 and PCT/CA2012/050180 (which are incorporated herein by reference). Enzymatic extraction is advantageous over mechanical extraction in that it results in an increased release of product with minimal release of contaminating plant proteins, with the major contaminants in the resulting extract being the enzymes used for cell wall disruption, which can be removed using adequate subsequent downstream steps.

[00123] Mechanical or enzymatic extracts were submitted to centrifugation to eliminate cellular debris, *Agrobacteria*, DNA and larger particles. Centrifuged extracts were then passed through filtration steps performed in order to remove solids in suspension, reduce bioburden, and stabilize and condition the extract prior to downstream processing. Although recovery of EV71 VLPs in the filtrate could not be evaluated in absence of a quantification assay, Western blot analyses indicated that VLP loss during filtration steps was minimal. The resulting clarified extract was further processed using tangential flow filtration (TFF) or directly loaded onto chromatographic media as suitable.

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[00124] The size of VLPs enables the use of TFF for efficient and selective elimination of the soluble proteins found in the clarified extract, including enzymes used for cell wall depolymerisation. The TFF step also concentrates VLPs and enables a buffer exchange in preparation for chromatography.

5 [00125] Several chromatography approaches (anion exchange, cation exchange, hydrophobic interaction chromatography (HIC) and pseudo-affinity), modes (bind or flow through) and buffer conditions (pH 5 to 8, conductivity from 10 to 80 mS/cm) were evaluated for their capacity to increase purity and reduce contaminating DNA and endotoxins, while preserving the desired characteristics of a
10 VLP. We have found that under certain conditions, the POROS[®] D (a weak anion exchange resin) used in flow through mode could provide the most efficient removal of DNA and endotoxins from concentrated EV71 VLPs.

[00126] A second TFF step was added following chromatography in the EV71 VLP purification process. The role of this TFF step was to concentrate and formulate
15 the product in the desired buffer. Pore size and operating conditions for this second TFF step were determined based on parameters identified for the first TFF. Finally, a drug substance with concentrated apparently pure EV71 particles was obtained following 0.22- μ m filtration. The product was formulated in PBS containing 0.01% Polysorbate 80.

20 *VLP characterization*

[00127] A first lot of EV71 VLPs was produced with the adapted process described above (lot no. 479-23-018) and the product was fully characterized (Table
25 3, lot no. 479-23-018). Purity was determined by densitometry from scans of Coomassie stained gels where only bands that showed positive signals on Western blots (anti VP1-VP2), and that could be further confirmed by mass spectrometry, were considered as part of product. Product quality profile analysis indicated that the preparation contained highly pure EV71 VLPs.

Table 3. Quality attributes of EV71 VLP, lot no. 479-23-018.

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Attribute	EV71 VLPs Initial process
Lot number	479-23-018
Purity	96.4%
Protein conc. (BCA, µg/ml)	1192.4
SEC-HPLC (% in void volume (high molecular weight structures))	100%
Light scattering Particle size (nm)	48.3
Electron microscopy	Round particles Approx. 30 nm Well dispersed
Tryptic mapping/MS	2
Number of impurities detected (p<0.05 and >2 peptides)	Ubiquitin (4 pep) Peroxidase (2 pep)
3 first impurities	
Bioburden (CFU/ml)	<10

* Preliminary estimates calculated from a single run.

Further analysis of the product by electron microscopy confirmed that purified EV71 VLPs were intact (Figure 6A) and tryptic mapping by mass spectrometry confirmed the purity of the product.

Example 4 - Process modifications

Purification of VLPs with intact VP1 by HIC

During initial screening of chromatographic approaches to purify EV71 VLPs, it had been noticed that HIC resins could separate the VLPs containing intact VP1 from particles containing fragmented VP1. Under certain conditions, while the particles containing LMW VP1 fragments were strongly bound to the resins, the intact particles were flowing through the column. This HIC step was therefore inserted as a polishing step following POROS® D chromatography. EV71 particles purified through this process were homogenous in size (light scattering), at close to 100% purity, with no protein contaminants detectable by mass spectrometry. Product quality attributes of

this product (lot no. 479-31-020) are presented in table 3, central column. *Modified extraction procedure.*

[00128] Plant extract may be clarified by acidification at pH around 5.2 or by heat treatment and the coagulate eliminated by centrifugation. The heat treatment was inserted between the mechanical extraction and centrifugation steps. Using a heat treatment of 10 minutes at 60°C (pH 8.0) eliminated more than 90% of soluble proteins without affecting the solubility of EV71 VLPs to a detectable level. The VP1 remained intact when extracted and clarified under these conditions.

[00129] The mechanical extraction at pH 8.0, combined with heat treatment-based clarification, was implemented for EV71 VLP purification process in replacement of the enzymatic extraction. A VLP lot has been produced with this process (lot no. 479-32-020). The characteristic of the product are presented in table 4 (third column). The results obtained showed that mechanical extraction, used in conjunction with heat-based clarification of proteins, represents an efficient primary recovery step that is fully compatible with the previously defined downstream steps. The resulting process is high yielding and generates an EV71 VLP product that is 98% pure. Light scattering profiles of the EV71 VLPs prepared from this process showed high homogeneity. Cryo TEM analysis of this product confirmed that the particles have the size and shape of EV71 VLPs (figure 8).

Table 4. Comparison of EV71 VLP characteristics for lots produced with processes comprising enzymatic extraction with and without HIC or mechanical extraction.

Attribute	Enzymatic extraction (pH 5.1) without HIC	Enzymatic extraction (pH 5.1) with HIC	Mechanical extraction (pH 8.0) with heat treatment
Lot number	479-35-020	479-31-020	479-32-020
Purity	95.5%	100%	98.2%
Protein conc. (BCA, µg/ml)	352.8	297.8	715.3
SEC-HPLC (% in void volume (high molecular weight structures))	100%	98.9%	100%
Electron microscopy	To be determined	Round particles 25-30 nm Well dispersed	Round particles 25-30 nm

Well
dispersed

* Preliminary estimates calculated from a single run for each process.

[00130] All citations are hereby incorporated by reference.

5 [00131] 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.

CLAIMS

1. A method of producing an Enterovirus-like particle (EVLP) in a plant, a portion of a plant, or a plant cell comprising:

a) introducing into the plant, the portion of the plant, or plant cell a first nucleic acid and a second nucleic acid, the first nucleic acid comprising a first regulatory region active in the plant operatively linked to a first nucleotide sequence encoding an Enterovirus polyprotein, wherein the Enterovirus polyprotein consists of Enterovirus 71 polyprotein P1 and the second nucleic acid comprising a second regulatory region active in the plant and operatively linked to a second nucleotide sequence encoding one or more than one Enterovirus 71 3CD protease;

b) incubating the plant, portion of the plant, or plant cell under conditions that permit the expression of the first and second nucleic acids to produce the Enterovirus 71 polyprotein P1 and the one or more than one Enterovirus 71 3CD protease, the Enterovirus 71 polyprotein P1 being processed into structural proteins VP1, VP3 and VP0, or VP1, VP2, VP3 and VP4, thereby producing the EVLP.

2. The method of claim 1, further comprising the step(s) of:

(d) harvesting the plant, the portion of the plant, or plant cell; and/or

(e) purifying the EVLP.

3. The method of claim 1, wherein the ratio of introduced amounts of the first nucleic acid relative to the second nucleic acid is between 20:1 and 0.5:1.

4. The method of claim 1, wherein the Enterovirus polyprotein comprises structural proteins VP1, VP2, VP3, VP4, or a combination thereof.

5. The method of claim 1, wherein the first nucleic acid and the second nucleic acid are on separate nucleic acid constructs.

6. The method of claim 1, wherein the first nucleic acid and the second nucleic acid are on a same nucleic acid construct.

7. The method of claim 1, wherein in the step of introducing (step a), the nucleic acid is transiently expressed in the plant.

8. The method of claim 1, wherein, in the step of introducing (step a), the nucleic acid is stably expressed in the plant.

9. A method of producing an Enterovirus-like particle (EVLP) in a plant, portion of a plant or plant cell comprising:

a) providing the plant, the portion of the plant or plant cell comprising a first nucleic acid comprising a first regulatory region active in the plant, the portion of the plant or plant cell operatively linked to a first nucleotide sequence encoding an Enterovirus polyprotein, wherein

the Enterovirus polyprotein consist of Enterovirus 71 polyprotein P1 and a second nucleic acid comprising a second regulatory region active in the plant, the portion of the plant or plant cell operatively linked to a second nucleotide sequence encoding one or more than one Enterovirus 71 3CD protease;

b) incubating the plant, the portion of the plant or plant cell under conditions that permit the expression of the nucleic acids to produce the Enterovirus 71 polyprotein P1 and the one or more than one Enterovirus 71 3CD protease, the Enterovirus 71 polyprotein P1 being processed into structural proteins VP1, VP3 and VP0, or VP1, VP2, VP3 and VP4, thereby producing the EVLP.

10. The method of claim 9, further comprising the step(s) of:

(d) harvesting the plant, the portion of the plant, or the plant cell; and/or

(e) purifying the EVLP.

11. The method of claim 9, wherein the ratio of introduced amounts of the first nucleic acid relative to the second nucleic acid is between 20:1 and 0.5:1.

12. The method of claim 9, wherein the Enterovirus polyprotein comprises structural proteins VP1, VP2, VP3, VP4, or a combination thereof.

13. The method of claim 9, wherein the first nucleic acid and the second nucleic acid are on separate nucleic acid constructs.

14. The method of claim 9, wherein the first nucleic acid and the second nucleic acid are on the same nucleic acid construct.

15. A plant extract comprising an Enterovirus-like particles (EVLPs) produced by the method of claim 1 or 9.

16. A composition for use in inducing an immune response against Enterovirus in a subject, the composition comprising the plant extract of claim 15 and a pharmaceutically acceptable carrier.

17. The plant extract of claim 15 for use in inducing immunity to an Enterovirus infection in a subject.

18. The plant extract of claim 15, wherein the plant extract is for oral, intradermal, intranasal, intramuscular, intraperitoneal, intravenous, or subcutaneous administration.

19. A polyclonal antibody prepared using the plant extract as described in claim 15.

20. The method of claims 1 or 9, wherein the first nucleic acid sequence comprises the first regulatory region operatively linked with one or more than one comovirus enhancer, the nucleotide sequence encoding the Enterovirus polyprotein, and one or more than one amplification element, and a third nucleic acid encoding a replicase is introduced into the plant

or portion of the plant.

21. The method of claims 1 or 9, wherein the second nucleic acid does not comprise one or more than one amplification element or one or more comovirus enhancer.

22. A plant cell comprising a first nucleic acid and a second nucleic acid, the first nucleic acid comprising a first regulatory region active in the plant cell and operatively linked to a first nucleotide sequence encoding an Enterovirus polyprotein, wherein the Enterovirus polyprotein consists of Enterovirus 71 polyprotein P1, and the second nucleic acid comprising a second regulatory region active in the plant cell and operatively linked to a second nucleotide sequence encoding one or more than one Enterovirus 71 3CD protease.

23. A plant extract comprising an Enterovirus-like particle (EVLP), the EVLP comprising Enterovirus 71 structural proteins VP1, VP2, VP3 and VP4.

24. A plant cell comprising an Enterovirus-like particle (EVLP), the EVLP comprising Enterovirus 71 structural proteins VP1, VP2, VP3 and VP4.