

PLANT EXPRESSION CONSTRUCTS COMPRISING AND USES THEREOF

5 FIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to Gemini-virus constructs capable of symptomless, systemic spread in plant hosts.

Plants may be genetically engineered for a variety of purposes including for the generation of plants with enhanced viral resistance, for the development of abiotic stress
10 tolerant plants, for commercially improved plants and for the expression of heterologous polypeptides for pharmaceutical and industrial purposes.

Present techniques for DNA delivery into plants include direct as well as indirect methods. However, each of these delivery methods is not without limitations. The direct DNA delivery systems such as particle bombardment, silicon carbide
15 whisker technology and electroporation tend to result in integration of multiple copies of transgenes and are considered to be limited, unpredictable and transient. Indirect approaches such as *Agrobacterium* oftentimes result in integration of multiple copies of the foreign DNA into the plant genome along with unwanted sequences from the vector 'backbone'.

20 Integration of foreign DNA into the plant genome to become a heritable trait raises many risks. Traits beneficial to crops may, through horizontal gene transfer or hybridization through breeding with wild relatives, provide wild plants with unwanted competitive advantages. Also, Transformation with *Agrobacterium* is a complex process which requires elimination of false positives arising from the growth of *Agrobacterium*
25 in host tissues, and selection of transformed plants. The use of antibiotic resistance as a marker in the development of transgenic crops has also raised concerns regarding the increase of antibiotic resistance in the environment through horizontal transfer of antibiotic resistance genes to soil micro-organisms. Scientists now have the means to remove marker genes before a crop plant is developed for commercial use, but these
30 means involve further costs and tedious procedures. In addition, several species or varieties of plants are still difficult to transform.

Infection of plants with modified viruses is simpler and quicker than the regeneration of stably transformed plants, since plant viruses are small and easy to manipulate, have the inherent ability to enter the plant cell, and will multiply to produce

a high copy number of the gene of interest. Viral vectors have been engineered for delivery of genetic material and expression of recombinant proteins in plants. Viral expression systems are considered transient expression systems since the viral vectors are not integrated into the genome of the host. However, viral vectors still hold many limitations. Plant viral vectors have the potential to cause disease in their plant hosts, they possess the ability to naturally spread between plants in the field, and in some cases, can be spread through pollen or seed to the next generation. Viral vectors are also limited in their systemic spread in the plant, in host ranges, expression stability, and in the size of insert which can be tolerated. Finally, like transgenic plants, modified viruses are classified as a Genetically Modified Organism (GMO) and thus are subject to regulatory and moral constraints.

Viruses belonging to the family Geminiviridae carry one or two circular single-stranded (ss) DNA genomes and are insect-transmissible. *Begomoviruses*, *Curtoviruses*, *Mastreviruses* and *Topocuviruses* are genera within this family which have been classified according to genome organization, insect transmissibility and host range. Begomoviruses can carry a monopartite or bipartite genome and are whitefly-transmissible. The association of satellite DNAs with monopartite begomoviruses is addressed further on.

Tomato yellow leaf curl virus (TYLCV) is a monopartite begomovirus. It carries six overlapping open reading frames (ORFs) transcribed bi-directionally from an intergenic region (IR; also termed "common region"), serving as the viral origin of replication and as a bi-directional promoter. The IR is about 300-bp long and carries the universal motif TAATATT/AC and a binding site for the replicase-associated protein (REP). Two ORFs are expressed in viral orientation (*V1*, *V2*) and four ORFs in the complementary orientation (*C1* to *C4*). Each gene product appears to participate in more than one function. The viral ssDNA is transmitted to the plant and converted into double-stranded (ds) DNA (replicative form) by the host machinery. The dsDNA replicates and expresses viral mRNAs and proteins. Viral genes are not involved in this stage of replication (dsDNA to dsDNA). However, when rolling circle replication (RCR) is initiated by REP, RCR intermediates may serve as templates for dsDNA replication, dependent on host gene activity. At the next stage of replication, the numerous viral dsDNAs serve as templates for RCR, producing progeny ssDNA. ORF

C1 (also termed *rep*) is essential for the initiation of RCR. The progeny ssDNA molecules are then encapsidated, transported to other tissues or transmitted to other plants. At least three viral gene products are implicated in viral movement within the plant: V2, the coat protein (CP) V1, and C4. The viral genes *V1*, *V2*, *C4* and probably
5 *C2* are implicated in symptom appearance and disease severity. In summary, five of the viral ORFs have no direct role in viral DNA replication or expression (some have auxiliary roles), whereas the sixth ORF (*rep*) is involved only in the rolling-circle phase. Movement and pathogenicity, however, require the activity of viral gene products.

The association of viral satellites with RNA viruses is a well-documented
10 phenomenon. Satellites, either encapsidated or in the form of naked nucleic acid, depend on a helper virus for their replication. A geminivirus-associated, 682-base-long DNA satellite was first reported by Dry et al. in 1997, Proc. Natl. Acad. Sci. USA 94, 7088-7093. This satellite was associated with an Australian type of TYLCV and did not share sequence homology with the helper virus. However, the satellite carried two
15 motifs that also reside in the IR of the helper virus: the universal stem-loop motif and the REP-binding motif. Other gemini-associated satellites have been discovered since then (Bridson et al., 2001, Virology 285, 234-243; Mansoor et al., 1999, Virology 259, 190-199; Zhou et al., 2003, J. Gen. Virol. 84, 237-247. All of these satellite DNAs (generally referred to as DNA β and DNA-1) are about half the size of the viral DNA,
20 and carry, in addition to the aforementioned motifs, an ORF termed $\beta C1$. The role of the putative $\beta C1$ protein has not yet been conclusively determined but it is probably involved in movement, nuclear localization and silencing suppression (Cui et al., 2005, J. Virol. 79, 10764-10775). In many cases of geminiviral infection, the satellite determines symptom severity. Geminiviral satellites are encapsidated and replicated via
25 factors provided by helper viruses. A satellite associated with a particular virus may be supported for replication by other geminiviruses as well.

International Patent Application WO2007/141790 teaches Gemini-virus based constructs wherein the inserted sequence to be expressed is flanked by a non-contiguous nucleic acid sequence encoding a Geminivirus replicase.

SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided a method of expressing a molecule of interest in a plant, the method comprising contacting roots of the plant in a solution comprising at least one
5 Geminivirus based expression construct so as to allow the at least one Geminivirus based expression construct to be absorbed by the roots, the expression construct comprising a polynucleotide encoding the molecule of interest, and further the expression construct being capable of systemic symptomless spread in a plant host, thereby expressing a molecule of interest in a plant.

10 According to an aspect of some embodiments of the present invention there is provided a method of expressing a molecule of interest in a plant, the method comprising grafting a section of a first plant infected with at least one Geminivirus based expression construct onto a section of a second plant, the expression construct comprising a polynucleotide sequence which encodes the molecule of interest, and
15 further the Geminivirus based expression construct being capable of systemic symptomless spread in a plant host, thereby expressing a molecule of interest in a plant.

According to some embodiments of the invention, the Geminivirus based expression construct is devoid of a polynucleotide sequence encoding a C2 and C3 coding sequence.

20 According to some embodiments of the invention, the Geminivirus based expression construct comprises a polynucleotide sequence encoding the molecule of interest, the polynucleotide sequence being flanked by a non-contiguous nucleic acid sequence encoding a Geminivirus replicase.

According to an aspect of some embodiments of the present invention there is
25 provided a Geminivirus based expression construct, capable of systemic symptomless spread in a plant host, the expression construct being devoid of a polynucleotide sequence encoding a C2 and C3 coding sequence.

According to an aspect of some embodiments of the present invention there is provided a method of expressing a molecule of interest in a plant cell comprising
30 introducing into the plant tissue the nucleic acid construct the present invention, the heterologous polypeptide being the molecule of interest, thereby expressing a molecule of interest in a plant cell.

According to some embodiments of the invention, the expression construct comprises:

- (i) a polynucleotide sequence encoding a Geminivirus intergenic region (IR);
- 5 (ii) a polynucleotide sequence encoding a modified Geminivirus coat protein (CP); and
- (iii) a polynucleotide sequence encoding a modified Geminivirus precoat protein (V2).

According to some embodiments of the invention, the expression construct
10 further comprises a polynucleotide sequence encoding a modified replicase protein (C1).

According to some embodiments of the invention, the modified replicase protein is as set forth in SEQ ID NO: 36.

According to some embodiments of the invention, the expression construct
15 further comprises a polynucleotide sequence encoding a modified C4 protein.

According to some embodiments of the invention, the expression construct is capable of replication in a prokaryotic cell.

According to some embodiments of the invention, the expression construct is incapable of plant to plant transmission by an insect vector.

20 According to some embodiments of the invention, the expression construct further comprises a heterologous polynucleotide sequence.

According to some embodiments of the invention, the heterologous polynucleotide is larger than 1 kb.

25 According to some embodiments of the invention, the heterologous polynucleotide is larger than 5 kb.

According to some embodiments of the invention, the heterologous polynucleotide comprises an operon.

According to some embodiments of the invention, the heterologous polynucleotide is adapted for gene silencing.

30 According to some embodiments of the invention, the expression construct comprises a bacterial polynucleotide sequence.

According to some embodiments of the invention, the modified Geminivirus V2 protein is as set forth in SEQ ID NO: 6.

According to some embodiments of the invention, the modified Geminivirus coat protein comprises an amino acid sequence as set forth in SEQ ID NO: 3.

5 According to some embodiments of the invention, the modified Geminivirus coat protein comprises a mutation or deletion in nucleotides encoding an N-terminal 100 amino acids.

According to some embodiments of the invention, the expression construct comprises a polynucleotide sequence as set forth in SEQ ID NO: 10 or 11.

10 According to some embodiments of the invention, the heterologous polynucleotide encodes a polypeptide selected from the group consisting of a reporter molecule, an antiviral molecule, a viral moiety, an antifungal molecule, an antibacterial molecule, an insect resistance molecule, a herbicide resistance molecule, a biotic or abiotic stress tolerance molecule, a pharmaceutical molecule, a growth inducing
15 molecule, and a growth inhibiting molecule.

According to some embodiments of the invention, the Geminivirus is a begomovirus.

According to some embodiments of the invention, the Geminivirus is a Tomato yellow leaf curl virus (TYLCV).

20 According to some embodiments of the invention, the expression construct is adapted for expression in a plant host selected from the group consisting of *Solanaceae*, *Cucurbitaceae*, *Umbelliferae*, *Liliaceae*, *Gramineae* (*Poaceae*), *Rosaceae*, *Musaceae*, *Vitacea*, and *Cruciferae*.

25 According to some embodiments of the invention, the molecule of interest is selected from the group consisting of a reporter molecule, an antiviral molecule, a viral moiety, an antifungal molecule, an antibacterial molecule, an insect resistance molecule, a herbicide resistance molecule, a biotic or abiotic stress tolerance molecule, a pharmaceutical molecule, a growth inducing molecule, a product of genes in a metabolic pathway and a growth inhibiting molecule.
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According to some embodiments of the invention, the genes in the metabolic pathway are encoded by an operon.

According to some embodiments of the invention, the plant is selected from the group consisting of a Solanaceae, a Cucurbitaceae, an Umbelliferae, a Liliaceae, a Gramineae (Poaceae), a Rosaceae *Musaceae*, *Vitacea* and a Cruciferae.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

In the drawings:

FIGs. 1A-C are schematic representations of the constructs IR-V2-CP (Figure 1A), IR-V2-CP-GFP (Figure 1B) and IR-GUS.

FIGs. 2A-F are images illustrating the replication and movement of IR-V2-CP-GFP in tomato plants. Figure 2A: negative control – untreated plants, Figure 2B: Plants treated with IR-GFP only, Figure 2C-F: Plants treated with IR-V2-CP-GFP.

FIGs. 3A-B are images illustrating the IR-V2-CP requirement for *in-trans* replication and movement of another satellite. Figure 3A: IR-GUS was injected into plants along with IR-V2-CP. Figure 3B: a control plant injected with IR-GUS only.

FIGs. 3C-F are photographs of ethidium bromide stained gels. Figure 3C: PCR analysis of DNA from plants treated with IR-V2-CP-GFP with TYLCV-CP primers. Lanes 2, 4, 6, 8: negative controls; DNA extracted from untreated plants. Lanes 3, 5, 7, 9: analysis of DNA from remote leaves of plants injected with IR-V2-CP-GFP. Figure 3D: Detection of TYLCV-CP transcripts by RT-PCR in plants treated with IR-V2-CP-

GFP. Lanes 2, 4, 6: RT-PCR was carried out with RNA template from various plants injected with IR-V2-CP-GFP. Lanes 3, 5, 7: same reactions carried out with the same RNA templates without reverse transcriptase. Figure 3E: PCR analysis of plants with GFP primers. Lanes 2, 5: plants were injected with IR-V2-CP-GFP. Lanes 3, 4: DNA was extracted from untreated plants. Figure 3F: PCR assays to rule out the presence of TYLCV in IR-V2-CP-GFP-injected plants. PCR was conducted with primers for TYLCV CP (product should appear in plasmid-treated and virus-infected plants) and with primers for TYLCV C2 (product should appear only in TYLCV-infected plants). Lane 6: negative control; PCR was conducted with DNA from untreated plants. Size markers are shown in the leftmost lane in Figures 3C-F.

FIG. 4 is a photograph of an ethidium bromide stained gel demonstrating the results of PCR analysis for GUS in rootstocks which had been grafted with scions carrying IL-60-BS + IR-GUS. Lanes 1 to 3: Different grafted tobacco plants. Lanes 4 to 6: Different grafted tomato plants. Lane 7: Negative control (no template). Lane 8: Size markers.

FIG. 5 is a photograph of an ethidium bromide stained gel demonstrating the results of PCR analysis for PRN in rootstocks which had been grafted with scions carrying IL-60-BS + IR-PRN. Lanes 1 to 3: Different grafted tobacco plants. Lanes 4 to 6: Different grafted tomato plants. Lane 7-9: Negative control (no template). Lane 10: Size markers

FIG. 6 is a photograph of an ethidium bromide stained gel demonstrating the results of PCR analysis for GUS from leaves of tomato plants into which IL-60-BS and IR-GUS had been transferred through the roots.

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to Gemini-virus constructs capable of symptomless, systemic spread in plant hosts and methods of expressing molecules of interest using same.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways.

Tomato yellow leaf curl virus (TYLCV) is a monopartite begomovirus. It carries six overlapping open reading frames (ORFs) transcribed bi-directionally from an intergenic region (IR; also termed "common region"), serving as the viral origin of replication and as a bi-directional promoter. The IR is about 300-bp long and carries the universal motif TAATATT/AC [SEQ ID NO: 9] and a binding site for the replicase-associated protein (REP). Two ORFs are expressed in viral orientation (*VI*, *V2*) and four ORFs in the complementary orientation (*C1* to *C4*).

Whilst analyzing the relevance of the genes of the TYLCV, the present inventors found that the virally oriented (sense) genes *VI* and *V2*, together with the viral integration region (IR), were sufficient for the virus's dsDNA replication (dsDNA to dsDNA), its mobilization throughout the plant and the expression of its genes. This was particularly surprising in light of the fact that mutations in *C4* were previously shown to result in lack of systemic spread and reduce levels of virus in tomato plants, and *C4* was therefore considered to be associated with movement (Jupin et al. (1994) Virology 204, 82-90).

However, the present inventors found that a 1486-bp IR-*V2*-CP fragment, devoid of functional *C1*, *C2*, *C3* and *C4*, was able to promote DNA replication and mobilization, but to exclude the rolling-circle phase of replication. Accordingly, this fragment prevented synthesis of single-stranded (ss) DNA. Consequently, replication, mobilization and expression occurred without causing disease symptoms.

Whilst reducing the present invention to practice, the present inventors showed that this construct could be used to express foreign DNA in a plant (Figures 2A-F and 3C-F). The present inventors further showed that this construct could be used to aid in replication and movement of another geminivirus based satellite (Figures 3A-B).

In addition, the present inventors discovered that geminivirus based expression vectors which are capable of systemic symptomless spread in a plant host are capable of being absorbed into a plant via its roots (Figures 5 and 6) or transferred from one plant to another via grafting (Figure 4).

Thus, according to one aspect of the present invention there is provided a Geminivirus based expression construct, capable of systemic symptomless spread in a plant host, the expression construct being devoid of a polynucleotide sequence encoding a *C2* and *C3* coding sequence.

As used herein, the phrase "systemic symptomless spread" refers to the ability of the plant virus-based vector of the present invention to spread, for example, into leaves not serving as the site of infection without inducing the characteristic geminivirus symptoms such as leaf yellowing, leaf curling, stunting of plant growth, or development of flowers or fruit.

Examples of susceptible host species include *Cynanchum acutum*, *Datura stramonium*, *Hyoscyamus desertorum*, *Lens culinaris*, *Lycopersicon esculentum*, *Lycopersicon pimpinellifolium*, *Malva nicaensis*, *Malva parviflora*, *Nicotiana benthamiana*, *Nicotiana glutinosa*, *Nicotiana tabacum*, *Phaseolus vulgaris* and *Sonchus oleraceus*, as well as insusceptible host species such as *Abelmoschus esculentus*, *Althaea rosea*, *Amaranthus retroflexus*, *Arachis hypogaea*, *Atriplex*, *Beta vulgaris*, *Calotropis aegyptia*, *Capparis aegyptia*, *Chenopodium amaranticolor*, *Cucumis sativus*, *Gomphrena globosa*, *Gossypium hirsutum*, *Hibiscus rosa-sinensis*, *Lavatera cretica*, *Lonicera*, *Lycium*, *Medicago sativa*, *Momordica balsamina*, *Nerium oleander*, *Nicotiana rustica*, *Ochradenus baccatus*, *Physalis floridana*, *Pisum sativum*, *Plumbago capensis*, *Polygonum equisetiforme*, *Portulaca oleracea*, *Prosopis farcta*, *Ricinus communis*, *Solanum incanum*, *Solanum villosum*, *Tamarix*, *Tribulus*, *Vicia faba*, *Withania somnifera*, *Xanthium strumarium* and *Zinnia elegans*.

Additional susceptible and insusceptible hosts are listed in <http://pheneDOTcpmcDOTcolumbiaDOTedu/ICTVdB/29030000.htm>

Preferred Geminiviruses which can be used with the present invention include the tomato yellow leaf curl virus (TYLCV) as well as other Begomoviruses (see, pheneDOTcpmcDOTcolumbia.edu/ICTVdB/29030000.htm). It will be appreciated that although some of the terminology utilized herein refers to the genes encoded by TYLCV, one of ordinary skill in the art would be more than capable of identifying and utilizing the genetic orthologues of other geminivirus species and strains.

As mentioned, the nucleic acid constructs of the present invention are devoid of functional C1, C2, C3 and C4 coding sequences. According to one embodiment, the construct is devoid of C2 and C3 coding sequences.

According to one embodiment, the nucleic acid construct of the present invention comprises modifications in the C1 and C4 genes. These modifications may be conservative or non-conservative.

According to one embodiment of this aspect of the present invention, the modifications in the C1 and C4 genes are such that the proteins encoded by same are non-functioning. Thus, the modification may be a truncation, a point mutation, a frame shift mutation, a partial deletion or a full deletion.

5 Accordingly, the present invention contemplates a construct which comprises a truncated C1 sequence (SEQ ID NO: 36) and a truncated C4 sequence (SEQ ID NO: 38) and is devoid of C2 and C3 completely.

 According to another embodiment of this aspect of the present invention, the nucleic acid construct of the present invention comprises modifications in the V1 gene
10 (encoding the coat protein (CP)) and/or the V2 (encoding the precoat protein) gene.

 For example, the part of the CP that is involved in viral movement and systemic spread in plants has been mapped to the C-terminal part of the CP (Noris. et.al.(1998) J. Virol. 72, 10050-10057). Accordingly, the present invention contemplates altering the N-terminal part of the CP in some cases. In an exemplary embodiment of the invention,
15 60 nucleotides (corresponding to positions 552-612) of the TYLCV may be deleted, causing the removal of 20 amino acids (positions 27 to 46) from the native viral CP. The resultant CP would still carry a bipartite nuclear-localization signal (NLS; amino acids 1-20), although a third part (KRR at position 41-43) of what may have been a tripartite NLS would be removed.

20 According to one embodiment, the sequence of the CP is as set forth in SEQ ID NO: 34.

 Alternatively, or additionally, point mutations may be introduced by single-base deletion into the V2 gene. For example, a single-base deletion may be introduced at position 640 of the native TYLCV-DNA, causing a frameshift which may be corrected
25 for – for example by adding a G to position 744 of the native viral DNA. Due to the resultant frameshift, a stretch of amino acids residing between positions 56 and 91 of the native CP would become different, with no apparent similarity to the corresponding stretch (positions 926 to 1031) of IR-V2-CP. Beyond that point, however, the CP sequences of TYLCV and IR-V2-CP would be almost identical. Due to the changes in
30 amino acids 56 to 91, the conserved sequence GCEGPCKVQS (SEQ ID NO: 33), carried by all geminoviruses tested to date (Kirthi et al. (2003) Arch. Virol. 148, 2369-2380), would be missing from IR-V2-CP. In many viruses (but not TYLCV), this

sequence is part of a zinc-finger motif required for attachment to single-stranded (ss) DNA (apparently for encapsidation), a property that is redundant for a vector.

Deletion at the N terminus of the CP typically results in a deletion at the C terminus of the overlapping ORF V2 ("pre-coat"). Thus, for example, the present invention contemplates constructs comprising a V2 gene as set forth in SEQ ID NO: 37,
5 encoding the polypeptide sequence as set forth in SEQ ID NO: 6.

It will be appreciated that in order for the expression construct of the present invention to be expressed, the expression construct typically comprises an IR region. This region serves, amongst other things as a bi-directional promoter.

10 The IR derived sequence can include for example, a nucleotide region defined by coordinates 1-314 or 62-314 of TYLCV (GenBank Accession number X15656). For example, the IR derived sequence may be set forth in SEQ ID NO: 35.

The present invention also contemplates modifications in the IR region. Typically, these modifications do not affect the promoter region or the ability of the IR
15 to direct expression in a plant over an extended period of time in the plant (e.g. longer than one month, 3 months, 6 months 1 year or even over the entire lifetime of the plant).

One example of an IR region which can be targeted for modification is the replication-associated protein binding domain (Akbar Behjatnia et al. Nucleic Acids Research, 1998, Vol. 26, No. 4, 925-931).

20 The aforescribed alterations are all consistent with a disarmed dsDNA construct which is capable of replicating (dsDNA to dsDNA) by attracting the host machinery to its origin of replication and retaining its mobility, but with no ability to produce progeny viral ssDNA.

An exemplary construct of the present invention is set forth in SEQ ID NO: 11,
25 comprising an IR region, a V2 gene and a CP gene only (see Figure 1A). It will be appreciated that the construct may also comprise additional sequences which do not encode functional polypeptides. Accordingly, another exemplary construct of the present invention as set forth in SEQ ID NO: 10.

As is further detailed in the Examples section which follows, the above described
30 construct can be carried out using molecular techniques such as PCR which are well known to the ordinary skilled artisan.

Preferably, the nucleic acid construct of the present invention carries one or more polynucleotide insertions so as to provide additional features to the nucleic acid construct of the present invention. Such an insertion can be several nucleotides, to several thousand nucleotides long. The insert can include a complete eukaryotic or prokaryotic expression vector a polylinker insert or a molecule having a biological activity. In any case, it should be noted that the nucleic acid construct of the present invention can carry inserts which increase the final geminivirus by 20-100 % and as much as 200 % beyond that of a wild type genome and yet, the nucleic acid construct of the present invention is capable of efficiently spreading throughout the host plant. The insert can encode alternative or additional functions including for example, bacterial replication, antibiotic resistance, affinity purification tags and the like.

One preferred use for the nucleic acid constructs of the present invention is plant expression of a polynucleotide or a polypeptide of interest. The polynucleotide which encodes the molecule of interest may be inserted into the expression construct described herein, wherein the IR region of the construct serves as a promoter therefore – see for example Figure 1B. Alternatively, the polynucleotide encoding the molecule of interest may be inserted on an additional construct which only comprises an IR sequence (i.e. a transactivatable expression vector). This set up may be particularly useful for expression of a dsRNA capable of down-regulating expression of a gene. Since the IR region acts as a bidirectional promoter, an IR region may be flanked on both sides by a polynucleotide sequence encoding RNA molecules, which following expression are capable of hybridizing to form a dsRNA. This set up is described in more detail herein below and in WO2007/141790, incorporated herein by reference.

Co-administration of the transactivatable expression vector together with the construct of the present invention would result in expression of the molecule of interest (see for example Figures 3A-B). Alternatively, the transactivation can be effected by stepwise introduction of the construct of the present invention and the transactivatable expression vector.

One of ordinary skill in the art is familiar with nucleic acids or proteins whose expression, controlled by the expression vector of the present invention, is advantageous. Furthermore, the skilled artisan is familiar with genes whose repression

or deletion, by means of expression of, for example, a suitable double-stranded RNA, or an antisense RNA, would lead to a desired effect.

Nucleic acid sequences whose expression under the control of the expression vector of the present invention has advantageous effects are exemplified below.

5 The expressed polynucleotide sequence can encode a molecule which would protect the plant from abiotic stress factors such as drought, heat or chill. Examples include antifreeze polypeptides from *Myoxocephalus Scorpius* (WO 00/00512), *Myoxocephalus octodecemspinosus*, the *Arabidopsis thaliana* transcription activator CBF1, glutamate dehydrogenases (WO 97/12983, WO 98/11240), calcium-dependent
10 protein kinase genes (WO 98/26045), calcineurins (WO 99/05902), casein kinase from yeast (WO 02/052012), farnesyltransferases (WO 99/06580; Pei Z M et al. (1998) Science 282:287-290), ferritin (Deak M et al. (1999) Nature Biotechnology 17:192-196), oxalate oxidase (WO 99/04013; Dunwell J M (1998) Biotechn Genet Eng Rev 15:1-32), DREB1A factor ("dehydration response element B 1A"; Kasuga M et al.
15 (1999) Nature Biotech 17:276-286), genes of mannitol or trehalose synthesis such as trehalose-phosphate synthase or trehalose-phosphate phosphatase (WO 97/42326) or by inhibiting genes such as trehalase (WO 97/50561).

 The expressed polynucleotide sequence could be a metabolic enzyme for use in the food-and-feed sector. Examples include, phytases (GenBank Acc. No.: A19451) and
20 cellulases.

 The expressed polynucleotide sequence can confer resistance to viruses, fungi, insects, nematodes and diseases, by directly attacking the pathogen, turning on the host defenses or by leading to an accumulation of certain metabolites or proteins. Examples of include glucosinolates (defense against herbivores), chitinases or glucanases and
25 other enzymes which destroy the cell wall of parasites, ribosome-inactivating proteins (RIPS) and other proteins of the plant resistance and stress reaction as are induced when plants are wounded or attacked by microbes, or chemically, by, for example, salicylic acid, jasmonic acid or ethylene, or lysozymes from nonplant sources such as, for example, T4-lysozyme or lysozyme from a variety of mammals, insecticidal proteins
30 such as *Bacillus thuringiensis* endotoxin, α -amylase inhibitor or protease inhibitors (cowpea trypsin inhibitor), lectins such as wheatgerm agglutinin, siRNA, antisense RNA, RNases or ribozymes. Further examples are nucleic acids which encode the

Trichoderma harzianum chit42 endochitinase (GenBank Acc. No.: S78423) or the N-hydroxylating, multi-functional cytochrome P-450 (CYP79) protein from Sorghum bicolor (GenBank Acc. No.: U32624), or functional equivalents thereof.

Accumulation of glucosinolates as protection from pests (Rask L et al. (2000) Plant Mol Biol 42:93-113; Menard R et al. (1999) Phytochemistry 52:29-35), the expression of Bacillus thuringiensis endotoxins (Vaech et al. (1987) Nature 328:33-37) or the protection against attack by fungi, by expression of chitinases, for example from beans (Broglie et al. (1991) Science 254:1194-1197), is advantageous. Resistance to pests such as, for example, the rice pest *Nilaparvata lugens* in rice plants can be achieved by expressing the snowdrop (*Galanthus nivalis*) lectin agglutinin (Rao et al. (1998) Plant J 15(4):469-77).

The expression of synthetic cryIA(b) and cryIA(c) genes, which encode lepidoptera-specific Bacillus thuringiensis delta-endotoxins can bring about a resistance to insect pests in various plants (Goyal R K et al. (2000) Crop Protection 19(5):307-312).

Additional genes which are suitable for pathogen defense comprise "polygalacturonase-inhibiting protein" (PGIP), thaumatococcal, invertase and antimicrobial peptides such as lactoferrin (Lee T J et al. (2002) J Amer Soc Hortic Sci 127(2):158-164).

The expressed polynucleotide sequence can bring about an accumulation of chemicals such as of tocopherols, tocotrienols or carotenoids. One example of such a polynucleotide is phytoene desaturase. Preferred are nucleic acids which encode the Narcissus pseudonarcissus phytoene desaturase (GenBank Acc. No.: X78815) or functional equivalents thereof.

The expressed polynucleotide sequence can be used for production of nutraceuticals such as, for example, polyunsaturated fatty acids (arachidonic acid, eicosapentaenoic acid or docosahexaenoic acid) examples include, fatty acid elongases and/or desaturases, or for production of proteins with improved nutritional value such as, for example, with a high content of essential amino acids (for example the high-methionine 2S albumin gene of the brazil nut). Preferred are polynucleotide sequence which encode the Bertholletia excelsa high-methionine 2S albumin (GenBank Acc. No.: AB044391), the Physcomitrella patens delta6-acyl-lipid desaturase (GenBank Acc. No.:

AJ222980; Girke et al. (1998) *Plant J* 15:39-48), the *Mortierella alpina* delta6-desaturase (Sakuradani et al. 1999 *Gene* 238:445-453), the *Caenorhabditis elegans* delta5-desaturase (Michaelson et al. 1998, *FEBS Letters* 439:215-218), the *Caenorhabditis elegans* A5-fatty acid desaturase (des-5) (GenBank Acc. No.: AF078796), the *Mortierella alpina* delta5-desaturase (Michaelson et al. *JBC* 273:19055-19059), the *Caenorhabditis elegans* delta6-elongase (Beaudoin et al. 2000, *PNAS* 97:6421-6426), the *Physcomitrella patens* delta6-elongase (Zank et al. 2000, *Biochemical Society Transactions* 28:654-657), or functional equivalents of these.

The expressed polynucleotide sequence can be used for production of high-quality proteins and enzymes for industrial purposes (for example enzymes, such as lipases) or as pharmaceuticals (such as, for example, antibodies, blood clotting factors, interferons, lymphokins, colony stimulation factor, plasminogen activators, hormones or vaccines, as described by Hood E E, Jilka J M (1999) *Curr Opin Biotechnol* 10(4):382-6; Ma J K, Vine N D (1999) *Curr Top Microbiol Immunol* 236:275-92). For example, it has been possible to produce recombinant avidin from chicken albumen and bacterial P-glucuronidase (GUS) on a large scale in transgenic maize plants (Hood et al. (1999) *Adv Exp Med Biol* 464:127-47. Review).

The expressed polynucleotide sequence can be used for obtaining an increased storability in cells which normally comprise fewer storage proteins or storage lipids, with the purpose of increasing the yield of these substances. Examples include, acetyl-CoA carboxylase. Preferred polynucleotide sequence are those which encode the *Medicago sativa* acetyl-CoA carboxylase (accase) (GenBank Acc. No.: L25042), or functional equivalents thereof.

Additional examples of expressible polynucleotides include Hepatitis B surface antigen [Kumar GBS et al., *PLANTA* 222 (3): 484-493, 2005], herbicide resistance [Duke, SO, *Pest Management Science* 61:211-218, 2005], interferon [Edelbaum, O. et al., *J. Interferon Res.* 12: 449-453, 1992], T7-RNA polymerase [Zeitoune et al., *Plant Science* 141:59-65., 1997].

Further examples of polynucleotide sequence which can be expressed by the expression vector of the present invention are mentioned for example in Dunwell J M, *Transgenic approaches to crop improvement*, *J Exp Bot.* 2000; 51 pages 487-96.

The expression vector of the present invention can also be employed for the reduction (suppression) of transcription and/or translation of target genes. Thus, the expression vector of the present invention can express nucleic acids which bring about PTGS (post transcriptional gene silencing) or TGS (transcriptional silencing) effects and thus a reduction of the expression of endogenous genes. Such reduction can be achieved for example by expression of an antisense RNA (EP-A1 0 458 367; EP-A1 0 140 308; van der Krol A R et al. (1988) *BioTechniques* 6(10):658-676; de Lange P et al. (1995) *Curr Top Microbiol Immunol* 197:57-75, inter alia) or of a double-stranded RNA, each of which has homology with the endogenous target gene to be suppressed. Also, the expression of a suitable sense RNA can bring about a reduction of the expression of endogenous genes, by means of what is known as co-suppression (EP-A1 0 465 572). Especially preferred is the expression of a double-stranded small interfering RNA (siRNA) for reducing the gene expression of a target gene via RNA interference (RNAi). WO 99/32619 and WO 99/53050 describe methods for inhibiting individual target genes using an RNA with double-stranded structure, where the target gene and the region of the RNA duplex have at least partial identity (see also: Montgomery M K et al. (1998) *Proc Natl Acad Sci USA* 95:15502-15507; Sharp P A (1999) *Genes & Development* 13(2):139-141; Fire A et al. (1998) *Nature* 391:806-11).

The following exemplifies applications where reduction of gene expression can be employed using the expression vector of the present invention.

Delayed fruit maturation or a modified maturation phenotype (prolonged maturation, later senescence) can be achieved for example by reducing the gene expression of genes selected from the group consisting of polygalacturonases, pectin esterases, beta-(1,4)glucanases (cellulases), beta.-galactanases (.beta.-galactosidases), or genes of ethylene biosynthesis, such as 1-aminocyclopropane-1-carboxylate synthase, adenosylmethionine hydrolase (SAMase), aminocyclopropane-1-carboxylate deaminase, aminocyclopropane-1-carboxylate oxidase, genes of carotenoid biosynthesis such as, for example, genes of pre-phytoene biosynthesis or phytoene biosynthesis, for example phytoene desaturases, and O-methyltransferases, acyl carrier protein (ACP), elongation factor, auxin-induced gene, cysteine(thiol) proteinases, starch phosphorylases, pyruvate decarboxylases, chalcone reductases, protein kinases, auxin-related gene, sucrose transporters, meristem pattern gene. Further advantageous genes

are described for example in WO 91/16440, WO 91/05865, WO 91/16426, WO 92/17596, WO 93/07275 or WO 92/04456. Especially preferred is the reduction of the expression of polygalacturonase for the prevention of cell degradation and mushiness of plants and fruits, for example tomatoes. Nucleic acid sequences such as that of the tomato polygalacturonase gene (GenBank Acc. No.: x14074) or its homologs are
5 preferably used for this purpose.

Improved protection against abiotic stress factors (heat, chill, drought, elevated moisture, pollutants, UV radiation). It is preferred to reduce the expression of genes which are implicated in stress reactions.

10 The reduction of the gene expression of genes encoding storage proteins (hereinbelow SPs) has numerous advantages, such as, for example, the reduction of the allergenic potential or modification regarding composition or quantity of other metabolites, such as, for example, oil or starch content.

Resistance to plant pathogens such as arachnids, fungi, insects, nematodes, protozoans, viruses, bacteria and diseases can be achieved by reducing the gene
15 expression of genes which are essential for the growth, survival, certain developmental stages (for example pupation) or the multiplication of a specific pathogen. Such a reduction can bring about a complete inhibition of the abovementioned steps, or else a delay of same. They can take the form of plant genes which for example make possible the penetration of the pathogen, but may also be homologous pathogen genes. The
20 transgenically expressed nucleic acid sequence (for example the double-stranded RNA) is preferably directed against genes of the pathogen. The antipathogenic agent which acts may be, in this context, the transgenically expressed nucleic acid sequence itself (for example the double-stranded RNA), but also the transgenic expression cassettes or
25 transgenic organisms. The plants themselves, in the form of a transgenic organism, may contain the agents and pass them on to the pathogens, for example in the form of a stomach poison. Various essential genes of a variety of pathogens are known to the skilled artisan (for example for nematode resistance WO 93/10251, WO 94/17194).

Virus resistance can be achieved for example by reducing the expression of a
30 viral coat protein, a viral replicase, a viral protease and the like. A large number of plant viruses and suitable target genes are known to the skilled artisan.

Reduction of undesired, allergenic or toxic plant constituents such as, for example, glucosinolates or patatin. Suitable target genes are described (in WO 97/16559, inter alia). The target genes which are preferred for reduction of allergenic proteins are described for example by Tada Y et al. (1996) FEBS Lett 391(3):341-345 or Nakamura R (1996) Biosci Biotechnol Biochem 60(8):1215-1221.

Delayed signs of senescence. Suitable target genes are, inter alia, cinnamoyl-CoA:NADPH reductases or cinnamoyl-alcohol dehydrogenases. Further target genes are described (in WO 95/07993, inter alia).

Reduction of the susceptibility to bruising of, for example, potatoes by reducing for example polyphenol oxidase (WO 94/03607) and the like.

Increase of the methionine content by reducing threonine biosynthesis, for example by reducing the expression of threonine synthase (Zeh M et al. (2001) Plant Physiol 127(3):792-802).

It will be appreciated that the nucleic acid construct of the present invention can also express homologues of the above described molecules that exhibit the desired activity (i.e., the biological activity). Such homologues can be, for example, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100 %, identical to any of the expressed sequences described above as determined using the BestFit software of the Wisconsin sequence analysis package, utilizing the Smith and Waterman algorithm, where gap weight equals 50, length weight equals 3, average match equals 10, and average mismatch equals -9.

Thus, the present invention provides a geminivirus based nucleic acid construct which spreads systemically throughout the host plant and yet does not induce symptoms therein.

In summary, the nucleic acid construct of the present invention can be utilized for any purpose. Examples of uses include the following:

- (i) plant expression of proteins (specific examples provided hereinabove) for various purposes including plant improvement, biopharming etc;
- (ii) plant expression of nucleic acid molecules (e.g. siRNA, specific examples provided hereinabove);

(iii) produce indicator plants which detect viral infection - a plant carrying a construct including a reporter molecule (e.g. fluorophore) attached to the IR region would express the reporter in when infected by a geminivirus; and

(iv) produce infection-resistant plants - a plant carrying a construct including an anti-viral or anti-plant molecule attached, for example, to the IR region would express such a molecule when infected by a geminivirus; such "immunity" or suicide scheme would only be active when the plant is infected; since the nucleic acid constructs of the present invention are preferably transient and not stably integrated into a genome of the host plant, such a trait would not be inherited by the progeny of the plant nor would it persist in commercial products of the plant.

The nucleic acid construct of the present invention can be utilized to stably or preferably transiently transform plant cells. In stable transformation, the nucleic acid molecule of the present invention is integrated into the plant genome, and as such it represents a stable and inherited trait. In transient transformation, the nucleic acid molecule is expressed by the cell transformed but not integrated into the genome, and as such represents a transient trait.

There are various methods of introducing foreign genes into both monocotyledonous and dicotyledonous plants (Potrykus, I. (1991). *Annu Rev Plant Physiol Plant Mol Biol* 42, 205-225; Shimamoto, K. et al. (1989). Fertile transgenic rice plants regenerated from transformed protoplasts. *Nature* (1989) 338, 274-276).

The principal methods of the stable integration of exogenous DNA into plant genomic DNA includes two main approaches:

(i) *Agrobacterium-mediated gene transfer*. See: Klee, H. J. et al. (1987). *Annu Rev Plant Physiol* 38, 467-486; Klee, H. J. and Rogers, S. G. (1989). *Cell Culture and Somatic Cell Genetics of Plants*, Vol. 6, *Molecular Biology of Plant Nuclear Genes*, pp. 2-25, J. Schell and L. K. Vasil, eds., Academic Publishers, San Diego, Cal.; and Gatenby, A. A. (1989). *Regulation and Expression of Plant Genes in Microorganisms*, pp. 93-112, *Plant Biotechnology*, S. Kung and C. J. Arntzen, eds., Butterworth Publishers, Boston, Mass.

(ii) *Direct DNA uptake*. See, e.g.: Paszkowski, J. et al. (1989). *Cell Culture and Somatic Cell Genetics of Plants*, Vol. 6, *Molecular Biology of Plant Nuclear Genes*, pp. 52-68, J. Schell and L. K. Vasil, eds., Academic Publishers, San Diego, Cal.; and

Toriyama, K. et al. (1988). *Bio/Technol* 6, 1072-1074 (methods for direct uptake of DNA into protoplasts). See also: Zhang et al. (1988). *Plant Cell Rep* 7, 379-384; and Fromm, M. E. et al. (1986). Stable transformation of maize after gene transfer by electroporation. *Nature* 319, 791-793 (DNA uptake induced by brief electric shock of plant cells). See also: Klein et al. (1988). *Bio/Technology* 6, 559-563; McCabe, D. E. et al. (1988). Stable transformation of soybean (*Glycine max*) by particle acceleration. *Bio/Technology* 6, 923-926; and Sanford, J. C. (1990). Biolistic plant transformation. *Physiol Plant* 79, 206-209 (DNA injection into plant cells or tissues by particle bombardment). See also: Neuhaus, J. M. et al. (1987). *Theor Appl Genet* 75, 30-36; and Neuhaus, J. M. and Spangenberg, G. C. (1990). *Physiol Plant* 79, 213-217 (use of micropipette systems). See U.S. Pat. No. 5,464,765 (glass fibers or silicon carbide whisker transformation of cell cultures, embryos or callus tissue). See also: DeWet, J. M. J. et al. (1985). "Exogenous gene transfer in maize (*Zea mays*) using DNA-treated pollen," *Experimental Manipulation of Ovule Tissue*, G. P. Chapman et al., eds., Longman, New York-London, pp. 197-209; and Ohta, Y. (1986). High-Efficiency Genetic Transformation of Maize by a Mixture of Pollen and Exogenous DNA. *Proc Natl Acad Sci USA* 83, 715-719 (direct incubation of DNA with germinating pollen).

The *Agrobacterium*-mediated system includes the use of plasmid vectors that contain defined DNA segments which integrate into the plant genomic DNA. Methods of inoculation of the plant tissue vary depending upon the plant species and the *Agrobacterium* delivery system. A widely used approach is the leaf-disc procedure, which can be performed with any tissue explant that provides a good source for initiation of whole-plant differentiation (Horsch, R. B. et al. (1988). "Leaf disc transformation." *Plant Molecular Biology Manual A5*, 1-9, Kluwer Academic Publishers, Dordrecht). A supplementary approach employs the *Agrobacterium* delivery system in combination with vacuum infiltration. The *Agrobacterium* system is especially useful for in the creation of transgenic dicotyledenous plants.

There are various methods of direct DNA transfer into plant cells. In electroporation, the protoplasts are briefly exposed to a strong electric field, opening up mini-pores to allow DNA to enter. In microinjection, the DNA is mechanically injected directly into the cells using micropipettes. In microparticle bombardment, the DNA is adsorbed on microprojectiles such as magnesium sulfate crystals or tungsten particles,

and the microprojectiles are physically accelerated into cells or plant tissues. Additional direct DNA transfer techniques include glass or silicone carbide whiskers (see, for example, Dunwell, *Methods Mol Biol.* 1999;111:375-82).

Following stable transformation, plant propagation then occurs. The most
5 common method of plant propagation is by seed. The disadvantage of regeneration by seed propagation, however, is the lack of uniformity in the crop due to heterozygosity, since seeds are produced by plants according to the genetic variances governed by Mendelian rules. In other words, each seed is genetically different and each will grow with its own specific traits. Therefore, it is preferred that the regeneration be effected
10 such that the regenerated plant has identical traits and characteristics to those of the parent transgenic plant. The preferred method of regenerating a transformed plant is by micropropagation, which provides a rapid, consistent reproduction of the transformed plants.

Micropropagation is a process of growing second-generation plants from a
15 single tissue sample excised from a selected parent plant or cultivar. This process permits the mass reproduction of plants having the preferred tissue and expressing a fusion protein. The newly generated plants are genetically identical to, and have all of the characteristics of, the original plant. Micropropagation allows for mass production of quality plant material in a short period of time and offers a rapid multiplication of
20 selected cultivars with preservation of the characteristics of the original transgenic or transformed plant. The advantages of this method of plant cloning include the speed of plant multiplication and the quality and uniformity of the plants produced.

Micropropagation is a multi-stage procedure that requires alteration of culture medium or growth conditions between stages. The micropropagation process involves
25 four basic stages: stage one, initial tissue culturing; stage two, tissue culture multiplication; stage three, differentiation and plant formation; and stage four, greenhouse culturing and hardening. During stage one, the tissue culture is established and certified contaminant-free. During stage two, the initial tissue culture is multiplied until a sufficient number of tissue samples are produced to meet production goals.
30 During stage three, the newly grown tissue samples are divided and grown into individual plantlets. At stage four, the transformed plantlets are transferred to a

greenhouse for hardening where the plants' tolerance to light is gradually increased so that they can continue to grow in the natural environment.

Transient transformation of, for example, leaf cells, meristematic cells, or the whole plant is also envisaged by the present invention.

5 Transient transformation can be effected by any of the direct DNA transfer methods described above or by mechanical or vector mediated viral infection using the plant viruses derived plasmid of the present invention.

As mentioned, the constructs of the present invention may also be transformed into a plant via grafting a section of one plant which comprises the construct onto a
10 section of another plant. Alternatively, the constructs of the present invention may be transformed into a plant by soaking its roots into a solution comprising the construct.

Thus, according to another aspect of the present invention there is provided a method of expressing a molecule of interest in a plant, the method comprising contacting roots of the plant in a solution comprising at least one Geminivirus based
15 expression construct so as to allow the at least one Geminivirus based expression construct to be absorbed by the roots, the expression construct comprising a polynucleotide encoding the molecule of interest, and further the expression construct being capable of systemic symptomless spread in a plant host, thereby expressing a molecule of interest in a plant.

20 According to yet another aspect of the present invention there is provided a method of expressing a molecule of interest in a plant, the method comprising grafting a section of a first plant infected with at least one Geminivirus based expression construct onto a section of a second plant, the expression construct comprising a polynucleotide sequence which encodes the molecule of interest, and further the Geminivirus based
25 expression construct being capable of systemic symptomless spread in a plant host, thereby expressing a molecule of interest in a plant.

As used herein, the term "grafting" refers to the joining together of the parts of plants so that they bind together and the sap can flow, thus forming a new plant that can grow and develop. A graft therefore consists of two parts: (i) the lower part is the
30 rootstock as referred to herein and essentially consists of the root system and a portion of the stem, and (ii) the upper part, the scion or graft, which gives rise to the aerial parts

of the plant. It will be appreciated that a single graft can comprise two parts of the same plant of alternatively a single graft can comprise parts of two different plants.

Preferably, the first plant and the second plant are compatible for grafting.

As used herein, the term "compatible" refers to the ability of the grafted
5 rootstock and plant tissue to grow together and survive. It is well known that compatible rootstock and plant tissue grafts do not have to be from the same plant species. For example, tomato scions can be grafted onto eggplant rootstock. Transgenic rootstock can be prepared using standard methods.

Grafting can be accomplished using standard materials and methods known in
10 the art. (See, for example, Black et al. (2003); Fernandez-Garcia et al. (2004); Edelstein et al. (2004); see Worldwide Website: www.dotparamount-seeds.com/Paramountonline/graftingdothtm; see Worldwide Website: www.dotagnetdotorg/library/article/eb480dothtml).

Exemplary plants which can be grafted according to this aspect of the present
15 invention include dicotyledonous plants, such as, for example, peas, alfalfa, tomato, tomatillo, melon, chickpea, chicory, clover, kale, lentil, soybean, tobacco, potato, sweet potato, radish, cabbage, rape, apple trees, grape, cotton, sunflower, citrus (including orange, mandarin, kumquat, lemon, lime, grapefruit, tangerine, tangelo, citron, and pomelo), pepper, bean, and lettuce. Plants within the scope of the present invention also
20 include conifers.

Examples of tomato rootstock that can be used to prepare pathogen resistant transgenic rootstock includes, but is not limited to, "PG3" and "Beaufort." Examples of tomato cultivars that can be used to provide scions for the present invention include, but are not limited to, "Monroe," "Belle," "Summer Set," "Match," "Trust," "Better Boy,"
25 "Celebrity," "Grace," "Heinz 1439," "Roma," "Rugers," "Ultra Girl," "2710," "BHN 665," "STM 0227," "STM 5206," "Boy oh Boy," "Jubilation," "Sunchief," and "Fabulous."

Contemplated geminivirus based expression constructs that may be grafted from one plant to another or soaked up by plant roots are those described herein above and
30 further those described in International Patent Application WO2007/141790.

The terms "comprises", "comprising", "includes", "including", "having" and their conjugates mean "including but not limited to".

The term "consisting of means "including and limited to".

The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable sub-combination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions illustrate some embodiments of the invention in a non limiting fashion.

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed.

(1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

METHODS AND MATERIALS

Cloning of TYLCV - A full 2.8-genome-length clone of the Israeli strain of TYLCV (SEQ ID NO: 4 GenBank accession # X15656) was produced as described in Navot, N., et al [Virology 185, 151-161 (1991)].

Construction of IL-60 – The IL-60 vector (SEQ ID NO: 2) of the present invention was constructed by making the following changes to the native TYLCV viral vector (SEQ ID NO: 4):

(i) a deletion of a stretch of 60 nucleotides (nos. 552 to 612 of SEQ ID NO: 1) encoding 20 amino acids near the N-terminus of the coat protein, TYLCV-CP (nos. 27 to 46 of SEQ ID NO: 5). Deletion was carried out by inverse PCR in accordance with Livneh, O. et al. [Euphytica 62:97-102 (1992)] using primers directed outward from the ends of the deleted segment [Inverse forward primer (unphosphorylated): OH-acaggcccatagaccgaaagccca; SEQ ID NO: 14, Inverse reverse (phosphorylated): P-tgggctgtcgaagttcagcct; SEQ ID NO: 15]. The self-ligated PCR product was cleaved with *SacI* to produce a single (linear) product, confirming that a circular form has been made (non-ligated, linear PCR products would have produced two fragments upon cleaving).

(ii) a PCR derived deletion of T in position 640 (of TYLCV) and addition of G at following position 744 thereby generating a frame shift in the TYLCV sequence (SEQ ID NO: 4) encoding positions 56-91 of native TYLCV-CP protein (SEQ ID NO: 5). Frame shift was achieved in 2 steps, first aimed at deleting the T and second at adding the G. The first step aimed at deleting the T at position 640 included two PCR steps, an initial and nested PCR. The initial PCR product was cut with *TaqI*, and a mutated (missing the T) nested PCR product, which was situated between 2 *TaqI* restriction sites, was ligated instead of the cut out piece. Initial PCR amplified a 439bp product flanked with *TaqI* restriction sites, and possessing two middle *TaqI* restriction sites. [forward primer:

ggctgaactcgcagcccatagcagcgcgtgctgctg (SEQ ID NO: 20), *BceI* recognition site is emphasized in bold, *TaqI* restriction site is underlined; reverse primer: gcggtactgggctcattatatcgaacatatt (SEQ ID NO: 21), *BmrI* recognition site is emphasized in bold, *TaqI* restriction sites are underlined]. Nested PCR amplified a product flanked by the same *TaqI* restriction site at the forward end (using the same forward primer- SEQ ID NO: 20) and another middle *TaqI* restriction site at the reverse end. The reverse primer also possessed the missing respective T [nested reverse primer: ggcttcgatacattctgtat↑ttctg (SEQ ID NO: 22), *TaqI* recognition site is emphasized in bold, arrow represents the position of the deleted T]. The initial PCR product was

cleaved with *TaqI* and run on a gel, to obtain 3 bands (from the 2 flanking and 2 middle *TaqI* restriction sites). The upstream piece, situated between the primers of the nested PCR, was removed. The remaining 2 bands were extracted from the gel and ligated to the mutated PCR product of the nested PCR to obtain the desired sequence (*Bcfl* to *TaqI* with a missing T). The second step, aimed at adding the G at position 744, involved an initial PCR with primers holding *BstDSI* (forward) and *MaeIII* (reverse) restriction sites [forward primer: ctgatgttcccc**gtgg**atgtgaaggcccat (SEQ ID NO: 23), *BstDSI* recognition site is emphasized in bold; reverse primer: ccacgag**taac**atcactaacaac**Ca**acaatac (SEQ ID NO: 24), *MaeIII* recognition site is emphasized in bold, added G (C in the reverse complement) is also emphasized in bold], to obtain a PCR product holding the additional G. The PCR product, and the product of the first step (*Bcfl* to *TaqI* with a missing T) were cleaved with *BstDSI* and *MaeIII*, and the cleavage product was replaced with the PCR product including the added G, by ligation. Finally, IL-60 was cleaved with *BceFI* and *BmrI*, a fragment was removed and replaced by the sequence obtained in the steps described above (*Bcfl* to *BmrI* with a missing T and an added G).
 iii) a deletion of 45 amino acids at the C terminus of native TYLCV V2 ("pre-coat"-SEQ ID NO: 6), caused by the deletion of the TYLCV CP described hereinabove.

Construction of IL-60-BS - the IL-60-BS vector (SEQ ID NO: 1) of the present invention was constructed by ligating a linearized (*Sac I*) Bluescript II-KS+ plasmid (Stratagene, La Jolla, CA, USA) into position 2443 of the IL-60 plasmid (SEQ ID NO: 2), interrupting the rep (rolling circle replication) protein (SEQ ID NO: 7) at position 93, within the N-terminus. The gene coding for C4 (symptom expression) was also interrupted by the BS insertion.

Construction of IR- GUS-pD - The IR- GUS-pD vector (SEQ ID NO: 13) was constructed by amplifying the IR region, pre-coat ORF and a part of the 5' UTR of the coat protein ORF- (positions 61 to 473 of TYLCV; accession # X15656) using forward primer 933: atacttggacacctaattggc (SEQ ID NO: 29) and reverse primer 934: agtcacggggcccttaca (SEQ ID NO: 30). This fragment was termed "IR-region". IR region was T/A cloned into the plasmid pDRIVE, to produce a plasmid called IR-pD (SEQ ID NO: 12). The coding sequence of *GUS* (bases 1466 to 3274 of GenBank accession # M14641) (SEQ ID NO: 31) was cleaved out of a GUS-carrying plasmid with *SacI* and

SaII and inserted into a *SaII*/*SacI* cleaved pDRIVE carrying the aforementioned IR region.

Construction of IR-PRN-pD – The IR-PRN-pD vector was constructed by placing the the entire PRN operon (as a single piece carrying all 4 genes) of *P. fluorescence* (corresponding to bases 424-6165 of GenBank accession # U74493; SEQ ID NO: 32) in place of GUS in IR-GUS-pD.

Propagation of IR-V2-CP, IR-V2-CP-GFP, IL-60 and IR- GUS-pD, and their administration to plants - *E. coli* cells were transformed with the above described constructs and propagated under ampicillin selection; the construct was extracted using standard procedures. IR-V2-CP was administered directly into plants, without mediation by *Agrobacterium*. The stem, or leaf petiole, of the recipient plant was punctured by a hypodermic needle. A capillary tube was inserted into the resultant hole, and approximately 2 microgram of DNA (in 100 μ l of 5 mM Tris-HCl; pH 8.5) were pipetted into the capillary tube until fully soaked by the plant.

RT-PCR analysis: RT-PCR analysis was carried out according to standard procedures (Sambrook and Russel, 2001). Primers used for analyzing expression of IR-V2-CP-GFP are set forth in Table 1.

Table 1

SEQ ID NO:	Primer design	Sequence 5'-3'	Description
SEQ ID NO: 16	Forward	attgggctgttccatagggc	For detection of CP (V1)
SEQ ID NO: 17	Reverse	gaaggctgaactcgacag	For detection of CP (V1)
SEQ ID NO: 18	Forward	tgtgtggacaggtaatgg	For detection of GFP
SEQ ID NO: 19	Reverse	ggccgaattcagtaaaggagaag	For detection of GFP

Primers used in order to detect GUS were as follows: Forward: ATTGATCAGCGTTGGTGGGA (SEQ ID NO: 25); Reverse: TGCGGTTCGCGAGTGAAGATC (SEQ ID NO: 26).

Primers used in order to detect PRN was as follows: Forward: GCGAACGAACACGATAGCAA (SEQ ID NO: 27); Reverse: CGTCAATGAGGGCGTGAAT (SEQ ID NO: 28).

Delivery of plasmids to plants by grafting - IL-60-BS and IR-GUS (or IR-PRN) were injected to tobacco and tomato plants as described herein above. Plants in which GUS (or PRN) have been replicated and spread (as indicated by PCR) were set aside for the following grafting experiments.

Scions of tobacco and tomato plants in which IR-GUS or IR-PRN were present, were grafted on untreated tobacco and tomato plants respectively (the rootstocks). The rootstocks were PCR analyzed for GUS and PRN one month following grafting. DNA was extracted from leaves remote from the point of grafting by at least 3 leaves.

Delivery of plasmids to plants by soaking - Roots of seedlings or young plantlets (tomato, tobacco, grapevines) were slightly trimmed. The roots were soaked in a 2-5 µg of each of IL-60-BS and IR-GUS dissolved in water. The plantlets were kept in the solution until all liquid was soaked up. Another volume of water was then added and after it has also been soaked up the plantlets were planted in pots. Replication and spread of GUS was tested periodically by PCR in upper leaves.

EXAMPLE 1

IR and the sense viral genes (V1 and V2) are sufficient for stabilization, movement and spread of artificial satellites in plant tissues

IR-carrying satellites are activated to replicate, move and be expressed, either by a helper virus or by the mutated disarmed vector IL-60-BS (see WO2007/141790, incorporated herein by reference). The following example now shows that the sense-transcribed genes of TYLCV alone, under IR regulation, are sufficient for self-replication, movement and expression. This genomic segment can promote replication, movement and expression of IR-carrying satellites *in trans* as well.

GFP was fused to the 3' end of CP in the IR-V2-CP construct (Figures 1A and 1B). Leaves remote from the point of injection were analyzed (Figures 2A-F). Upon injection into tomato plants, GFP fluorescence was observed mainly in the nuclei of phloem companion cells (FIGs. 2C-D). It was also observed at the cell periphery,

suggesting movement across plasmodesmata (FIGs. 2A, E), as well as in mesophyll cells (FIGs. 2A, F).

PCR analysis indicated the presence of both TYLCV CP and GFP sequences, as well as TYLCV transcripts in treated plants (Figures 3C-E). The absence of other
5 TYLCV sequences (Figure 3F) indicated that the plants were TYLCV-free. These results indicate that IR-V2-CP-GFP replicates and moves on its own. GFP was present in the nuclei of companion cells, at the cell periphery and, apparently, across plasmodesmata.

The IR-V2-CP construct can induce replication and movement of another DNA
10 *in trans*, providing the DNA carries an IR. As shown in Figures 3A-B, the artificial satellite IR-GUS (Figure 1C) was mobilized and expressed GUS in the presence of IR-V2-CP.

EXAMPLE 2

Delivery of IL-60-BS to plants by grafting

15 The results are illustrated in Figures 4 and 5. Specifically, these Figures illustrate that both IR-GUS (Figure 4) and IR-PRN (Figure 5) were transferred from the scion to the rootstock where they replicated and spread.

EXAMPLE 3

Delivery of IL-60-BS by through the roots

20 Figure 6 demonstrates that IR-GUS was transferred through the roots and replicated and spread in the plants. The same result was achieved when other IR vectors (IR-GFP and IR-PRN) were transferred through the roots of additional plants including tomato, tobacco, grapevine and periwinkle (data not shown).

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Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all
30 such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or
5 identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention. To the extent that section headings are used, they should not be construed as necessarily limiting.

WHAT IS CLAIMED IS:

1. A method of expressing a molecule of interest in a plant, the method comprising contacting roots of the plant in a solution comprising at least one Geminivirus based expression construct so as to allow said at least one Geminivirus based expression construct to be absorbed by said roots, said expression construct comprising a polynucleotide encoding the molecule of interest, and further said expression construct being capable of systemic symptomless spread in a plant host, thereby expressing a molecule of interest in a plant.

2. The method of claim 1, wherein said Geminivirus based expression construct is devoid of a polynucleotide sequence encoding a C2 and C3 coding sequence.

3. The method of claim 1, wherein said Geminivirus based expression construct comprises a polynucleotide sequence encoding the molecule of interest, said polynucleotide sequence being flanked by a non-contiguous nucleic acid sequence encoding a Geminivirus replicase.

4. A method of expressing a molecule of interest in a plant, the method comprising grafting a section of a first plant infected with at least one Geminivirus based expression construct onto a section of a second plant, said expression construct comprising a polynucleotide sequence which encodes the molecule of interest, and further said Geminivirus based expression construct being capable of systemic symptomless spread in a plant host, thereby expressing a molecule of interest in a plant.

5. The method of claim 4, wherein said Geminivirus based expression construct is devoid of a polynucleotide sequence encoding a C2 and C3 coding sequence.

6. The method of claim 4, wherein said Geminivirus based expression construct comprises a polynucleotide sequence encoding the molecule of interest, said polynucleotide sequence being flanked by a non-contiguous nucleic acid sequence encoding a Geminivirus replicase.

7. A Geminivirus based expression construct, capable of systemic symptomless spread in a plant host, the expression construct being devoid of a polynucleotide sequence encoding a C2 and C3 coding sequence.

8. The expression construct of claim 7 comprising:

- (i) a polynucleotide sequence encoding a Geminivirus intergenic region (IR);
- (ii) a polynucleotide sequence encoding a modified Geminivirus coat protein (CP); and
- (iii) a polynucleotide sequence encoding a modified Geminivirus precoat protein (V2).

9. The expression construct of claim 8, further comprising a polynucleotide sequence encoding a modified replicase protein (C1).

10. The expression construct of claim 9, wherein said modified replicase protein as set forth in SEQ ID NO: 36.

11. The expression construct of claim 8, further comprising a polynucleotide sequence encoding a modified C4.

12. The expression construct of claim 7, being capable of replication in a prokaryotic cell.

13. The expression construct of claim 7, being incapable of plant to plant transmission by an insect vector.

14. The expression construct of claim 7, further comprising a heterologous polynucleotide sequence.

15. The expression construct of claim 14, wherein said heterologous polynucleotide is larger than 1 kb.

16. The expression construct of claim 14, wherein said heterologous polynucleotide is larger than 5 kb.

17. The expression construct of claim 14, wherein said heterologous polynucleotide comprises an operon.

18. The expression construct of claim 14, wherein said heterologous polynucleotide is adapted for gene silencing.

19. The expression construct of claim 7, further comprising a bacterial polynucleotide sequence.

20. The expression construct of claim 8, wherein said modified Geminivirus V2 protein is as set forth in SEQ ID NO: 6.

21. The expression construct of claim 8, wherein said modified Geminivirus coat protein comprises an amino acid sequence as set forth in SEQ ID NO: 3.

22. The expression construct of claim 8, wherein said modified Geminivirus coat protein comprises a mutation or deletion in nucleotides encoding an N-terminal 100 amino acids.

23. The expression construct of claim 8 comprising a polynucleotide sequence as set forth in SEQ ID NO: 10 or 11.

24. The expression construct of claim 14, wherein said heterologous polynucleotide encodes a polypeptide selected from the group consisting of a reporter molecule, an antiviral molecule, a viral moiety, an antifungal molecule, an antibacterial molecule, an insect resistance molecule, a herbicide resistance molecule, a biotic or abiotic stress tolerance molecule, a pharmaceutical molecule, a growth inducing molecule, and a growth inhibiting molecule.

25. The expression construct of claim 7, wherein the Geminivirus is a begomovirus.

26. The expression construct of claim 7, wherein the Geminivirus is a Tomato yellow leaf curl virus (TYLCV).

27. The expression construct of claim 7, wherein the expression construct is adapted for expression in a plant host selected from the group consisting of *Solanaceae*, *Cucurbitaceae*, *Umbelliferae*, *Liliaceae*, *Gramineae* (*Poaceae*), *Rosaceae*, *Musaceae*, *Vitacea*, and *Cruciferae*.

28. A method of expressing a molecule of interest in a plant cell comprising introducing into the plant tissue the nucleic acid construct of claim 14, said heterologous polypeptide being the molecule of interest, thereby expressing a molecule of interest in a plant cell.

29. The method of claim 28, wherein the molecule of interest is selected from the group consisting of a reporter molecule, an antiviral molecule, a viral moiety, an antifungal molecule, an antibacterial molecule, an insect resistance molecule, a herbicide resistance molecule, a biotic or abiotic stress tolerance molecule, a pharmaceutical molecule, a growth inducing molecule, a product of genes in a metabolic pathway and a growth inhibiting molecule.

30. The method of claim 29, wherein the genes in the metabolic pathway are encoded by an operon.

31. The method of claim 28, wherein the plant is selected from the group consisting of a Solanaceae, a Cucurbitaceae, an Umbelliferae, a Liliaceae, a Gramineae (Poaceae), a Rosaceae *Musaceae*, *Vitacea* and a Cruciferae.