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[Continued on next page]

(54) Title NOVEL PLANT VIRUS

Figuic 1

(57) Abstract The invention relates to the field of virology The invention provides an isolated plant virus (ToCh V) named Tomato chocolate virus (ToCh V), and components thereof The invention further relates to methods of producing a ToCh V-resistant plant comprising the steps of identifying a ToCh V-resistant donor plant, crossing said ToCh V-resistant donor plant with a recipient plant, and selecting from an offspring plant a resistant plant

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Title: Novel plant virus

FIELD OF THE INVENTION

The present invention is in the field of plant disease. More in particular, the invention relates to a new plant virus isolated from tomato, to methods for detecting said virus, to methods of detecting resistant plants and to methods for producing resistant plants.

BACKGROUND OF THE INVENTION

The tomato *Solanum lycopersicum* (formerly *Lycopersicon esculentum)* is susceptible to a large number of viral species. Some of the most prominent tomato viruses include *Tomato spotted wilt virus* (TSWV; genus *Tospovirus);* *Pepino mosaic virus* (PepMV; genus *Potexvirus),* and *Tomato yellow leaf curl virus* (TYLCV; genus *Begomovirus).* The damage that these diseases inflict on the plant range from discoloration of leaves and necrotic lesions, to severe crop loss and death of the plant.

The ability to provide resistant plants is of utmost importance to commercial breeders, and for some of the economically most damaging viruses, resistant plant varieties have been produced. However, from time to time, new viruses emerge that may inflict considerable damage on crops.

In 1996 a new tomato virus was reported which had infected tomato plants in the USA and Italy since 1993, and was named *Tomato infectious chlorosis virus* (TICV; genus *Crinivirus;* Duffus *et al.,* 1996). Another new tomato virus of the same genus was reported in 1998. This virus was shown to have infected tomato plants in the USA since 1989 and was named *Tomato chlorosis virus* (ToCV; Wisler *et al.,* 1998). Both these new viruses proved to be spread by a whitefly, the insect being a very effective disease-transmission vector.

It is generally believed that the geographic distribution of known

viruses will increase and that new viruses will continue to appear, partly as a result of recombination of different viruses to form new strains or new viruses. The development of resistant cultivars can play an important role in the successful management of these diseases.

Recently, a new virus was discovered on tomato plants from Spain, which caused symptoms that could not be attributed to any known virus. The plants exhibited necrotic lesions on leaves and brown rings on fruits and showed reduced growth. Serological tests (ELISA) indicated the presence of *Pepino mosaic virus* (PepMV). Electron microscopic investigations indeed revealed the rod-like particles typical for potexviruses. However, also spherically shaped viral particles were found in infected leaf tissue. The inventors were able to separate the new virus from the complex with PepMV. The new virus was tentatively named Tomato torrado virus (ToTV) (WO 2006/085749).

Following the discovery of the ToTV virus, a new and very related virus was isolated from a tomato plant from the state of Sinaloa in Mexico. This plant showed symptoms locally known as 'marchitez disease', including severe leaf necrosis, beginning at the base of the leaflets, and necrotic rings on the fruits. (This disease should not be confused with another virus known to cause what is called in Mexico "marchitez manchada del tomate", the causative agent of which is tomato spotted wilt virus (TSWV) or Virus de Ia Marchitez Manchada del Tomate (VMMT), [see e.g. De La Torre-Almaraz *et al.* Agrociencia 36: 211-221. 2002]).Virus particles isolated from the infected plants are isometric with a diameter of approximately 28 nm. The viral genome consists of two (+)ssRNA molecules of 7221 (RNAl) and 4906 nucleotides (RNA2). The viral capsid contains three coat proteins of 35, 26 and 24 kDa respectively. The above-mentioned characteristics; symptoms, morphology, number and size of coat proteins, and number of RNAs, are similar to the previously described Tomato torrado virus (ToTV). Sequence analysis of the entire viral genome shows that this new virus is related to, but

distinct from ToTV and, together with ToTV belongs to a new plant virus genus. For this new virus the name tomato marchitez virus (ToMarV) was proposed (PCT/NL2008/050076).

Following the discovery and successful isolation of ToMarV, the inventors further discovered diseased plants in Guatemala suffering from a disease known as "Chocolate spot disease" or shortly "chocolate". Initial experiments using ToTV-specific amplification primers appeared to indicate the presence of ToTV-specific RNA sequences in several diseased plants. However, the causative virus could not be isolated and the referred viral agent could not be detected in subsequent experiments. Herein, we report on the discovery and successful isolation of a new virus.

For being able to trace its origin, monitor its epidemiology and prevent possible spreading of the disease, it is of great importance to be able to recognise the disease in an early stage. Only then sufficient measures can be taken to isolate plants and initiate phytosanitairy precautions. At this moment no diagnostic tools are available. Consequently, there is a need for developing diagnostic tools for this disease. Furthermore, at present there are no plants known that harbour specific resistance to this new virus, while there is a need for developing such resistant plants.

SUMMARY OF THE INVENTION

The invention provides in a first aspect a plant virus tentatively named Tomato chocolate virus (ToChV), deposited by the application under depositors reference "ToChV-GOl" on 8 December 2008 with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), in Braunschweig, Germany. This deposit received accession number DSM 22139.

The virus causes disease-symptoms in tomato plants as well as in other plants, and may cause the symptoms by itself, or in a complex with other viruses or diseases.

The disease symptoms may vary from rugosis in the youngest tomato

leaves and a beginning chlorosis, to a clear chlorosis and necrosis, beginning at the basis of the leaflets (see Figure 1). The growth of infected plants may be reduced compared to non-infected plants.

The above description relates to plants newly infected with the isolated virus and need not necessarily reflect the exact symptoms encountered in the field. Factors such as plant race or variety, development stage, additional disease pressure, and abiotic factors *(e.g.* temperature and relative humidity) will eventually determine the expression and characteristics of the symptoms.

The viral particles are spherical in shape with a diameter of approximately 28 - 34 nm (see Figure 2). Virus particles consist of at least three putative capsid proteins of approximately 24, 26 and 35 kDa (see Figure 3A). Upon purification, the viral genome appears to consist of two RNA molecules of approximately 6 kb and of approximately 8 kb (see Figure 3B). The purified virus was also used to inoculate tomato plants to fulfil Koch's postulates. The inoculated tomato plants (cultivar Moneymaker) showed typical symptoms approximately two weeks after inoculation.

ToChV is mechanically transmissible to several *Nicotiana* species, inlcuding *N. tabacum Xanthii* plants (See Table 2 in Example 1). A standard inoculation buffer *(e.g.* a 0.03 M phosphate buffer at pH 7.7) is suitable.

In another aspect, the present invention provides a virus comprising the nucleic acid sequence of SEQ ID NO:1, 2, and 3 as defined in Figures 4, 5 and 6 respectively, and sequences having a nucleotide sequence homology of at least 70%, more preferably, at least 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% thereto and/or being the causal agent of Chocolate spot disease in tomatoes. Such viruses are also encompassed by the term ToChV as used herein.

The viral genome of ToChV consists of 2 RNA molecules, one of which is referred to herein as RNAl having the sequence provided as SEQ ID NO:1 herein (Fig. 4), the other of which is referred to herein as RNA2 having the sequence provided as SEQ ID NO:2 herein (Fig. 5). It has been found that

another, very closely related variant may be present in infected plants. This ToChV variant is referred to herein as ToChV-type 2. This ToChV variant (ToChV-G02) has a similar or identical RNAl sequence, but an RNA2 sequence having a homology with type 1 of 91.1% on nucleotide-level and 96.4% on amino acid (aa)-level. RNA2 of type 1 comprises two ORF's: RNA2-ORF1: 189 aa en RNA2-ORF2: 1192 aa.

Hence the present invention provides a virus comprising the nucleic acid sequence of any one or both RNAs of deposits ToChV-GOl and ToChV-G02, and sequences having a nucleotide sequence homology of at least 70%, more preferably, at least 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% thereto, as such viruses are also encompassed by the term ToChV as used herein.

In another aspect, the present invention provides an isolated virus belonging to the presumptive novel species of Tomato chocolate virus (ToChV), of which the plant virus as deposited on 8 December 2008 under depositors reference ToChV-GOl with the DSMZ, Braunschweig, Germany (DSM 22139) is the type strain, wherein said isolated virus identifiable as phylogenetically corresponding to said novel genus by determining a nucleic acid sequence of a suitable fragment of the genome of said virus and testing it in phylogenetic analyses wherein maximum likelihood trees are generated and finding it to be phylogenetically more closely related to the plant virus as deposited than it is related to a virus isolate of a non-ToChV reference strain or closest relative, such as ToMarV or ToTV.

The plant virus as deposited on 22 December 2008 under depositors reference ToChV-G02 with the DSMZ, Braunschweig, Germany (DSM 22202), and the plant virus as deposited on 21 July 2009 under depositors reference ToChV-G02 with the DSMZ, Braunschweig, Germany are variant strains to ToChV-GOl, in that they possess a variant type RNA2.

In a preferred embodiment of this aspect, said virus is the causative agent of (herein also referred to as being associated with) a disease that causes

necrotic lesions in tomato and which virus shows to be more closely related to the virus as deposited as described above than to any other virus, based on numerical taxonomic analysis of one or more of the taxonomic descriptors:

- Virion properties, including morphological and physical properties of virions, properties of the genome and properties of the proteins;
- Genomic organization and replication;
- Antigenic properties; and
- Biological properties, including natural and experimental host range, pathogenicity, tissue trophism, mode of transmission in nature, vector relationships and geographical distribution.

In another aspect, the present invention relates to an isolated or recombinant nucleic acid comprising a nucleic acid sequence selected from SEQ ID NO:1, 2 and 3, sequences having a nucleotide sequence homology of at least 70%, more preferably, at least 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% to SEQ ID NO:1, 2 and 3 and their complementary strands.

In another aspect, the present invention relates to an expression vector comprising the above isolated or recombinant nucleic acid.

In yet another aspect, the present invention relates to a polynucleotide capable of hybridizing under stringent conditions to a nucleic acid according to the invention.

In yet another aspect, the present invention relates to a polypeptide encoded by an open reading frame (ORF) in the nucleic acid sequence of SEQ ID NO:1, 2 or 3 and complementary strands thereof. Herein provided is the sequence of RNA-I, including any and all open reading frames therein. RNA-I of ToTV and ToMarV were previously found to encode for the helicase and the RNA-dependent RNA polymerase. The skilled person will appreciate that the ORFs for these polypeptides can be easily determined in the context of the disclosures for ToTV and ToMarV from the sequence as provided herein. Also, provided herein is the sequence of RNA-2 (both of type 1 (fig. 5) and type 2 (fig.

6)), including any and all open reading frames therein. With the availability of the deposited strain ToChV-G02, the skilled person is capable of determining the full polypeptide sequence encoded in RNA-2 of ToChV-G02. Also, these polypeptides are contemplates as aspects of the present invention.

Preferably, said polypeptide is selected from the group consisting of the 24, 26 and 35 kDa capsid proteins, the helicase, the RNA-dependent RNA polymerase and the putative movement protein (MP) of ToChV.

In yet another aspect, the present invention relates to an antigen comprising a polypeptide according to the present invention.

In yet another aspect, the present invention relates to an antibody specifically directed against the antigen according to the present invention.

In another aspect, the invention provides a method for producing an antibody against ToChV comprising the steps of: a) providing a ToChV virus, or a (recombinant) protein or peptide fragment thereof ; b) immunizing an appropriate vertebrate host with said virus, protein or peptide fragment, and c) harvesting from blood (including serum) or splenocytes of said vertebrate host antibodies against said virus, protein or peptide fragment. In a preferred embodiment said method further comprises the steps of d) selecting one antibody-producing splenocyte, e) fusing said splenocyte to an immortalized hybridoma cell line and f) allowing said hybridoma fusion to produce monoclonal antibodies.

In another aspect, the invention provides a method for identifying a viral isolate as a ToChV virus comprising reacting said viral isolate or a component thereof with an antibody according to the invention.

In another aspect, the invention provides a method for identifying a viral isolate as a ToChV virus comprising reacting said viral isolate or a component thereof with a polynucleotide according to the invention.

In another aspect, the invention provides a method for detecting the presence of ToChV in a sample comprising determining in said sample the

presence of a ToChV virus or component thereof by reacting said sample with a polynucleotide or an antibody according to the invention.

In another aspect, the invention provides a method for identifying a ToChV-resistant plant comprising the steps of: a) exposing a plant or plant part to an infective dosage (an amount of virus or virus titre sufficient to cause disease symptoms upon a suitable incubation period of between 1 and 10 days) of ToChV, and b) identifying said plant as a ToChV-resistant plant when, after said exposure, either i) disease-symptoms in said plant or plant part remain absent or are delayed in expression or are at least reduced in severity relative to a susceptible control plant, and/or ii) ToChV virus or ToChV genomic sequences are not present in said plant or plant part or the presence of ToChV virus is at least quantitatively reduced in said plant relative to a susceptible control plant.

Step a) includes an incubation period for a duration sufficiently long to allow for the establishment of detectable disease-symptoms in susceptible control plants exposed to a comparable infective dosage of virus. By performing this method all forms of resistance, including full resistance, partial resistance, hypersensitivity and tolerance may be identified in a plant. In order to confirm tolerance, the (systemic) presence of the virus in the plant (cells) must be confirmed. Step b) may involve performing a method for detecting the presence of ToChV in a sample of said plant or plant part according to the present invention wherein use is made of an antibody or polynucleotide according to the invention in standard methods for nucleotide hybridization assays or immunoassays, well known to the skilled person. Alternatively, step b) may comprise contacting a part of said exposed plant with a susceptible indicator plant. In this way, one may detect the presence of a systemic or local infection in said exposed plant through observing the emergence of disease in the indicator plant or even a further contacted indicator plant contacted with said first contacted indicator plant.

In another aspect, the invention provides a method of producing a ToChV-resistant plant comprising the steps of identifying a ToChV-resistant donor plant by performing either of the above methods for identifying a ToChV-resistant plant according to the invention, crossing said ToChV-resistant donor plant with a recipient plant (which recipient plant may be either ToChV-susceptible or ToChV-resistant, but is suitably a ToChV-resistant plant in case the resistant phenotype is brought about by a recessive gene), and selecting from an offspring plant (e.g. an F_1, an F2, and a selfed plant) a resistant plant by performing a method for identifying ToChV resistance in a plant as described above. In the instance that the resistance trait is a recessive trait, resistant plants may be found among offspring plants of the selfings of the Fl or F2 or still further generations. In preferred embodiments of this aspect, said ToChV-resistant donor plant and the recipient plant are plants of the family *Solanaceae* or family *Cucurbitaceae.* In other preferred embodiments of this aspect, said recipient plant is a tomato plant, eggplant plant, pepper plant, melon plant, watermelon plant or cucumber plant, more preferably a plant of the species *Solanum lycopersicum,* most preferably an *S. lycopersicum* line that possesses commercially desirable characteristics.

In another aspect, the invention provides a ToChV-resistant plant, preferably a tomato plant, eggplant plant, pepper plant, melon plant, watermelon plant or cucumber plant, or part thereof, such as a seed, obtainable by a method of producing a ToChV-resistant plant according to the invention.

In another aspect, the invention provides a diagnostic kit for detecting the presence of ToChV in a sample or for identifying ToChV-resistance in a plant comprising a virus, a polynucleotide, a polypeptide, an antigen and/or an antibody according to the invention.

In another aspect, the invention provides the use of a virus, a polynucleotide, a polypeptide, an antigen or an antibody according to the invention for the production of a diagnostic composition.

In another aspect, the invention provides a diagnostic composition comprising a virus, a polynucleotide, a polypeptide, an antigen or an antibody according to the invention.

In another aspect, the invention provides the use of ToChV, or parts of the ToChV viral genome, as an expression vector.

In another aspect, the invention provides the use of ToChV, or parts of the ToChV viral genome, for producing pathogen-derived resistance in plants.

In another aspect, the invention relates to the use of an attenuated form of a ToChV virus, or its genome, or parts thereof, for premunition of a plant.

LEGENDS TO THE FIGURES

Figure 1 shows symptoms induced by ToChV on tomato accession 'Moneymaker' two weeks after inoculation with purified particles of ToChV, showing systemic necrosis, beginning at the base of the leaflets.

Figure 2 shows electron microscopy picture of ToChV-particles. Panel A: electron microscopy image of virus particles in crude sap of *N. hesperis* '67A'. Particle size is approximately 34 nm in diameter; Panel B: Electron microscopy image of purified Tomato chocolate virus (diameter of virions is approximately 28 nm).

Figure 3 shows the results of electrophoresis of purified virions using coomassie staining (A) and the result of electrophoresis of the RNA preparation on a denaturing gel using ortho-toluidine staining (B).

Figure 4 shows the nucleotide sequence of RNA-I of both variants referred to herein as ToChV-GOl and ToChV-G02, which sequence is herein referred to as SEQ ID NO:1.

Figure 5 shows the nucleotide sequence of RNA-2 of ToChV-GOl in the description referred to as SEQ ID NO:2.

Figure 6 shows the nucleotide sequence of RNA-2 of ToChV-G02 in the description referred to as SEQ ID NO:3.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used herein the term "torradovirus", is to be interpreted as referring to the new genus of viruses sharing RNA homology levels of at least 60-99% as indicated herein, unless expressly stated or intended otherwise (for instance when ToChV, ToMarV and ToTV are compared), the term "ToTV" should be interpreted as referring to the species ToTV-EOl. This broad genus includes at least the species ToTV-EOl (DSM 16999) as well as the PRI-TMarV0601 (DSM 19656). The genus is also referred to herein as torradovirus and is defined by the homology levels between RNA sequences of the viral genome (or translated into DNA sequences); or the homology levels between amino acid sequences of open reading frames within the viral genome.

As used herein the term "ToChV" is to be interpreted as referring to a species within the new genus of toradoviruses which genus shares the RNA homology levels as indicated herein, unless expressly stated or intended otherwise (for instance when ToChV, ToMarV and ToTV are compared), the term ToChV should be interpreted as including reference to the species ToChV-GOl or ToChV-G02. The term "ToChV" is also to be interpreted as referring to a virus belonging to this novel species within the new genus of ToTV viruses which genus wherein said novel species shares homology levels on nucleotide or amino acid sequence levels generally accepted among virologists to indicate different isolates (strains) belonging to the same species. Such intraspecific homology levels are usually above 40, 50, 60, 70, or even 80 to 90%, for instance above 95% for nucleotide sequences, and possibly even higher for amino acid sequences.

As used herein the term "ToChV-resistant", is to be interpreted as referring to the resistance of a plant, in particular a tomato plant to the

establishment of an infection by ToChV, or the establishment of a disease caused by a viral species of the new genus of viruses sharing the RNA homology levels as indicated herein, unless expressly stated or intended otherwise.

When reference is made herein to polynucleotides or polypeptides of ToChV, the term "ToChV-specific fragment" as used herein refers to a nucleic acid fragment having a sequence that is specific for the species of ToChV viruses as described herein. With the term "specific" is meant that the nucleic acid is capable of hybridizing specifically under stringent hybridization conditions to the nucleic acid of said virus.

As used herein, the term "plant part" indicates a part of a plant, including single cells and cell tissues such as plant cells that are intact in plants, cell clumps and tissue cultures from which plants can be regenerated. Examples of plant parts include, but are not limited to, single cells and tissues from pollen, ovules, leaves, embryos, roots, root tips, anthers, flowers, fruits, stems shoots, and seeds; as well as pollen, ovules, leaves, embryos, roots, root tips, anthers, flowers, fruits, stems, shoots, scions, rootstocks, seeds, protoplasts, calli, and the like.

The term "sample" includes a sample from a plant, from a plant part or from a transmission vector, or a soil, water or air sample.

The term "transmission vector" as used herein refers to the disease-spreading agent or substance. Transmission vectors of ToChV in the field may comprise, but are not limited to animals such as *Arthropoda* (in particular those of the classes *Insecta* and *Arachnida), Nematoda* (in particular those of the class *Adenophore* ά), but also larger animals such as for instance birds, rabbits and mice, fungi (i.e. phylum *Eumycota,* in particular fungi of the class *Phy cornycota),* (parasitic) plants (including members of the family *Cuscutaceae),* pollen, seed, water, soil particles, and even human hands, equipment and shoes.

The term "offspring" plant refers to any plant resulting as progeny from a vegetative or sexual reproduction from one or more parent plants or descendants thereof. For instance an offspring plant may be obtained by cloning or selfing of a parent plant or by crossing two parent plants and include selfings as well as the F1 or F2 or still further generations. An F1 is a first-generation offspring produced from parents at least one of which is used for the first time as donor of a trait, while offspring of second generation (F2) or subsequent generations (F3, F4, etc.) are specimens produced from selfings of Fl's, F2's etc. An F1 may thus be (and usually is) a hybrid resulting from a cross between two true breeding parents (true-breeding is homozygous for a trait), while an F2 may be (and usually is) an offspring resulting from self-pollination of said F1 hybrids.

The term "resistant", as used herein, refers to a plant that is capable of resisting the multiplication in its cells and/or the (systemic) movement of the virus to other cells and/or the development of disease symptoms after infection with said virus, which virus is capable of infecting and multiplying in corresponding non-resistant or susceptible varieties of said plant. The term is used to include such separately identifiable forms of resistance as "full resistance", "immunity", "partial resistance", "hypersensitivity" and "tolerance".

"Full resistance" is referred to as complete failure of the virus to develop after infection, and may either be the result of failure of the virus to enter the cell (no initial infection) or may be the result of failure of the virus to multiply in the cell and infect subsequent cells (no subliminal infection, no spread). The presence of full resistance may be determined by establishing the absence of viral particles or viral RNA in cells of the plant, as well as the absence of any disease symptoms in said plant, upon exposure of said plant to an infective dosage of virus (i.e. after 'infection'). Among breeders, this phenotype is often referred to as "immune". "Immunity" as used herein thus refers to a form of

resistance characterized by absence of viral replication even when virus is actively transferred into cells by e.g. electroporation.

An "infective dosage" is defined as a dosage of viral particles or virus nucleic acid capable of infecting a plant, which dosage may vary between plants and between ToChV-isolates tested. Theoretically, an amount of about 1 to 10 to an amount of about 500-5000 viral particles of said virus or the nucleic acids thereof will be sufficient. Infection in this way may be achieved by mechanical inoculation of purified virus particles or virus nucleic acid on plants.

"Partial resistance" is referred to as reduced multiplication of the virus in the cell, as reduced (systemic) movement of the virus, and/or as reduced symptom development after infection. The presence of partial resistance may be determined by establishing the systemic presence of low titres of viral particles or viral RNA in the plant and the presence of decreased or delayed disease-symptoms in said plant upon exposure of said plant to an infective dosage of virus. Virus titres may be determined by using a quantitative detection method (e.g. an ELISA method or a quantitative reverse transcriptase-polymerase chain reaction [RT-PCR]). Among breeders, this phenotype is often referred to as "intermediate resistant".

The term "hypersensitive" refers to a form of resistance whereby the infection remains local and does not systemically spread, for instance due to local necrosis of infected tissue or lack of systemic movement beyond inoculated tissue. Hypersensitive plants show local, but severe disease symptoms and the local presence of the virus can be established in such plants.

"Tolerant" is used herein to indicate a phenotype of a plant wherein disease-symptoms remain absent upon exposure of said plant to an infective dosage of virus, whereby the presence of a systemic or local viral infection, virus multiplication, at least the presence of viral genomic sequences in cells of said plant and/or genomic integration thereof can be established. Tolerant plants are therefore resistant for symptom expression but symptomless

carriers of the virus. Sometimes, viral sequences may be present or even multiply in plants without causing disease symptoms. This phenomenon is also known as "latent infection". Some DNA and RNA viruses, may become undetectable following a primary infection only to reappear later and produce acute disease. In latent infections, the virus may exist in a truly latent non-infectious occult form, possibly as an integrated genome or an episomal agent (so that viral particles cannot be found in the cytoplasm, while PCR protocols may indicate the present of viral nucleic acid sequences) or as an infectious and continuously replicating agent. A reactivated virus may spread and initiate an epidemic among susceptible contacts. The presence of a "latent infection" is indistinguishable from the presence of a "tolerant" phenotype in a plant.

The term "susceptible" is used herein to refer to a plant having no resistance to the virus resulting in entry of the virus into the plant's cells and multiplication and systemic spread of virus, resulting in disease symptoms. The term "susceptible" is therefore equivalent to "non-resistant". A susceptible plant exhibits normal virus titres in its cells upon infection. Susceptibility may thus be determined by establishing the presence of normal (i.e. relative to other viral infections in plants) titres of viral particles or of viral RNA in cells of the plant and the presence of normal disease symptoms (i.e. relative to the disease symptoms as herein described for the plant from which ToChV was first isolated) in said plant upon exposure of said plant to an infective dosage of virus.

The term "sensitive" reflects the symptomatic reaction of a susceptible plant upon virus infection. The reaction or symptoms can be more or less severe depending on the level of sensitivity of the plant. If the plant is injured or even killed by the virus, said plant is qualified as "sensitive".

Plants artificially inoculated with attenuated virus strains are subsequently protected from closely related virulent viruses. As protective viruses, either naturally occurring mild strains or an attenuated strain (an

artificially-induced mild mutant) may be used. Preferably, in order to attain premunition of a plant against ToChV, an attenuated strain of ToChV may be used that is symptomless or that shows at least reduced symptom expression in an infected plant, relative to a virulent strain of ToChV. Methods of producing attenuated virus may for instance include random mutagenesis of the ToChV genome and screening for strains with symptom attenuation. Express reference is made to the methods for producing attenuated plant viruses as described in the articles of Takeshita *et al,* 2001; Lu *et al,* 2001; Hagiwara, *et al,* 2002; and Hirata *et al,* 2003.

As used herein, the term "tomato" means any plant, line or population of *Lycopersicon* or *Solanum* that includes, but is not limited to, those presented in the Listing below. Recently, nomenclature of *Lycopersicon* has been changed.

The new nomenclature for *Lycopersicon* is provided in the following Listing (from: Peralta, Knapp & Spooner, unpublished monograph (see: http://www.sgn.cornell.edu "New nomenclature for Lycopersicon")).

Name in tomato monograph (Peralta *et al.*, in preparation for publication in *Systematic Botany Monographs*)	***Lycopersicon* equivalent**
Solanum juglandifolium Dunal	*Lycopersicon juglandifolium* (Dunal) J.M.H. Shaw
Solanum ochranthum Dunal	*Lycopersicon ochranthum* (Dunal) J.M.H. Shaw
Solanum sitiens I.M. Johnst.	*Lycopersicon sitiens* (I.M. Johnst.) J.M.H. Shaw
Solanum lycopersicoides Dunal	*Lycopersicon lycopersicoides* (Dunal in DC.) A. Child ex J.M.H. Shaw
Solanum pennellii Correll	*Lycopersicon pennellii* (Correll) D'Arcy
Solanum habrochaites S. Knapp & D.M Spooner	*Lycopersicon hirsutum* Dunal
Solanum 'N peruvianum' to be described by Peralta (4 geographic races: humifusum, lomas, Marathon, Chotano-Yamaluc)	Part of *Lycopersicon peruvianum* (L.) Miller (incl. var.humifusum and Marathon races)
Solanum 'Callejon de Huaylas' to be described by Peralta	Part of *Lycopersicon peruvianum* (L.) Miller (from Ancash, alogn Río Santa)
Solanum neorickii D.M. Spooner, G.J. Anderson & R.K. Jansen	*Lycopersicon parviflorum* C.M. Rick, Kesicki, Fobes & M. Holle
Solanum chmielewskii (C.M. Rick, Kesicki, Fobes & M. Holle) D.M. Spooner, G.J. Anderson & R.K. Jansen	*Lycopersicon chmielewskii* C.M. Rick, Kesicki, Fobes & M. Holle
Solanum corneliomuelleri J.F. Macbr. (1 geographic race: Misti nr. Arequipa)	Part of *Lycopersicon peruvianum* (L.) Miller; also known as *Lycopersicon glandulosum* C.F. Mull.
Solanum peruvianum L.	*Lycopersicon peruvianum* (L.) Miller
Solanum chilense (Dunal) Reiche	*Lycopersicon chilense* Dunal
Solanum cheesmaniae (L. Riley) Fosberg	*Lycopersicon cheesmaniae* L. Riley (published as *cheesmanii*)
Solanum galapagense S. Darwin & Peralta	Part of *Lycopersicon cheesmaniae* L. Riley (previously known as forma or var. *minor*)
Solanum lycopersicum L.	*Lycopersicon esculentum* Miller
Solanum pimpinellifolium L.	*Lycopersicon pimpinellifolium* (L.) Miller

An "expression vector" is defined as a nucleic acid molecule containing a gene, usually a heterologous gene, that is expressed in a host cell. Typically, this gene comprises a protein encoding sequence. Gene expression is always placed under the control of a promoter, and such a gene is said to be "operably linked to" the promoter. The term "heterologous" refers to a DNA molecule, or

a population of DNA molecules, that does not exist naturally within a given host cell.

The term "polynucleotide" as used herein, is interchangeable with the term "nucleic acid", and refers to a nucleotide multimer or polymeric form of nucleotides having any number of nucleotides, *e.g.*, deoxyribonucleotides or ribonucleotides, or compounds produced synthetically *{e.g.* PNA as described in U.S. Pat. No. 5,948,902 and the references cited therein) and can be either double- or single-stranded. A polynucleotide can hybridize with naturally occurring polynucleotides in a sequence specific manner analogous to that of two naturally occurring polynucleotides, *e.g.* can participate in Watson-Crick base pairing interactions. The term also includes modified, for example by methylation and/or by capping, and unmodified forms of the polynucleotide.

The terms "ribonucleic acid" and "RNA" as used herein mean a polymer composed of ribonucleotides.

The terms "deoxyribonucleic acid" and "DNA" as used herein mean a polymer composed of deoxyribonucleotides.

The term "oligonucleotide" refers to a short sequence of nucleotide monomers (usually 6 to 100 nucleotides) joined by phosphorous linkages *{e.g.,* phosphodiester, alkyl and aryl-phosphate, phosphorothioate), or non-phosphorous linkages *{e.g.,* peptide, sulfamate and others). An oligonucleotide may contain modified nucleotides having modified bases *{e.g.,* 5-methyl cytosine) and modified sugar groups *{e.g.,* 2'-O-methyl ribosyl, 2'-O-methoxyethyl ribosyl, 2'-fluoro ribosyl, 2'-amino ribosyl, and the like). Oligonucleotides may be naturally-occurring or synthetic molecules of double- and single-stranded DNA and double- and single-stranded RNA with circular, branched or linear shapes and optionally including domains capable of forming secondary structures *{e.g.,* stem-loop, pseudo knots and kissing loop structures).

The term "nucleotide sequence homology" as used herein denotes the presence of homology between two polynucleotides. Polynucleotides have

"homologous" sequences if the sequence of nucleotides in the two sequences is the same when aligned for maximum correspondence. Sequence comparison between two or more polynucleotides is generally performed by comparing portions of the two sequences over a comparison window to identify and compare local regions of sequence similarity. The comparison window is generally from about 20 to 200 contiguous nucleotides. The "percentage of sequence homology" for polynucleotides, such as 50, 60, 70, 80, 90, 95, 98, 99 or 100 percent sequence homology may be determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may include additions or deletions *(i.e.* gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by: (a) determining the number of positions at which the identical nucleic acid base occurs in both sequences to yield the number of matched positions; (b) dividing the number of matched positions by the total number of positions in the window of comparison; and (c) multiplying the result by 100 to yield the percentage of sequence homology. Optimal alignment of sequences for comparison may be conducted by computerized implementations of known algorithms, or by visual inspection. Readily available sequence comparison and multiple sequence alignment algorithms are, respectively, the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.,* 1990; Altschul *et al.,* 1997) and ClustalW programs, both available on the internet. Other suitable programs include, but are not limited to, GAP, BestFit, Plot Similarity, and FASTA in the Wisconsin Genetics Software Package (Genetics Computer Group (GCG), Madison, WI, USA) (Devereux *et al.,* 1984).

As used herein, "substantially complementary" means that two nucleic acid sequences have at least about 65%, preferably about 70%, more preferably about 80%, even more preferably 90%, and most preferably about 98%, sequence complementarity to each other. This means that primers and probes

must exhibit sufficient complementarity to their template and target nucleic acid, respectively, to hybridise under stringent conditions. Therefore, the primer and probe sequences need not reflect the exact complementary sequence of the binding region on the template and degenerate primers can be used. For example, a non-complementary nucleotide fragment may be attached to the 5'-end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer has sufficient complementarity with the sequence of one of the strands to be amplified to hybridize therewith, and to thereby form a duplex structure which can be extended by the polymerizing means. The non-complementary nucleotide sequences of the primers may include restriction enzyme sites. Appending a restriction enzyme site to the end(s) of the target sequence would be particularly helpful for cloning of the target sequence. A substantially complementary primer sequence is one that has sufficient sequence complementarity to the amplification template to result in primer binding and second-strand synthesis. The skilled person is familiar with the requirements of primers to have sufficient sequence complementarity to the amplification template.

The term "hybrid" in the context of nucleic acids refers to a double-stranded nucleic acid molecule, or duplex, formed by hydrogen bonding between complementary nucleotide bases. The terms "hybridise" or "anneal" refer to the process by which single strands of nucleic acid sequences form double-helical segments through hydrogen bonding between complementary bases.

The term "hybrid" in the context of plant breeding refers to a plant that is the offspring of genetically dissimilar parents produced by crossing plants of different lines or breeds or species.

The term "probe" refers to a single-stranded oligonucleotide sequence that will recognize and form a hydrogen-bonded duplex with a complementary sequence in a target nucleic acid sequence analyte or its cDNA derivative.

The term "primer" as used herein refers to an oligonucleotide which is capable of annealing to the amplification target allowing a DNA polymerase to attach, thereby serving as a point of initiation of DNA synthesis when placed under conditions in which synthesis of primer extension product is induced, i.e., in the presence of nucleotides and an agent for polymerization such as DNA polymerase and at a suitable temperature and pH. The (amplification) primer is preferably single stranded for maximum efficiency in amplification. Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the agent for polymerization. The exact lengths of the primers will depend on many factors, including temperature and composition (A/T en G/C content) of primer. A pair of bi-directional primers consists of one forward and one reverse primer as commonly used in the art of DNA amplification such as in PCR amplification.

It will be understood that "primer", as used herein, may refer to more than one primer, particularly in the case where there is some ambiguity in the information regarding the terminal sequence(s) of the target region to be amplified. Hence, a "primer" includes a collection of primer oligonucleotides containing sequences representing the possible variations in the sequence or includes nucleotides which allow a typical base pairing.

The oligonucleotide primers may be prepared by any suitable method. Methods for preparing oligonucleotides of specific sequence are known in the art, and include, for example, cloning and restriction of appropriate sequences, and direct chemical synthesis. Chemical synthesis methods may include, for example, the phospho di- or tri-ester method, the diethylphosphoramidate method and the solid support method disclosed in *e.g.* U.S. Pat. No. 4,458,066. The primers may be labeled, if desired, by incorporating means detectable by

for instance spectroscopic, fluorescence, photochemical, biochemical, immunochemical, or chemical means.

Template-dependent extension of the oligonucleotide primer(s) is catalyzed by a polymerizing agent in the presence of adequate amounts of the four deoxyribonucleotide triphosphates (dATP, dGTP, dCTP and dTTP, i.e. dNTPs) or analogues, in a reaction medium which is comprised of the appropriate salts, metal cations, and pH buffering system. Suitable polymerizing agents are enzymes known to catalyze primer- and template-dependent DNA synthesis. Known DNA polymerases include, for example, *E. coli* DNA polymerase I or its Klenow fragment, T4 DNA polymerase, and Taq DNA polymerase. The reaction conditions for catalyzing DNA synthesis with these DNA polymerases are known in the art.

The products of the synthesis are duplex molecules consisting of the template strands and the primer extension strands, which include the target sequence. These products, in turn, serve as template for another round of replication. In the second round of replication, the primer extension strand of the first cycle is annealed with its complementary primer; synthesis yields a "short" product which is bound on both the 5'- and the 3'-ends by primer sequences or their complements. Repeated cycles of denaturation, primer annealing, and extension result in the exponential accumulation of the target region defined by the primers. Sufficient cycles are run to achieve the desired amount of polynucleotide containing the target region of nucleic acid. The desired amount may vary, and is determined by the function which the product polynucleotide is to serve.

The PCR method is well described in handbooks and known to the skilled person.

After amplification by PCR, the target polynucleotides may be detected by hybridization with a probe polynucleotide which forms a stable hybrid with that of the target sequence under stringent to moderately stringent hybridization and wash conditions. If it is expected that the probes will be

essentially completely complementary (i.e., about 99% or greater) to the target sequence, stringent conditions will be used. If some mismatching is expected, for example if variant strains are expected with the result that the probe will not be completely complementary, the stringency of hybridization may be lessened. However, conditions are chosen which rule out nonspecific/adventitious binding. Conditions which affect hybridization, and which select against nonspecific binding are known in the art, and are described in, for example, Sambrook *et al.,* (2001). Generally, lower salt concentration and higher temperature increase the stringency of binding. For example, it is usually considered that stringent conditions are incubations in solutions which contain approximately 0.IxSSC, 0.1% SDS, at about 65°C incubation/wash temperature, and moderately stringent conditions are incubations in solutions which contain approximately l-2xSSC, 0.1% SDS and about 50°-65°C incubation/wash temperature. Low stringency conditions are 2xSSC and about 30°-50°C.

The terms "stringency" or "stringent hybridization conditions" refer to hybridization conditions that affect the stability of hybrids, *e.g.,* temperature, salt concentration, pH, formamide concentration and the like. These conditions are empirically optimised to maximize specific binding and minimize non-specific binding of primer or probe to its target nucleic acid sequence. The terms as used include reference to conditions under which a probe or primer will hybridize to its target sequence, to a detectably greater degree than other sequences *{e.g.* at least 2-fold over background). Stringent conditions are sequence dependent and will be different in different circumstances. Longer sequences hybridise specifically at higher temperatures. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridises to a perfectly matched probe or primer. Typically, stringent conditions will be those in which the salt

concentration is less than about 1.0 M Na^+ ion, typically about 0.01 to 1.0 M Na^+ ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes or primers *(e.g.* 10 to 50 nucleotides) and at least about 60°C for long probes or primers *(e.g.* greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringent conditions or "conditions of reduced stringency" include hybridization with a buffer solution of 30% formamide, 1 M NaCl, 1% SDS at 37°C and a wash in 2xSSC at 40°C. Exemplary high stringency conditions include hybridization in 50% formamide, IM NaCl, 1% SDS at 37°C, and a wash in O.lxSSC at 60°C. Hybridization procedures are well known in the art and are described by *e.g.* Ausubel *et al.,* 1998 and Sambrook *et al,* 2001.

The term "antigen" refers to a substance capable of triggering an immune response in a vertebrate, resulting in production of an antibody as part of the defence against said substance. Antigens can be virus proteins that can provoke the antibody production in for instance blood cells, cells of lymph nodes, and spleen of vertebrates.

The term "antibody" includes reference to antigen binding peptides and refers to antibodies, monoclonal antibodies, to an entire immunoglobulin or antibody or any functional fragment of an immunoglobulin molecule. Examples of such peptides include complete antibody molecules, antibody fragments, such as Fab, F(ab')2, complementarity determining regions (CDRs), VL (light chain variable region), VH (heavy chain variable region), and any combination of those or any other functional portion of an antibody peptide. The term "antibody" refers to a polypeptide substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof which specifically bind and recognize an analyte (antigen). However, while various antibody fragments can be defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized *de novo* either chemically or by utilizing recombinant DNA methodology. Thus, the term

antibody, as used herein, also includes antibody fragments such as single chain Fv, chimeric antibodies *(i.e.,* comprising constant and variable regions from different species), humanized antibodies *(i.e.,* comprising a complementarity determining region (CDR) from a non-human source) and heteroconjugate antibodies *(e.g.,* bispecific antibodies).

The terms "substantially pure" and "isolated", are used interchangeably and describe a protein, peptide or nucleic acid which is substantially separated from other (sub)cellular components which naturally accompany it. The term embraces a nucleic acid or protein which has been removed from its naturally occurring environment, and includes recombinant or cloned DNA isolates and chemically synthesized analogues or analogues biologically synthesized by heterologous systems. Generally, the term refers to a purified protein and nucleic acid having a purity of at least about 75%, for example 85%, 95% or 98% by weight. Minor variants or chemical modifications typically share the same polypeptide or nucleotide sequence. A substantially pure protein or nucleic acid will typically comprise about 85 to 100% (w/w) of a protein or nucleic acid sample, more usually about 95%, and preferably will be over about 99% pure. Protein or nucleic acid purity or homogeneity can be indicated by a number of means well known in the art, such as polyacrylamide gel electrophoresis of a protein sample, followed by visualizing a single polypeptide band on a polyacrylamide gel upon staining, or by agarose gel electrophoresis of a nucleic acid sample, followed by visualizing a single polynucleotide band on an agarose gel upon staining. "Staining" may either refer to the use of a-specific peptide or nucleic acid stains such as silver and Coomassie stains, or ethidium bromide and SYBR® stains, or may refer to the use of specific peptide or nucleic acid stains such as contacting the peptide with an antibody and visualizing the antibody using a labeled secondary antibody *(e.g.* conjugated to alkaline phosphatase) in the case of proteins or peptides, or contacting the nucleic acid with a complementary probe labelled for visualizing the presence of hybridization between the nucleic acid and the

probe. For certain purposes higher resolution can be provided by using high performance liquid chromatography (HPLC) or a similar means for purification. Such methods are in the area of common general knowledge (see *e.g.* Katz, *et al.,* 1998)

Identification and taxonomy.

The invention provides an isolated virus comprising at least one nucleic acid sequence selected from SEQ ID NO:1, 2 and 3 and sequences having a nucleotide sequence homology of at least 60%, 65%, 70%, 75%, 80%, 85%, 90% or preferably 95% thereto. As stated above, polynucleotides have "homologous" sequences if the sequence of nucleotides in the two sequences is the same when aligned for maximum correspondence. BLAST searches using nucleotide sequences obtained from the ToChV virus isolates as disclosed herein revealed significant homologies with viral sequences of both ToTV and ToMarV.

The ToChV virus isolates share virion characteristics and genome organization with ToTV-EOl, but based on levels of nucleotide and amino acid sequence identities the two viruses are related but distinct. Also, ToChV is related but distinct from ToMarV.

Tomato chocolate virus (ToChV) is a new picorna-like plant virus, related to, but distinct from, tomato torrado virus (ToTV) and Tomato Marchitez virus (ToMarV). Based on standard virological criteria, all three viruses appear to belong to the same genus. ToTV is the tentative new type species of a novel genus of plant viruses: *Torradovirus.*

It should be understood that homologies may be large when two sequences are compared over a small comparison window since local regions of sequence similarity can often be found when two long nucleotide sequences are compared. However, the skilled person is aware that sequence homology requires the establishment of common motifs between the sequences, among which the sequence identity may locally be as high as 35 to 100 %, but may be as low as 10-20% in other parts of the genomic sequence of the same ORF.

Thus, when reference is made herein that a sequence has a nucleotide sequence homology of at least 50% to SEQ ID NO:1, 2 or 3, this may refer to the sequence homology between regions amongst common motifs, where homology is greatest, but also between the coding sequence of two homologous proteins. Note that sequence homologies may differ between the various genes in the genome (see Examples).

As an indication of the relatedness between the newly identified ToChV virus isolates and other viruses (including viruses to be compared therewith) a phylogenetic analysis may normally be performed based on (part of the) the genomic sequence information of the viruses.

Several such analyses are presented in the Examples below. The information obtained thus far indicates that ToChV shows the highest level of homology with ToMarV. Data on vector transmission suggest that ToChV might be transmitted by whiteflies while the natural vectors of CRLV and Sadwaviruses are unknown. On the basis of the presently available information, including sequence information as well as additional taxonomical information as presented in Table 1, below, the newly found virus is a novel species within the *Torradovirus* genus.

Tabel 1: Taxonomic descriptors for the newly discovered ToChV virus (as listed by the Internationally accepted methods from the handbook *"Matthew's Plant Virology" ('Matthew's Plant Virology"*, Fourth Edition, Roger Hull (ed.) Academic Press, San Diego, p 15, Table 2.1 therein) (The numbering of the Table in Matthews' is adhered to in Table 1 below).

I Virion Properties
A. Morphological properties of virions
1. 28 - 34 nm in size
2. Spherical (icosahedral) in shape
3. Envelop absent
B. Physical properties of the virions

(not investigated)
C. Properties of the genome
1. RNA type nucleic acid
2. Single stranded RNA
3. Linear RNA
4. Positive sense coding
5. At least 2 segments (RNA-1: SEQ ID NO:1 and RNA-2: SEQ ID NO:2 or 3)
6. 6 kb and 8 kb
7. 5' terminal cap unknown
8. 5' terminal covalently linked polypeptide unknown
9. Poly(A) tract present at both segments
10. Nucleotide sequence comparisons:
D. Properties of the proteins
1. 3 x CP; Hel; RdRp; Protease; putative MP
2. CPs: 35, 26 and 24 kDa
3. (For functional activities, see above and Examples below)
4. (For amino acid sequence comparison see Examples below)
II. Genomic organization and replication
1. Genomic organization
6. Cytopathology: At least the epidermis of mesophyll cells or accompanying cells of phloem
III. Antigenic properties.
1. No known serological relationships
IV. Biological properties
1a. Natural host range: Tomato;
1b. Experimental host range:

Tested alternative host plants for ToChV	**Local symptoms / systemic symptoms**
Chenopodium quinoa	no symptoms
Nicotiana benthamiana	systemic chlorosis and malformation
Nicotiana clevelandii	Systemic chlorosis
Nicotiana glutinosa	systemic vein clearing and chlorosis
Nicotiana hesperis 67A	Local chlorotic or necrotic lesions. Systemic vein clearing, chlorosis and malformation
Nicotiana occidentalis 37B	systemic vein clearing and chlorosis
Nicotiana occidentalis P1	Local chlorotic or necrotic lesions. Systemic veinclearing and chlorosis

Nicotiana tabacum 'White Burley'	Systemic veinclearing and chlorosis
Nicotiana tabacum Xanthii	systemic chlorosis
Physalis floridana	systemic mosaic

2. Pathogenicity

Symptoms on natural host plant: Necrotic lesions finalizing in burn-like, full necrosis of plant material and death of the plant; concentric rings of necrotic spots on fruits.

Association with disease: Chocolate spot

3. Tissue trophism: At least the epidermis of mesophyll cells or accompanying cells of phloem

4. Mode of transmission in nature unknown

5. Vector relationships: Presumably white fly

6. Geographical distribution: Mediterranean; America's (Central America, Mexico and Southern part of North America)

It was recently postulated that genomic sequences obtained by PCR-amplification from diseased tomato plants from Mexico and Guatemala, which disease is locally known by the names of "Chocolate spot disease", "Chocolate" or "Marchitez" were identical to those of the herein described ToTV from Spain (see PCT/NL2008/050076). However, upon re-examination, the tentative causal agent associated with this "Marchitez" from Mexico and "Torrado" from Spain are distinct from the presently isolated ToChV from Guatemala. Koch's postulates for Chocolate spot disease were fulfilled using the presently isolated strains ToChV-GOl and ToChV-G02.

As more viral sequences become available, both from non-torradoviruses that may or may not be closely related thereto, or from viruses closely related to or essentially resembling the torradovirus genus, phylogenetic analysis will prove a valuable way of determining the breadth of the taxon or clade of the torradovirus genus based on phylogenetic relatedness between isolates.

Phylogenetic relatedness may for instance be determined based on any one or all of the nucleotide sequences of RNA 1 and/or RNA 2 of the viral genome or on capsid protein (gene) sequence data. Phylogenetic analyses are well known to the skilled person and may for instance comprise analysis by distance-based tree-reconstruction *{e.g.* neighbor joining), maximum likelihood or parsimony analysis methods by using such programs as ClustalX

(Thompson et al., 1997), PAUP (Swofford, 2000) or PHYLIP (Felsenstein, 1989).

In order to perform an analysis of phylogenetic relatedness between a novel isolate, the sequences of ToChV as provided herein, and reference sequences from viral strains from, for instance, GenBank, EMBL, or DDBJ databases, genomic RNA from said novel isolate may be extracted directly from infected plants and genomic sequences may be amplified therefrom. Reverse transcription with PCR amplification methods (RT-PCR) may for instance be conducted using degenerate oligonucleotide primers, such primers being for instance capable of acting as amplification primer for amplification of nucleic acid sequences from the genomes of divergent ToChV isolates as well as from the genomes of closely related viral species. Preferably, but not necessarily full-length genomic amplification products may thus be obtained from reference strains *(e.g.* divergent isolates), test-strains (ToChV-suspected isolates) and closely related species. Preferably, specific genetic regions of interest are amplified for comparison. The amplification products (DNA) may then be sequenced by for instance direct double-stranded nucleotide sequencing using fluorescently labeled dideoxynucleotide terminators (Smith et al., 1986) with the degenerate oligonucleotide primers used for RT-PCR. Nucleotide sequence editing, analysis, optional prediction of amino acid sequences, and alignments may be conducted with software packages available, such as with the LaserGene sequence analysis package version 5 (DNASTAR, Inc., Madison, Wis.) and IntelliGenetics GeneWorks version 2.5.1 (IntelliGenetics, Mountain View, Calif.) software. Phylogenetic analyses may then be completed with phylogenetic analysis using for instance parsimony (PAUP) software with a neighbor-joining algorithm using absolute distances following a heuristic search and 1,000 bootstrap replicates, and a phylogenetic tree may be generated by parsimony analysis of the aligned contiguous nucleotide or amino acid sequences, whereby in such trees the numbers generally represent bootstrap confidence levels. Following 1,000 replications, a

confidence level of above 60%, preferably above 70%, more preferably above 80%, 90%, 95% or 98%, within a phylogenetic tree, are to be considered sufficient proof of correct phylogenetic inference (placement of isolates in a certain clade), provided that the tree is sufficiently branched whereby optionally branching may be improved by rooting to a suitably out-group species. In this way it can be determined which isolates are most closely related to the ToChV sequences as provided herein. Relatedness is generally expressed in terms of percentage sequence similarity, herein termed sequence homology.

Although phylogenetic analyses provide a convenient method of identifying a virus in case of sufficient nucleic acid homology, with known viruses several other possibly more straightforward albeit somewhat more coarse methods for identifying said virus or viral proteins or nucleic acids from said virus are herein also provided. As a rule of thumb, a ToChV virus can be identified by the percentages of homology of the viral proteins or nucleic acids to be identified in comparison with viral proteins or nucleic acids identified herein by sequence. It is generally known that virus species, especially RNA virus species, often constitute a quasi species wherein a cluster of said viruses displays heterogeneity among its members. Thus it is expected that each isolate may have a somewhat different percentage homology with the sequences of the isolate as provided herein. Therefore, other viral isolates that exhibit sufficient sequence homology to ToChV (e.g. more than 70%, 80%, 90%, 95%, 98%, or 99% sequence homology) are considered to belong to the same viral species. The ToChV virus of the present invention is therefore a virus having at least 70 or 75% homology, preferably at least 80%, more preferably at least 85%, still more preferably at least 90%, still more preferably at least 95%, still more preferably at least 98%, and most preferably at least 99% homology to the protein or nucleic acid sequences provided herein and causes ToChV-induced disease-symptoms in *Solanaceae*, more particularly in *Solarium* and *Nicotiana*, which disease-symptoms may or may not be similar to

those described herein (in particular in Table 1). In *Cucurbitaceae* the virus may not cause visible symptoms although the virus may be capable of propagation in the plants.

When one wishes to compare a separate virus isolate with the protein or nucleic acid sequences described herein, the invention provides an isolated virus (ToChV) identifiable as phylogenetically corresponding to ToChV by determining a protein or nucleic acid sequence of said separate virus isolate and determining that said protein or nucleic acid sequence has a percentage sequence homology of at least 70%, preferably at least 75%, more preferably at least 80%, still more preferably at least 90%, still more preferably at least 95%, still more preferably at least 98%, and most preferably at least 99% to the sequences as listed herein.

Viral isolates having individual proteins or nucleic acids with higher homology than these mentioned maximum values are considered as phylogenetically corresponding and thus taxonomically corresponding to ToChV virus, and generally the proteins will be encoded by a nucleic acid sequence structurally corresponding with a sequence listed herein. Herewith the invention provides a virus phylogenetically corresponding to the isolated virus of which the sequences are listed herein.

It should be noted that, similar to other viruses, a certain degree of variation can be expected to be found between ToChV-viruses isolated from different sources.

Also, the nucleotide or amino acid sequence of the ToChV virus or fragments thereof as provided herein for example show less than 95%, preferably less than 90%, more preferably less than 80%, more preferably less than 70% and most preferably less than 60% nucleotide sequence homology, or less than 95%, preferably less than 90%, more preferably less than 80%, more preferably less than 70% and most preferably less than 60% amino acid sequence homology with the respective nucleotide or amino acid sequence of any non-ToChV virus or closest relative such as a torradovirus.

Sequence divergence of ToChV strains around the world may be somewhat higher, in analogy with other plant viruses.

The invention provides an isolated virus (ToChV) identifiable as phylogenetically corresponding thereto by determining a nucleic acid sequence of a suitable fragment of the genome of said virus and testing it in phylogenetic analyses wherein maximum likelihood trees are generated using for instance 1000 bootstraps as described above and finding it to be phylogenetically more closely related to a virus isolate comprising the sequences of SEQ ID NO:1, 2 or 3 as listed herein for ToChV than it is related to a virus isolate of a non-ToChV reference strain or closest relative.

Suitable nucleic acid genome fragments each useful for such phylogenetic analyses are for example any portion of nucleic acid sequences of the 6 kb or 8 kb RNA fragments (respectively termed herein RNA2 and RNAl) as described in the Example.

With the provision of the sequence information of this ToChV virus, the invention provides diagnostic means and methods to be employed in the detection of ToChV virus in a sample. Preferably, the detection of ToChV virus is performed with reagents that are most specific for ToChV virus. This by no means however excludes the possibility that less specific but sufficiently cross-reactive reagents are used instead, for example because they are more easily available and sufficiently address the task at hand.

The invention for example provides a method for detecting the presence of ToChV in plants, preferably in tomato plants, more preferably in plants of *S. lycopersicum.* Said method may for instance comprise determining in said sample the presence of a ToChV virus or component thereof by reacting said sample with a ToChV-specific nucleic acid or antibody according to the invention. Although contact-infection of an indicator plant is also a suitable method for detecting the presence of virus in a test plant.

The invention provides the partial nucleotide sequence of a novel isolated virus (herein also called ToChV virus) and ToChV virus-specific

components or synthetic analogues thereof. Additional genomic sequences of the ToChV virus to those provided herein may be determined by sequencing methods known to the skilled person. Genomic sequence determination is well within reach of the skilled person now that the present invention provides the ToChV virus, as well as partial genomic sequences thereof. These methods comprise for instance those described in the Example below. In general such sequencing methods include the isolation of the viral genome nucleic acids by nucleic acid isolation procedures, and the determination of the nucleotide sequence of the isolated nucleic acid, for instance by dideoxy chain termination methods (Sanger *et al.*, 1977) optionally preceded by reverse transcription of RNA into DNA.

The invention provides among others an isolated or recombinant nucleic acid or virus-specific functional fragment thereof obtainable from a virus according to the invention. The isolated or recombinant nucleic acids comprise the sequences as listed herein or sequences of homologues, which are able to hybridise with those under stringent conditions. In particular, the invention provides primers and/or probes suitable for identifying a ToChV virus nucleic acid. Additional probes and primers capable of hybridising to a nucleic acid sequence of the ToChV virus may be developed by methods known to the skilled person.

Expression vectors and expression of viral encoding genes.

Furthermore, the invention relates to an expression vector comprising a nucleic acid according to the invention. To begin with, expression vectors such as plasmid vectors containing (parts of) a double stranded sequence of the ToChV viral genome, viral vectors containing (parts of) the genome of ToChV (for example, but not limited to vaccinia virus, retroviruses, baculovirus), or ToChV virus containing (parts of) the genome of other viruses or other pathogens are part of the present invention.

The expression vector may comprise a ToChV genomic sequence or part thereof that is under control of or operatively linked to a regulatory element, such as a promoter. The segment of DNA referred to as the promoter is responsible for the regulation of the transcription of DNA into mRNA. The expression vector may comprise one or more promoters suitable for the expression of the gene, preferably for the expression of viral protein-encoding gene, in plant cells, fungal cells, bacterial cells, yeast cells, insect cells or other eukaryotic cells. Expression vectors of the invention are very useful to provide antigens of the virus in gene expression systems.

Also, the invention pertains to a host cell comprising a nucleic acid or an expression vector according to the invention. Plasmid or viral vectors containing the nucleic acids encoding protein components of ToChV virus may be generated in prokaryotic cells for the expression of the components in relevant cell types (plant cells, fungal cells, bacteria, insect cells, plant cells or other eukaryotic cells). Plasmid or viral vectors containing full-length or partial copies of the ToChV virus genome may be generated in prokaryotic cells for the expression of viral nucleic acids *in vitro* or *in vivo.*

Methods for isolation and purification of ToChV.

The ToChV virus may be isolated from infected plants or other sources by any method available. Isolation may comprise purifying or partially purifying ToChV viral particles from a suitable source. A wide range of methods is available for virus isolation and purification (for instance, see Dijkstra and De Jager, 1998). Purification of ToChV may for instance be performed by using standard procedures for *e.g.* nepoviruses or luteoviruses (with the aid of organic solvents) (see *e.g.* Walker, 2004). Although such protocols may result in loss of infectivity of the virus, these procedures may still be useful to obtain virus material for other purposes.

Preferably, in order to maintain viral integrity, a mild purification method is used to purify ToChV. Such a mild purification method may for

instance comprise the procedure as exemplified in the Example below.

Infectivity of ToChV after purification may be checked by inoculation onto a sensitive plant.

Methods for purification of ToChV associated proteins and amino acid sequencing.

Having prepared purified or partially purified ToChV virus it is possible to prepare a substantially pure preparation of a viral-associated protein (e.g. virus-encoded proteins). Although numerous methods and strategies for protein purification are known in the art it will be most convenient to purify ToChV viral proteins, such as viral coat proteins, by either electrophoresis using for instance a sodium dodecylsulphate-polyacrylamide gel (SDS-PAGE) or by affinity chromatography. Each of these methods will be described below.

Proteins of interest of ToChV may be separated by electrophoresis using for instance Tricine-SDS-PAGE (Schagger and Von Jagow, 1987) or Glycine-SDS-PAGE (Laemmli, 1970). Other electrophoresis systems that are capable of resolving the various proteins comprised in the virus isolate, or transcribed from its genome and expressed in a suitable expression system, may of course also be employed, such as non-denaturing gel electrophoresis. The area of the PAGE gel including the target protein may be excised and the target polypeptides may be eluted therefrom, for instance by using an Elutrap® device (Schleicher & Schuell, Dassel, Germany). A target protein may be identified by its mobility relative to reference polypeptides in a gel. To increase purity the eluted protein may be run on a second SDS-PAGE gel and eluted a second time. The protein or peptide contained in the excised gel fragment may then be eluted again and is suitable for use in immunization or in protein sequencing.

Proteins of interest of ToChV may also be purified by affinity chromatography using an antibody (such as a monoclonal antibody) that specifically binds to a ToChV protein. The antibody may be covalently coupled

to solid supports such as celluloses, polystyrene, polyacrylamide, cross-linked dextran, beaded agarose or controlled pore glass using bifunctional coupling agents that react with functional groups on the support and functional groups *(i.e.,* reactive amino acid side chains) on the antibody molecule. Such methods are readily available to the skilled person. The resulting antibody-bearing solid phase is contacted with purified or partially purified virus under reducing conditions using pH, ionic strength, temperature and residence times that permit the protein of interest to bind to the immobilized antibody. The virus or protein is eluted from the column by passing an eluent that dissociates hydrogen bonds through the bed. Buffers at specific pH or NaCl solutions above about 2 M are commonly used eluents.

Methods for carrying out affinity chromatography using antibodies as well as other methods for immunoaffinity purification of proteins (such as viral capsid proteins) are well known in the art (see *e.g.,* Harlow and Lane, 1988).

With the teachings provided herein, the skilled person is capable of isolating a virus-specific protein of ToChV, determining the amino acid sequence of for instance the N-terminal part of said protein, designing a set of degenerate probes (for the degeneracy of the genetic code) to hybridise with the DNA coding for a region of said protein, using these probes on an array of genes in a genomic library produced from the virus, obtaining positive hybridisations and locating the corresponding genes. The skilled person is then capable of identifying the structural region of the gene and optionally upstream and downstream sequences thereof. Thereafter the skilled person is capable of establishing the correct sequence of the amino acid residues that form the protein.

Antibody production

Antibodies, either monoclonal or polyclonal, can be generated to a purified or partially purified protein or peptide fragment of the ToChV virus in a variety of ways known to those skilled in the art including injection of the

protein as an antigen in animals, by hybridoma fusion, and by recombinant methods involving bacteria or phage systems (see Marks *et al.,* 1992a; Marks *et al.,* 1992b; Lowman *et al.,* 1991; Lerner *et al.,* 1992, each of which reference discloses suitable methods).

Antibodies against viral particles, proteins or peptides of the virus may be produced by immunizing an appropriate vertebrate, preferably mammalian host, *e.g.,* rabbits, goats, rats, chicken and mice with the particles, proteins or peptides alone or in conjunction with an adjuvant. Usually two or more immunizations will be involved, and the blood or spleen will be harvested a few days after the last injection. For polyclonal antisera, the immunoglobulins may be precipitated, isolated and (affinity) purified. For monoclonal antibodies, the splenocytes will normally be fused with an immortalized lymphocyte, *e.g.,* a myeloid line, under selective conditions for hybridomas. The hybridomas may then be cloned under limiting dilution conditions and their supernatants screened for antibodies having the desired specificity. Techniques for producing (monoclonal) antibodies and methods for their preparation and use in various procedures are well known in the literature (see *e.g.* U.S. Pat. Nos. 4,381,292, 4,451,570, and 4,618,577; Harlow and Lane, 1988; Ausubel, *et al.,* 1998; Rose *et al.,* 1997; Coligan *et al.,* 1997). Typically, an antibody directed against a virus-associated protein will have a binding affinity of at least $IxIO^5$-$IxIO^7$ M^{-1}.

A recombinant protein derived from the ToChV virus, such as may be obtained by expressing a protein-encoding genomic sequence of the virus in a suitable expression system, is preferred as the antigen. However, purified proteins may also be used. Antigens suitable for antibody detection include any ToChV protein that combines with any ToChV-specific antibody of a mammal exposed to or infected with ToChV virus. Preferred antigens of the invention include those that bring about the immune response in mammals exposed to ToChV, which therefore, typically are recognised most readily by antibodies of

a mammal. Particularly preferred antigens include the capsid proteins of ToChV. Structural proteins from purified virus are the most preferred.

Methods for cloning genomic sequences, for manipulating the genomic sequences to and from expression vectors, and for expressing the protein encoded by the genomic sequence in a heterologous host are well-known, and these techniques can be used to provide the expression vectors, host cells, and the cloned genomic sequences encoding antigens, which sequences are to be expressed in a host to produce antibodies for use in diagnostic assays (see for instance Sambrook *et al.,* 2001 and Ausubel, *et al.,* 1998).

A variety of expression systems may be used to produce ToChV antigens. For instance, a variety of expression vectors suitable to produce proteins in *E. coli, B. subtilis,* yeast, insect cells, plant cells and mammalian cells have been described, any of which might be used to produce a ToChV antigen suitable to produce an anti-ToChV antibody or fragment thereof. Of course ToChV itself may also be used as an expression vector for this purpose.

One use of antibodies of the invention is to screen cDNA expression libraries for identifying clones containing cDNA inserts that encode proteins of interest or structurally-related, immuno-cross-reactive proteins. Such screening of cDNA expression libraries is well known in the art (see *e.g.* Young and Davis, 1983), to which reference is made in this context, as well as other published sources. Another use of these antibodies is for use in affinity chromatography for purification of ToChV proteins. These antibodies are also useful for assaying for ToChV infection.

The present invention thus provides a ToChV-specific viral protein or a fragment thereof hereinafter termed proteinaceous molecule. Useful proteinaceous molecules are for example derived from any of the genomic sequences or fragments thereof derivable from a virus according to the invention. Such proteinaceous molecules, or antigenic fragments thereof, as provided herein, are for example useful in diagnostic methods or kits and in diagnostic compositions. Particularly useful are those proteinaceous molecules

that are encoded by recombinant nucleic acid fragments that are identified for eliciting ToChV virus specific antibodies, whether *in vivo (e.g.* for providing diagnostic antibodies) or *in vitro (e.g.* by phage display technology or another technique useful for generating synthetic antibodies or parts thereof).

Also provided herein are antibodies, be it natural polyclonal or monoclonal, or synthetic antibodies *(e.g.* (phage) library-derived binding molecules) that specifically react with an antigen comprising a proteinaceous molecule or ToChV virus-specific functional fragment thereof, such as a capsid protein according to the invention.

Methods for identifying a viral isolate as a ToChV virus

Apart from the detection of ToChV virus, which involves diagnostic methods, the present invention also relates to methods for identification, i.e. confirmation that the isolate is ToChV. Such methods may be based on phylogenetic inference as described above, and determining the level of nucleotide or amino acid sequence homology between an unidentified viral isolate and one or more reference strains of confirmed ToChV virus and non-ToChV virus. Such methods may for instance comprise sequencing (part of) the genome of a viral isolate or of a capsid protein and comparing the level of homology of that sequence to the sequences as provided herein for ToChV. An isolate having more than 70% sequence homology with SEQ ID NO:1, 2, or 3 as provided herein is considered taxonomically corresponding to ToChV or belonging to the ToChV viral taxon. Such a virus is part of the present invention.

In order to identify a virus as a Tomato chocolate virus (ToChV), one may also make use of the taxonomic descriptors as presented in Table 1 above, and find by comparison that a new isolate belongs to the presumptive novel species, of which the ToChV strain identified by the nucleic acid sequences of SEQ ID NO: 1 and 2 provided herein, and deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH on 8 December 2008

under depositors reference number ToChV-GOl (DSM 22139) may be assigned as the type strain. Thus, it is not essential that a sequence comparison is performed in order to assess that a virus is ToChV. Rather, a method of identifying a virus as a Tomato chocolate virus (ToChV) may comprise the steps of assessing the presence of the combination of taxonomic descriptors selected from the group consisting of

a) morphological descriptors, such as spherical, non-enveloped virion particles of approximately 28 - 34 nm in diameter;

b) genome properties descriptors, such as having single stranded linear positive sense RNA virus properties based on two RNA segments that comprise poly(A)-tails, that encode polyproteins of 6 and 8 kDa, respectively, and that comprise coding regions or motifs for 3 capsid proteins, helicase, protease, RdRP and putative movement protein and which RNA segments and/or polyproteins and/or motifs have homologies based on sequence comparison essentially as described herein, and

c) biological properties descriptors, such as producing the necrotic lesions and burn-like symptoms in tomato, having a host range, vector relationship and/or geographical distribution essentially as described herein, and being associated with the diseases of tomato plants locally known under such names as chocolate spot.

The combination of taxonomic descriptors that results in a positive identification of a virus isolate as being a Tomato chocolate virus (ToChV) is that combination which shows the isolate as being more closely related (based on numerically taxonomic methods well known to the skilled artisan) to ToChV as described herein, than to other viruses, and wherein said isolate preferably produces the disease symptoms in tomato typical of chocolate as described herein.

Thus, a virus which, based on numerical taxonomic analysis of taxonomic descriptors essentially as defined in Table 1, shows to be more closely related to the virus as defined in claim 1 herein than to any other virus

known at the time of filing of the present application, and which virus is associated with a disease that causes necrotic lesions in tomato, is considered herein to be a ToChV and falls within the scope of the present invention.

In this way the invention provides a viral isolate identifiable with a method according to the invention as a plant virus taxonomically corresponding to a virus identifiable as likely belonging to the ToChV viral taxon. Depending upon the phylogenetic relatedness, or distinctness with other viral taxa, the ToChV viral taxon may be an isolate, a species, a genus or even a family of virus.

Alternatively, methods for identifying a viral isolate as a ToChV virus may be based on symptomatology, i.e. recognition of the virus by its disease-symptoms.

However, in a preferred embodiment, antibodies of the invention are used in a method for identifying a viral isolate as a ToChV virus, provided cross-reactivity of such antibodies with related non-ToChV strains has been effectively ruled out. Such methods comprise the step of reacting said viral isolate or a component thereof with an antibody as provided herein. Reacting is herein referred to as allowing the occurrence of antibody-antigen bonding. This can for example be achieved by using purified or non-purified ToChV virus or parts thereof (proteins, peptides). Preferably, infected cells or cell cultures are used to identify viral antigens using any suitable immunological method. Specifically useful in this respect are antibodies raised against ToChV viral capsid proteins.

Other preferred methods for identifying a viral isolate as a ToChV virus comprise reacting said viral isolate or a component thereof with a virus specific polynucleotide according to the invention, which polynucleotide is capable of hybridizing under stringent conditions to a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, 2, and 3, sequences having a nucleotide sequence homology of at least 60% to SEQ ID NO:1, 2, and 3, and their complementary strands and ToChV-specific fragments thereof. Such a

hybridization reaction may be performed in any format available to the skilled person and will generally involve tissue printing, dot blot methods, Southern/Northern blotting or hybridization, in situ hybridization, PCR, RT-PCR and the like.

Immunological detection methods.

Methods of the invention in which antigens are detected can in principle be performed by using any immunological method, such as for instance classical immunofluorescence (IF), immunohistochemical techniques or comparable antigen detection assay formats. Preferred ToChV detection methods based on detection of the viral coat protein may for instance comprise such methods as precipitation and agglutination tests, radio-immunoassay (RIA), immunogold labeling, immunosorbent electron microscopy (ISEM), enzyme-linked immunosorbent assay (ELISA), Western blotting and immunoblotting. Examples of types of immunoassays that can utilize antibodies of the invention are competitive and non-competitive immunoassays in either a direct or indirect format. Antibodies can be utilized in immunoassays in the liquid phase or bound to a solid phase carrier. In addition, the antibodies in these immunoassays can be detectably labeled in various ways. Those skilled in the art will know, or can readily discern, suitable immunoassay formats without undue experimentation. Assay formats are well known in the literature and are described, for example, in Harlow and Lane (1988).

A variety of immunoassay formats may be used to select antibodies specifically reactive with a particular polypeptide according to the invention, such as ToChV viral coat proteins. For example, solid-phase ELISA immunoassays are routinely used for this purpose. See Harlow and Lane (1988), for a description of immunoassay formats and conditions that can be used to determine selective binding.

Antibodies can be bound to many different carriers and used to detect the presence of the target molecules. Alternatively, antigens may be bound to many different carriers and used to detect the presence of the antibody. Examples of well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses and magnetite. The nature of the carrier can be either soluble or insoluble for purposes of the invention. Those skilled in the art will know of other suitable carriers for binding antibodies, or will be able to ascertain such using routine experimentation.

Western blot assays are described generally in Harlow and Lane (1988). According to this method the viral proteins (and other proteins in the virus preparation) are separated by gel electrophoresis and transferred to a solid phase *(i.e.,* a membrane such as nitrocellulose). The immobilized antigen is subsequently reacted with an antibody and detection system *(e.g.,* an alkaline phosphatase-conjugated second antibody). As will be apparent to those of skill, it will be advantageous to include appropriate negative and positive control materials (such as substantially purified antigen or ToChV virus) in the assay.

Enzyme-Linked Immuno-Sorbent Assays (ELISA) are described generally in Harlow and Lane (1988). The assay involves the reaction of a viral component *(e.g.,* a capsid protein) with an antibody. In one embodiment the sample may comprise a plant tissue which is ground and reacted to the antibody that has been coated onto a solid phase such as a test plate. If the virus is present in the sample, an enzyme-labeled specific antibody will bind to the antibody-virus complex, and it will be detected by an enzyme substrate reaction that produces a color reaction. Preferred methods of ELISA analysis are direct double antibody sandwich (DAS) ELISA (Clark and Adams, 1977), DAS indirect ELISA (Vela *et al.* 1986), or TAS-ELISA. It will be apparent to those skilled in the art that in an ELISA or any other type of assay it will sometimes be desirable to determine the presence of more than one viral capsid protein in a single reaction, for example by mixing two or more

antibodies with different specificities and assaying for either or all of the protective capsid-associated proteins of the invention.

Nucleic acid based detection methods

ToChV is composed of at least two ribonucleic acids (RNAs). There are strong indications for the presence of three protective capsid proteins. The methods described above are focused on immunological detection of viral proteins for the detection of the virus. By recombinant DNA technology it is possible to produce probes that directly or indirectly hybridize to the viral RNAs (or their complement), or cDNA produced therefrom by reverse transcription, and which can be used in assays for the detection of the virus. Nucleic acid amplification techniques allow the amplification of fragments of viral nucleic acids, which may be present in very low amounts.

In order to develop nucleic acid-based detection methods, virus-specific sequences must be determined for which primers or probes may then be developed. To detect ToChV by nucleic acid amplification and/or probe hybridization, the capsid protein of ToChV may be sequenced or, alternatively, the viral genomic RNA may be isolated from purified virus, reverse transcribed into cDNA and directly cloned and/or sequenced. Using either the cloned nucleic acid as a hybridization probe, using sequence information derived from the clone, or by designing degenerative primers based on the amino acid sequence of the ToChV protein, nucleic acid hybridization probes and/or nucleic acid amplification primers may be designed an used in a detection assay for detecting the presence of the virus in a sample as defined herein.

Methods of the invention in which nucleic acids are detected can in principle be performed by using any nucleic acid amplification method, such as the Polymerase Chain Reaction (PCR; Mullis and Faloona, 1987; U.S. Pat. No. 4,683,195; 4,683,202; and 4,800,159) or by using amplification reactions such as Ligase Chain Reaction (LCR; Barany, 1991; EP 0 320 308), Self-Sustained Sequence Replication (3SR; Guatelli *et al.*, 1990), Strand Displacement

Amplification (SDA; Walker *et al.*, 1992; U.S. Pat. Nos. 5,270,184 and 5,455,166), Transcriptional Amplification System (TAS; Kwoh *et al*, 1989), Q-Beta Replicase (Lizardi *et al*, 1988), Rolling Circle Amplification (RCA; U.S. Pat. No. 5,871,921), Nucleic Acid Sequence Based Amplification (NASBA; Compton, 1991), Cleavase Fragment Length Polymorphism (U.S. Pat. No. 5,719,028), Isothermal and Chimeric Primer-initiated Amplification of Nucleic Acid (ICAN), Ramification- extension Amplification Method (RAM; U.S. Pat. Nos. 5,719,028 and 5,942,391) or other suitable methods for amplification of nucleic acids.

Since the virus is an RNA virus (i.e. the sequences of Fig. 4 is the DNA equivalent of the viral RNA genome), a suitable detection method may comprise isolating the viral nucleic acids from a sample, for instance from an infected plant, by using methods known *per se* to the skilled person (e.g. Chomczynski and Sacchi, 1987; Boom *et al*, 1990) or commercially available systems (e.g. the RNeasy total RNA isolation kit or RNeasy plant RNA isolation kit from QIAGEN GmbH, Hilden, Germany, or the High-Pure-RNA-Isolation-Kit® (Roche Diagnostics, a division of F. Hoffmann-La Roche Ltd, Basel, Switzerland).

Total RNA may for instance be extracted from leaf material or protoplasts of plant cells and the total RNA, or specifically the viral genomic RNA, or a part thereof, may then be reverse transcribed into cDNA by using for instance an *Avian myeloblastosis virus* (AMV) reverse transcriptase or *Moloney murine leukemia virus* (M-MuLV) reverse transcriptase. A suitable method may for instance include mixing into a suitable aqueous buffering system (e.g. a commercially available RT buffer) a suitable amount of total RNAs (e.g. 1 to 5 μg), a suitable amount (e.g. 10 pmol) of a reverse transcription primer, a suitable amount of dNTPs and the reverse transcriptase, denaturing the nucleic acids by boiling for 1 min, and chilling them on ice, followed by reverse transcription at for instance 45°C for 1 h as

recommended for the specific reverse transcriptase used, to obtain cDNA copies of the viral sequences.

As a reverse transcription primer a polynucleotide according to the present invention may be used, for instance an 18-25-mer oligonucleotide comprising a nucleotide sequence complementary to the ToChV genomic sequence or preferably at least capable of hybridizing under stringent conditions to the nucleic acid sequence of SEQ ID NO:1, 2, and 3, or a ToChV-specific fragment thereof. Alternatively, an a-specific polyT primer (oligo dT primer) may be used in order to start reverse transcription from polyA RNA motifs.

Following the RT-step, the cDNA obtained may be PCR amplified by using for instance Pfu and Taq DNA polymerases and amplification primers specific for the viral genomic cDNA sequences. Also complete commercially available systems may be used for RT-PCR (e.g. the Access and AccessQuick™ RT-PCR Systems of Promega [Madison WI, USA], or the Titan™ One Tube RT-PCR System or two-step RT-PCR systems provided by Roche Diagnostics [a division of F. Hoffmann-La Roche Ltd, Basel, Switzerland]).

In order to amplify a nucleic acid with a small number of mismatches to one or more of the amplification primers, an amplification reaction may be performed under conditions of reduced stringency *(e.g.* a PCR amplification using an annealing temperature of 38°C, or the presence of 3.5 mM MgCk). The person skilled in the art will be able to select conditions of suitable stringency.

The primers herein are selected to be "substantially" complementary *(i.e.* at least 65%, more preferably at least 80% perfectly complementary) to their target regions present on the different strands of each specific sequence to be amplified. It is possible to use primer sequences containing *e.g.* inositol residues or ambiguous bases or even primers that contain one or more mismatches when compared to the target sequence. In general, sequences that exhibit at least 65%, more preferably at least 80% homology with the target

DNA or RNA oligonucleotide sequences, are considered suitable for use in a method of the present invention. Sequence mismatches are also not critical when using low stringency hybridization conditions.

The detection of the amplification products can in principle be accomplished by any suitable method known in the art. The amplified fragments may be directly stained or labelled with radioactive labels, antibodies, luminescent dyes, fluorescent dyes, or enzyme reagents. Direct DNA stains include for example intercalating dyes such as acridine orange, ethidium bromide, ethidium monoazide or Hoechst dyes.

Alternatively, the DNA or RNA fragments may be detected by incorporation of labelled dNTP bases into the synthesized fragments. Detection labels which may be associated with nucleotide bases include *e.g.* fluorescein, cyanine dye, digoxigenin (DIG) or bromodeoxyuridine (BrdUrd).

When using a probe-based detection system, a suitable detection procedure for use in the present invention may for example comprise an enzyme immunoassay (EIA) format (Jacobs *et al.,* 1997). For performing a detection by manner of the EIA procedure, either the forward or the reverse primer used in the amplification reaction may comprise a capturing group, such as a biotin group for immobilization of target DNA PCR amplicons on *e.g.* a streptavidin coated microtiter plate wells or streptavidin coated Dynabeads® (Dynal Biotech, Oslo, Norway) for subsequent EIA detection of target DNA-amplicons. The skilled person will understand that other groups for immobilization of target DNA PCR amplicons in an EIA format may be employed.

Probes useful for the detection of the target nucleic acid sequences as disclosed herein preferably bind only to at least a part of the nucleic acid sequence region as amplified by the nucleic acid amplification procedure. Those of skill in the art can prepare suitable probes for detection based on the nucleotide sequence of the target nucleic acid without undue experimentation as set out herein. Also the complementary nucleotide sequences, whether DNA

or RNA or chemically synthesized analogues, of the target nucleic acid may suitably be used as type-specific detection probes in a method of the invention, provided that such a complementary strand is amplified in the amplification reaction employed.

Suitable detection procedures for use herein may for example comprise immobilization of the amplicons and probing the nucleic acid sequences thereof by *e.g.* Northern and Southern blotting. Other formats may comprise an EIA format as described above. To facilitate the detection of binding, the specific amplicon detection probes may comprise a label moiety such as a fluorophore, a chromophore, an enzyme or a radio-label, so as to facilitate monitoring of binding of the probes to the reaction product of the amplification reaction. Such labels are well known to those skilled in the art and include, for example, fluorescein isothiocyanate (FITC), β-galactosidase, horseradish peroxidase, streptavidin, biotin, digoxigenin, ^{35}S, ^{14}C, ^{32}P or ^{125}I. Other examples will be apparent to those skilled in the art.

Detection may also be performed by a so called reverse line blot (RLB) assay, such as for instance described by Van den Brule *et al.* (2002). For this purpose RLB probes are preferably synthesized with a 5' amino group for subsequent immobilization on *e.g.* carboxyl-coated nylon membranes. The advantage of an RLB format is the ease of the system and its speed, thus allowing for high throughput sample processing.

The use of nucleic acid probes for the detection of RNA or DNA fragments is well known in the art. Mostly these procedures comprise the hybridization of the target nucleic acid with the probe followed by post-hybridization washings. Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For nucleic acid hybrids, the Tm can be approximated from the equation of Meinkoth and Wahl (1984): Tm = 81.5 °C + 16.6 (log M) + 0.41 (% GC)-0.61 (% form)-500/L; where M is the molarity of monovalent cations, % GC is the percentage of guanosine and cytosine

nucleotides in the nucleic acid, %form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The Tm is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. Tm is reduced by about 1 °C for each 1 % of mismatching; thus, the hybridization and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with > 90% identity are sought, the Tm can be decreased 10°C. Generally, stringent conditions are selected to be about 5 °C lower than the thermal melting point (Tm) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1,2,3, or 4 °C lower than the thermal melting point (Tm); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10 °C lower than the thermal melting point (Tm); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20 °C lower than the thermal melting point (Tm). Using the equation, hybridization and wash compositions, and desired Tm, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a Tm of less than 45 °C (aqueous solution) or 32 °C (formamide solution) it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen, 1993; Ausubel *et al*, 1998.

In another aspect, the invention provides oligonucleotide probes for the detection of ToChV RNA or cDNA. The detection probes herein are selected to be "substantially" complementary to a single stranded RNA molecule, or to one of the strands of the double stranded nucleic acids generated by an amplification reaction of the invention. Preferably the probes are substantially complementary to the, optionally immobilized *{e.g.* biotin labelled) antisense strands of the amplicons generated from the target RNA or DNA.

It is allowable for detection probes of the present invention to contain one or more mismatches to their target sequence. In general, sequences that exhibit at least 65%, more preferably at least 80% homology with the target oligonucleotide sequences are considered suitable for use in a method of the present invention.

ToChV resistant plants

The invention further relates to a method for identifying a ToChV-resistant plant, or part thereof. There are various possibilities of identifying ToChV-resistant plants. In a first set of embodiments of such a method, active/infectious virus or full-length infectious clones may be used, whereas in an alternative embodiment only virus-detection means are used.

A first step of a method for identifying a ToChV-resistant plant using active/infectious virus comprises exposing a plant or plant part, such as a leaf or stem segment, to a infective dosage of ToChV, the aim of which is to achieve an infection. The exposure may in many cases involve the establishment of physical contact. An infective dosage may vary between plants and between ToChV-isolates tested. Theoretically, an amount of about 1 to 10 to an amount of about 500-5000 viral particles of said virus or the nucleic acids thereof will be sufficient. Infection in this way may be achieved by mechanical inoculation of purified virus particles or virus nucleic acid on healthy plants.

Alternatively, infection may be achieved by, for instance:

- growing a healthy scion on a ToChV-infected rootstock, or vice versa;
- exposing a healthy plant to transmission vectors containing the virus (including infected plants, *e.g.* parasitic plants like *Cuscuta* spp.);
- introducing into a healthy plant an expression vector harbouring a coding region of the ToChV virus genome;
- the use of agro-infectious clones, such as *Agrobacterium tumefaciens* strains containing an expression vector harbouring a coding region of the ToChV virus genome.

In the context of the present invention, methods for exposing a plant or plant part to an infective dosage of ToChV are not limited to any particular method.

As stated, infection may comprise mechanical inoculation of the virus on healthy plants. For instance, a portion of a diseased leaf may be rubbed directly onto a leaf of a plant that is to be infected. In an alternative procedure, an inoculum may for instance be prepared by grinding virus-containing plant tissue, preferably young leaves showing symptoms, with a mortar and pestle, or any other suitable type of homogeniser, in for instance a buffer suitable for inoculation *(e.g.* a 0.03 M phosphate buffer, pH 7.7). After grinding, the obtained homogenate (the sap) is preferably filtered, *e.g.* through cheese cloth. The sap may then be inoculation, for instance by gently contacting leaves with an amount of the sap. The leaves are preferably pre-treated in order to damage the lower epidermis and enhance entry of the virus. This may for instance be achieved by pre-dusting the leaves with carborundum powder. Excessive wounding is preferably avoided. Preferably a carborundum powder is used having microscopically small angular particles of silicon carbide (400-500 mesh). Carborundum powder may also be added directly to the sap, in which case the pre-treatment is omitted. The sap may, for instance, be applied by the forefinger, a pad of sap-soaked foam or fabric, or even with the pestle used for grinding, a glass spatula, a stiff brush, or a spray gun. After inoculation, the leaves are preferably immediately washed with water.

A second step of a method for identifying a ToChV-resistant plant comprises identifying said plant as a ToChV-resistant plant when, after said exposure, either i) disease-symptoms in said plant or plant part remain absent or are delayed in expression or are at least reduced in severity or are localized relative to a susceptible and/or sensitive control plant, and/or ii) ToChV virus or ToChV genomic sequences are not present in said plant or plant part or the presence of ToChV virus is at least quantitatively reduced in said plant

relative to a susceptible control plant. As used herein the term localized means limited to the inoculated leaf.

Determining the development of ToChV-induced disease-symptoms in infected plants may be performed by quantitative methods, *e.g.* wherein the period required for the development of discernible *{e.g.* visible) disease-symptoms is noted, or by qualitative methods wherein, after a certain period has lapsed, the plant is inspected for symptom expression and the presence or severity of the symptoms is indicated.

In addition to determining the development of ToChV-induced disease-symptoms or as an alternative thereto, depending on the type of ToChV-resistance to be detected, the presence of the virus is detected in the plant or plant part. In order to detect the absence of virus in the test plants, any method may in principle be used. For instance, a method may be employed wherein a ToChV specific antibody, primer-set or probe according to the present invention is used. Alternatively, a portion of the test plant may be brought into contact with a susceptible indicator plant *{e.g. N. hesperis* '67A') to establish whether virus is present or absent in the test plant. The skilled person will understand that for such methods it is important to decontaminate the surface of the test plant, in order to distinguish between a transmission vector, a tolerant test-plant and a resistant test plant, since only the presence of virus in the plant cells needs to be established.

In performing the second step of a method for identifying a ToChV-resistant plant, the following results may be obtained. If, after successful inoculation (e.g. after the establishment of a plant-virus contact under conditions that would result in infection in a susceptible and sensitive control plant):

i) disease-symptoms remain absent; or viral particles, or viral RNA cannot be detected: the plant is resistant;

ii) disease-symptoms are delayed or reduced in severity; or systemic low titres of viral particles or viral RNA can be detected: the plant is partially resistant;

iii) disease-symptoms are severe, but remain local, limited to the inoculated leaf and do not systemically spread beyond inoculated tissue; or viral particles, or viral RNA can only be detected locally: the plant is hypersensitive;

iv) if disease-symptoms remain absent; and viral particles, or viral RNA can be detected: the plant is tolerant.

v) if the plant develops disease-symptoms and has high systemic virus titres, then the plant is susceptible and sensitive. Examples of such plants are the plants from which the virus of the present invention was isolated. These plants may serve as suitable control plants in methods of the present invention.

For the purpose of producing resistant plants, and from a viewpoint of phytosanitation, only outcome i), ii) and iii) may be considered of interest. For the purpose of obtaining plants suitable for the production of symptomless crops and products, outcome iv) may also be of particular commercial interest.

In an alternative embodiment of a method for identifying a ToChV-resistant plant only virus-detection means are used. For instance, a ToChV-resistant plant may be identified in the field by observing or identifying a symptomless plant among symptomatic plants and determining the absence of virus in said plant by performing any of the virus detection methods according to the present invention. In fact, this corresponds to a method for identifying a ToChV-resistant plant wherein step a) of exposing a plant or plant part to a infective dosage of ToChV, is performed passively (e.g. naturally). When such a method is performed it is preferred that a method for detecting the presence of ToChV in a sample according to the present invention is used wherein the presence of a ToChV virus or component thereof is performed by reacting said sample with a polynucleotide or an antibody according to the present

invention. Preferably, a method of identifying a ToChV-resistant plant requires the use of either the virus of the present invention or a polynucleotide or antibody according to the present invention.

The invention further relates to a method of producing a ToChV-resistant plant, or part thereof. Once a ToChV-resistant plant has been identified, this plant may serve as a donor plant of genetic material which is to be transferred from said donor plant to a recipient plant in order to provide said recipient plant with the genetic material. Transfer of genetic material from a donor plant to a recipient plant may occur by any suitable method known in the art. The genetic material will in most cases be genomic material. It is important however, that at least the resistance- conferring parts of the donor plant's genome are transferred. In the absence of methods for determining which parts of the donor plant's genome confer the ToChV resistance, the transfer may suitably occur by transferring complete chromosomes. Preferably, the ToChV-resistant plant serves as a male or female parent plant in a cross for producing resistant offspring plants, the offspring plant thereby receiving genomic material from the resistant donor and acting as the recipient plant. Although a susceptible parent in crosses is *sensu stricto* not necessarily a recipient plant, such a susceptible parent will herein also be included in the term recipient plant.

In a method for producing a ToChV-resistant plant, protoplast fusion can also be used for the transfer of resistance- conferring genomic material from a donor plant to a recipient plant, i.e. as a manner of crossing said plants. Protoplast fusion is an induced or spontaneous union, such as a somatic hybridization, between two or more protoplasts (cells of which the cell walls are removed by enzymatic treatment) to produce a single bi- or multi-nucleate cell. The fused cell, that may even be obtained with plant species that cannot be interbred in nature, is tissue cultured into a hybrid plant exhibiting the desirable combination of traits. More specifically, a first protoplast can be obtained from a tomato plant or other plant line that exhibits resistance to

infection by ToChV. For example, a protoplast from a ToChV-resistant (tomato, eggplant, pepper, melon, watermelon or cucumber) line may be used. A second protoplast can be obtained from a susceptible second plant line, optionally from another plant species or variety, preferably from the same plant species or variety, that comprises commercially desirable characteristics, such as, but not limited to disease resistance, insect resistance, valuable fruit characteristics, etc. The protoplasts are then fused using traditional protoplast fusion procedures, which are known in the art to produce the cross.

Alternatively, embryo rescue may be employed in the transfer of resistance-conferring genomic material from a donor plant to a recipient plant i.e. as a manner of crossing said plants. Embryo rescue can be used as a procedure to isolate embryo's from crosses wherein plants fail to produce viable seed. In this process, the fertilized ovary or immature seed of a plant is tissue cultured to create new plants (this method is described in detail in Pierik, 1999).

A method of producing a ToChV-resistant plant thus comprises in one embodiment the steps of identifying a ToChV-resistant donor plant as described herein above and crossing said ToChV-resistant donor plant with a recipient plant, as described above, thereby producing resistant offspring plants.

A method of producing a ToChV-resistant plant further comprises the step of selecting from offspring plants a resistant plant by performing a method for identifying a ToChV-resistant plant as described earlier.

Preferably, said ToChV-resistant donor plant is a plant of the family *Solanaceae* or *Cucurbitaceae,* even more preferably a tomato plant, an eggplant plant, a pepper plant, melon plant, watermelon plant or a cucumber plant.

Preferably, said recipient plant is a plant of the family *Solanaceae* or *Cucurbitaceae,* even more preferably a tomato plant, an eggplant plant, a pepper plant, a melon plant, a watermelon plant or a cucumber plant. Still

more preferably, said recipient plant is a tomato plant of the species *Solanum lycopersicum,* more preferably an *S. lycopersicum* plant that possess commercially desirable characteristics. The recipient plant may be a ToChV-susceptible plant, a ToChV sensitive plant or a ToChV resistant recipient plant. As explained above, the choice of the plant will primarily be determined by whether the resistance trait is dominant or recessive. The skilled person is aware of the various methodologies available to resolve such issues.

Also an aspect of the present invention is a ToChV-resistant plant, or a part thereof, obtainable by a method of the invention.

As stated, a preferred embodiment of a method for producing a ToChV-resistant plant comprises the transfer by introgression of said resistance-conferring nucleic acid sequence from a ToChV-resistant donor plant into a recipient plant by crossing said plants. Resistant plants developed according to this preferred embodiment can advantageously derive a majority of their traits from the recipient plant, and derive ToChV-resistance from the donor plant.

In one method, which is referred to as pedigree breeding, a donor plant that exhibits resistance to ToChV is crossed with a recipient plant that preferably exhibits commercially desirable characteristics, such as, but not limited to, disease resistance, insect resistance, valuable fruit characteristics, etc. The resulting plant population (representing the Fi hybrids) is then self-pollinated and allowed to set seed (F2 seeds). The F2 plants grown from the F2 seeds are then screened for resistance to ToChV. The population can be screened in a number of different ways, preferably by performing a method of the present invention for visual inspection.

Because the identification of ToChV-resistant plants has only at first been possible by the present invention, the method for producing the resistant plant is an aspect of the invention. Also an aspect of the present invention is a ToChV-resistant plant, or a part thereof, obtainable by a method of the invention.

The present invention provides for methods of preventing the spreading of ToChV infection in tomato plants by providing resistant tomato plants as well as by eliminating plants that carry the ToChV virus. These measures may form a part of a general strategy to improve phytosanitation in relation to ToChV virus. Tolerant plants may thus be identified and eliminated in order to eliminate such sources of the ToChV virus.

In one embodiment of a method for producing a ToChV-resistant plant, or part thereof, the present invention provides a method of producing a ToChV-tolerant plant. A tolerant plant may provide valuable crop, fruits and seeds, since, although the plant may harbour the virus, it does not exhibit disease symptoms. Such a method will involve the identification of tolerant plants, and the use of such tolerant plants as sources or donors of the desired genetic material. The aim is not to provide a plant capable of withstanding entry or multiplication of the virus in its cells, but to provide a plant which does not suffer from symptoms.

Thus, the present invention relates to a method for identifying a ToChV-tolerant plant, comprising the steps of a) exposing a plant or plant part to a infective dosage of ToChV, and b) identifying said plant as a ToChV-tolerant plant when, after said exposure, disease-symptoms in said plant or plant part remain absent, and ToChV is present in said plant or plant part.

Determining the development of ToChV-induced disease-symptoms in infected plants may be performed by quantitative methods, *e.g.* wherein the period required for the development of discernible *(e.g.* visible) disease-symptoms is noted, or by qualitative methods wherein, after a certain period has lapsed, the plant is inspected for the absence of symptom expression or the reduction in severity of the symptoms is indicated.

In a preferred embodiment, the presence of ToChV in said plant or plant part is determined in step b) by performing a method comprising determining in said plant or plant part the presence of a ToChV virus or component thereof

by reacting said plant or plant part with a polynucleotide according to the invention or an antibody according to the invention.

Diagnostic Kits

Methods and means provided herein are particularly useful in a diagnostic kit for diagnosing a ToChV virus infection by virological diagnosis. Such kits or assays may for example comprise a virus, a nucleic acid, a proteinaceous molecule or fragment thereof, and/or an antibody according to the invention.

The invention also provides a diagnostic kit for diagnosing a ToChV infection comprising a ToChV virus, a ToChV virus-specific nucleic acid, proteinaceous molecule or fragment thereof and/or an antibody according to the invention, and preferably a means for detecting said ToChV virus, ToChV virus-specific nucleic acid, proteinaceous molecule or fragment thereof and/or an antibody, said means for example comprising an excitable group such as a fluorophore or enzymatic detection system used in the art (examples of suitable diagnostic kit format comprise IF, ELISA, neutralization assay, RT-PCR assay, hybridisation assays). Suitable detection assays include direct and indirect assays, sandwich assays, solid phase assays such as those using plates or beads among others, and liquid phase assays. Assays suitable include those that use primary and secondary antibodies, and those that use antibody binding reagents such as protein A. Moreover, a variety of detection methods can be used in the invention, including colorimetric, fluorescent, phosphorescent, chemiluminescent, luminescent and radioactive methods.

To determine whether an as yet unidentified virus component or synthetic analogue thereof such as nucleic acid, proteinaceous molecule or fragment thereof can be identified as ToChV-virus-specific, it suffices to analyse the nucleic acid or amino acid sequence of said component, for example for a stretch of said nucleic acid or amino acid, preferably of at least 10, more preferably at least 25, more preferably at least 40 nucleotides or amino acids

(respectively), by sequence homology comparison with the provided ToChV viral sequences and with known non-ToChV viral sequences (preferably the closest phylogenetic relative of ToChV is used) using for example phylogenetic analyses as provided herein. Depending on the degree of relationship with said ToChV or non-ToChV viral sequences, the component or synthetic analogue can be identified.

A kit for detecting a ToChV virus may, depending on the assay format, include one or more antibodies specific for a protein, preferably specific for at least one capsid protein of ToChV, and preferably also includes a substantially purified ToChV protein or anti idiotypic antibody for use as a positive control.

Antiviral agents

The invention also provides methods to obtain an antiviral agent useful in the treatment of ToChV infection in plants comprising establishing a cell culture or experimental plant comprising a virus according to the invention, treating said culture or plant with an candidate antiviral agent, and determining the effect of said agent on said virus or its infection of said culture or plant. An example of such an antiviral agent comprises a ToChV-neutralising antibody, or functional component thereof, as provided herein, but antiviral agents of other nature may be obtained as well.

There are different antiviral agents used in plants, such as chemical products, bacteria, fungus, insects and virus. Most of them are related to systemic acquired resistance (SAR). The present invention contemplates the use of the ToChV genome or a part thereof as an inductor of systemic acquired resistance in plants. The systemic acquired resistance may be directed to ToChV or to other diseases. In this aspect, ToChV, its genome, or resistance-conferring parts thereof can be used as antiviral agent.

The invention also provides use of an antiviral agent according to the invention for the preparation of a treatment composition, in particular for the treatment of ToChV infection in plants, and provides a pharmaceutical

composition comprising an antiviral agent according to the invention, useful in a method for the treatment or prevention of a ToChV virus infection, said method comprising providing such a treatment composition to an individual plant.

The invention also relates to a plant model usable for testing of treatment methods and/or compositions. It has appeared that several *Nicotiana* species can be infected with the ToChV virus, thereby showing disease-symptoms dissimilar to those found in tomato plants suffering from the ToChV virus. Subjecting plants of *Nicotiana* to an antiviral treatment either before or during infection with the virus may have predictive value for application of such an antiviral agent in tomato plants.

The invention also relates to the use of ToChV, or parts of the ToChV viral genome, as expression vector, for instance for use in virus-induced gene silencing (VIGS). VIGS is a technology that exploits an RNA-mediated antiviral defence mechanism in plants. In plants infected with unmodified viruses the mechanism is specifically targeted against the viral genome. By using viral expression vectors carrying inserts derived from host genes the mechanism can also be used to target against the corresponding plant RNAs. VIGS has been used widely in plants for analysis of gene function and has been adapted for high-throughput functional genomics. Until now most applications of VIGS have been in *Nicotiana benthamiana*. However, the present invention contemplates the use of ToChV as new expression vector systems that allows for the analysis of gene function in other plants, such as tomato or other species of the family *Solanaceae*, such as pepper and potato and in species of the family *Cucurbitaceae*.

The invention is further explained in the Example without limiting it thereto.

EXAMPLES

Example 1. Characterization of Tomato chocolate **virus** (ToChV)

Introduction

Tomato chocolate virus (ToChV) was recovered from diseased Tomato plants from Guatemala and was designated as isolate ToChV-GOl. The virus was maintained in the greenhouse by mechanical inoculation to *N. tabacum* 'White Burley', *N. benthamiana, N. occidentalis* 'Pl', *N. occidentalis* '37B' or *N. hesperis* '67A'.

In the present experiments some of the intrinsic properties of ToChV were determined, such as particle structure and size, number and sizes of coat proteins, and composition of the viral genome. Also the symptoms after inoculation of the virus onto indicator plants were examined as well as the complete sequence of the RNA-I strain of the virus.

Methods and results

Mechanical inoculation onto indicator plants

ToChV could be inoculated onto indicator plants by mechanical inoculation using a standard 0.3 M phosphate buffer, pH 7.7. Depending on virus titer in the inoculum, symptoms appeared 4-7 days after inoculation. Typical symptoms on indicator plants are listed in table 2.

Table 2: Symptoms on indicator plants after inoculation with Tomato chocolate virus (ToChV).

Indicator plant	***Symptoms***
Chenopodium quinoa	No symptoms
Nicotiana benthamiana	Systemic chlorosis and malformation
Nicotiana clevelandii	Systemic chlorosis
Nicotiana glutinosa	Systemic veinclearing and chlorosis
Nicotiana hesperis 67A	Local chlorotic/necrotic lesions, Systemic veinclearing, chlorosis and malformation
Nicotiana occidentalis 37B	Systemic veinclearing and chlorosis
Nicotiana occidentalis P1	Local chlorotic/necrotic lesions, Systemic veinclearing and chlorosis
Nicotiana tabacum White Burley	Systemic veinclearing and chlorosis
Nicotiana tabacum Xanthii	Systemic chlorosis
Physalis floridana	Systemic mosaic

Virus purification

Purification attempts started using the protocols used earlier for isolation of Tomato torrado virus (ToTV; see WO 2006/085749) and Tomato marchitez virus (ToMarV; see PCT/NL2008/050076). Presence of the virus in the different fractions was verified by inoculation onto indicator plants *(N. occidentalis* 'Pl' or *N. hesperis* '67A') and appearance of typical chlorotic symptoms. Symptoms generally appeared 4-7 days post inoculation (dpi).

The virus was still infective after homogenization, alternating low speed and high speed centrifugation and sucrose cushion centrifugation. After the following sucrose gradient centrifugation, infectivity was found in the entire lower 2/3 part of the gradient. The virus was not found in a single fraction, suggesting that the virus aggregated and/or was attached to plant components. Fractionation of this material on a Cs_2SO_4 buoyant density gradient centrifugation never resulted in visible virus-containing bands.

Several attempts were made to optimize the purification protocol for ToChV by changing the host plant, buffer system, purification temperature, the use of different organic solvents (Chloroform and n-Butanol), the use of Triton X-IOO and Sulfobetaine, and centrifugation speeds.

In all cases the virus remained infective until the Cs_2SO_4 gradient, as could be seen from inoculation of samples from each purification step on indicator plants *(N. occidentalis* 'Pl' or *N. hesperis* '67A'), except for when n-Butanol was used in the purification protocol (infectivity was lost upon addition of n-Butanol). However, differences in abundance of symptoms varied, suggesting differences in virion concentration in the inoculum.

Virus concentration in young leaves of *N. hesperis* "67A' seemed to be the highest. A purification protocol using Triton X-IOO resulted in rather clean (not green) preparations after the sucrose cushion step. Inoculation of samples of different purification steps always resulted in severe symptoms in indicator plants just 4 days after inoculation, suggesting that the virus concentration was high. Nevertheless, inoculation from Cs_2SO_4 gradient fractions, after dialysis to remove the cesium salt, never resulted in infection of indicator plants. Also no infection could be obtained after centrifugation in CsCl or RbCl gradients.

It was found that sucrose gradient density centrifugation yielded the best results. Centrifugation for 15 hrs in a 20-50% sucrose gradient at 25000 rpm resulted in an almost complete separation between virus and plant parts. The virus was detected (by inoculation and by EM) in the lowest part of the gradient.

The virus-containing part of the gradient was concentrated by centrifugation and the pellet was resuspended in Tris buffer. This suspension contained high amounts of spherical virus particles (figure 2 B) of approximately 28 nm in diameter. This size is similar to that observed for ToTV and ToMarV, but is smaller than observed in crude sap preparations of

ToChV. It was proposed that this virus can swell and shrink due to different pH conditions, as was earlier reported for other spherical viruses.

Inoculation of this suspension resulted in infection of indicator plant *N. hesperis* '67A', and symptoms appeared within 4 days.

The Tris buffer suspension was used for RNA extraction and polyacrylamide gel electrophoresis (PAGE). The purification protocol was as follows.

All initial centrifugation steps were performed at 6°C, sucrose gradient and concentration were performed at 15°C. An amount of 100 gr of systemically infected leaves of *N. hesperis* was homogenized in 500 ml extraction buffer (Tris, pH8 + 2OmM Na_2SO_3, 10 mM Na-DIECA en 5 mM Na-EDTA) and squeezed through cheesecloth. The homogenate was centrifuged for 10 min in a Sorvall GSA at 3000 rpm. The supernatant was collected and centrifuges for 30 min in a Beckman R45 at 25000 rpm. The supernatant was collected and 1% Triton X-100 was added and the mixture was stirred for 30 min.

The mixture was placed on a 30% sucrose cushion and centrifuge for 3 h at 32000 rpm in a Beckman R45. The pellet was resuspended in 4 ml Tris buffer pH 8.0 and the virus suspension was loaded onto a 20-50% sucrose gradient and centrifuged for 15 h at 25000 rpm in a Beckman SW41. The fractions just above the pellet (1 ml per tube) were collected to provide the isolated and purified virions. The virions were concentrated by centrifugation for 3 hr at 35000 rpm in a Beckman SW55

PAGE (Polyacrylamide gel electrophoresis)

Viral proteins were separated by subjecting purified virus particles to 12% denaturing polyacrylamide gel electrophoresis (SDS-PAGE) [Laemmli, 1970], and visualized by silver staining.

Electrophoresis of the purified virions on a 12% polyacrylamide gel revealed multiple bands when stained with a sensitive silver staining, indicating that the purified virus preparation was not entirely free from plant proteins.

Using a coomassie staining clear bands were visible of 24, 26 and 35 kDa (see fig. 3A). These bands are of the sizes that fit well in the range of sizes of the three coat proteins of Torradoviruses. Also some bands of higher molecular sizes were visible, most likely plant proteins.

RNA extraction and evaluation

Electrophoresis of RNA obtained from pellets after the sucrose cushion on a denaturing agarose gel, revealed a faint band of about 6-7 kb. Presumably, as judged by its size, this is likely to be viral RNA. No smaller sized RNA band could be seen on the gel, except from a large quantity of small RNA's in the front.

RNA extracted from the sucrose gradient fraction yielded about 3-4 microgram RNA per purification (from 100 gr of leaves). Electrophoresis of the RNA preparation on a denaturing gel revealed two RNA bands of approximately 8 and 6 kb. There were hardly any small RNA's visible in the front, indicating that this preparation of RNA was much less contaminated with small plant RNAs than the preparation from the earlier purifications. The amount of RNA in the 8 kb band (RNAl) was lower than the amount of RNA in the 6 kb band (RNA2). The 8 kb band could not be properly visualized using ortho-toluidine staining of the denaturing RNA gel (Figure 3B).

Based on the above results, it was concluded that ToChV has a bipartite genome with RNA sizes of approximately 8 and 6 kb. This also fits well with the RNA properties of the genus *Torradovirus.*

Electron microscopy

Virus suspensions were mounted on formvar/carbon coated grids, stained with 2% uranyl acetate and examined in a Philips CM12 electron microscope.

RT-PCR and 5'RACE

PCR fragments were obtained by one-tube RT-PCR (Access RT-PCR system, Promega) .RT-PCRs were initiated using a universal oligo dT primer [Van der Vlugt *et al.* (1999). *Phytopathology* 89: 148-155] and various primers derived from the ToTV RNA 1 and RNA 2 sequences (respectively GenBank accession numbers DQ388879 and DQ388880). The 5' regions of the ToChV RNAs were determined by walking towards the 5' end of the viral genome through repeated use of a 5' RACE kit (Roche) in combination with the Expand high fidelity PCR system (Roche), essentially as described previously [Ongus JR, *et al.* (2004). *J Gen Virol* 85: 3747-3755, Valles SM, *et al.* (2004) *Virology* 328: 151-157]. cDNA primers for the 5'-RACE strategy and primer sets for additional RT-PCR reactions were based on newly obtained ToChV sequence data.

PCR and sequence analysis

All PCR products (of the 5' RACE) were purified using the QIAquick PCR Purification Kit (Qiagen) and directly sequenced.

Sequence analysis was performed with an Applied Biosystems 3100 Genetic Analyser, using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham) and the primers that were used for amplification. For longer PCR fragments specific primers were used for primer walking sequencing.

Nucleotide and amino acid sequence data were analyzed and assembled using the DNASTAR package (Lasergene).

Sequence comparisons with other viruses were performed with programs from the PHYLIP package. Multiple alignments and phylogenies

were performed with the CLUSTAL X program after bootstrapping in 1000 replicates. Neighbour-joining concensus phylogenies were viewed by the NJplot program [Thompson JD, *et al.* (1997) *Nucleic Acids Res* 25: 4876-4882] and printed by using TreeView [Page RDM (1996) *Computer Applications in the Biosciences* 12: 357-358].

RT-PCR with ToTV- and ToMarV-specific primers was performed directly on extracted RNA from the resuspended pellet after sucrose cushion centrifugation. Only primer pair M9f - MlOr (sequences are TGCAACCCATCCAGTTGCGG and TTTGGCTACCACATACTCC, respectively) generated a fragment of approximately 600 bp. The sequence of this PCR product was analyzed. This resulted in a sequence of 498 bp, which was compared to sequences in the NCBI database and to the sequence of ToTV, ToMarV and Tomato apex necrosis virus (ToANV). Homology was found on RNAl of the torradoviruses in the helicase region (Appr. 77% identical on nucleotide level, 86% on amino acid level).

The complete sequence of RNA-I was obtained and is displayed herein as Figure 4. This RNAl sequence was shared by both ToChV isolate variants (GOl and G02) described herein. Figure 5 displays the sequence of RNA2 for isolate GOl and Figure 6 displays the sequence of RNA2 for isolate G02.

2.6. Back-inoculation onto tomato plants

Purified virus was used to inoculate tomato plants (cultivar Moneymaker). The virions were directly inoculated from the final sucrose gradient onto three tomato plants, previously dusted with carborundum. Two weeks after inoculation all three plants showed symptoms. Two plants showed rugosis in the youngest leaves and a beginning chlorosis. One plant showed a clear chlorosis and necrosis, beginning at the basis of the leaflets.

3. Conclusions

A purification protocol for ToChV was developed. Initially used on systemically infected leaves, purified fractions were highly infectious but contained only limited number of virus particles (as judged by EM) thus virus yields were low. Young systemically infected leaves appeared to contain more virus particles and appeared to be a better source for obtaining useful yields of ToChV particles. ToChV was not resistant against buoyant density gradient centrifugation using CsCl, Cs_2SO_4, or RbCl and is most likely degraded in these gradients. Partially purified virus fractions that contained no or only low amounts of plant RNA could be obtained by using a sucrose gradient.

Purified virus fractions were used for inoculation of indicator plants and proved to be infective. In addition, purified virus was also used to inoculate tomato plants and produced the characteristic disease symptoms thereby fulfilling Koch's postulates. The inoculated tomato plants (cultivar Moneymaker) showed typical symptoms approximately two weeks after inoculation.

Electron microscopy revealed that the purified fractions contained virus particles of approximately 28 nm in diameter. In crude sap preparations particles of approximately 34 nm in diameter were found. These size differences may well be caused by swelling and shrinking of the virus particles due to pH changes.

The purified particles were subjected to PAGE. Apart from some plant proteins, three putative viral coat proteins were observed. The coat protein sizes were approximately 24, 26 and 35 kDa.

RNA extraction and electrophoresis showed that the genome of ToChV is bipartite. RNAl is approximately 8 kb and present in low amounts in the purified fraction. RNA2 is approximately 6 kb in size and more abundant in the purified fraction.

Sequence analysis of the PCR product obtained with primer pair M9f - MlOr showed a clear homology with the helicase domain of ToTV, ToMarV and

ToANV. Sequence identity in this conserved region is rather low (77% at nucleotide (nt) level and 86% at amino acid (aa) level)

From the above characteristics it is concluded that ToChV is a new member (species) of the new genus *Torradovirus.*

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11

11

Claims

1. A plant virus named Tomato chocolate virus (ToChV), deposited under depositors reference ToChV-GOl on 8 December 2008 with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, in Braunschweig, Germany under accession number DSM 22139, or deposited under depositors reference ToChV-G02 on 22 December 2008 under accession number DSM 22202 and on 21 July 2009 with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, in Braunschweig, Germany.

2. A virus comprising the nucleic acid sequence of SEQ ID NO:1, 2, or 3 as defined in Figures 4, 5, or 6, respectively, and sequences having a nucleotide sequence homology of at least 70% thereto, and/or being the causative agent of Chocolate spot disease in tomatoes.

3. An isolated virus belonging to the presumptive novel species of Tomato chocolate virus (ToChV), of which the plant virus DSM 22139 as defined in claim 1 is the type strain, wherein said isolated virus is identifiable as phylogenetically corresponding to said novel genus by determining a nucleic acid sequence of a suitable fragment of the genome of said virus and testing it in phylogenetic analyses wherein maximum likelihood trees are generated and finding it to be phylogenetically more closely related to the plant virus as defined in claim 1 than it is related to a virus isolate of a non-ToChV reference strain or closest relative.

4. A virus according to claim 2, wherein said virus, based on numerical taxonomic analysis of one or more of the taxonomic descriptors:

- Virion properties, including morphological and physical properties of virions, properties of the genome and properties of the proteins;

- Genomic organization and replication;
- Antigenic properties; and
- Biological properties, including natural and experimental host range, pathogenicity, tissue trophism, mode of transmission in nature, vector relationships and geographical distribution,

shows to be more closely related to the virus as defined in claim 1 than to any other virus, and wherein said virus is associated with a disease that causes necrotic lesions in tomato.

5. An isolated or recombinant nucleic acid comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, 2, and 3, sequences having a nucleotide sequence homology of at least 70% to SEQ ID NO: 1, 2, and 3, and their complementary strands.

6. An expression vector comprising a nucleic acid according to claim 5.

7. A polynucleotide capable of hybridizing under stringent conditions to a nucleic acid according to claim 5.

8. A polypeptide encoded by an open reading frame (ORF) in the nucleic acid sequence selected from the group consisting of i) SEQ ID NO:1, 2, and 3, and ii) complementary strands thereof.

9. A polypeptide according to claim 8, wherein said polypeptide is selected from the group consisting of the 24, 26 and 35 kDa capsid proteins, the helicase, the RNA-dependent RNA polymerase and the putative movement protein (MP) of ToChV.

10. An antigen comprising a polypeptide according to claim 8 or 9.

11. A capsid protein isolated from the viral capsid of the virus according to any one of claims 1-4.

12. An antibody specifically directed against the antigen according to claim 10 or 11.

13. A method for producing an antibody against the virus of any one of claims 1-4 comprising the steps of

a) providing the virus of any one of claims 1-4, or a protein or peptide fragment thereof;

b) immunizing an appropriate vertebrate host with said virus, protein or peptide fragment, and

c) harvesting from blood or splenocytes of said vertebrate host antibodies against said virus, protein or peptide fragment.

14. A method according to claim 13, further comprising the steps of selecting one antibody-producing splenocyte, fusing said splenocyte to an immortalized hybridoma cell line and allowing said hybridoma fusion to produce monoclonal antibodies.

15. A method for identifying a viral isolate as a virus according to any one of claims 1-4 comprising reacting said viral isolate or a component thereof with an antibody according to claim 12.

16. A method for identifying a viral isolate as a virus according to any one of claims 1-4 comprising reacting a viral isolate or a component thereof with a polynucleotide according to claim 7.

17. A method for detecting the presence of a virus as defined in any one of claims 1-4 in a sample comprising determining in said sample the presence of

a virus as defined to any one of claims 1-4 or component thereof by reacting said sample with a polynucleotide according to claim 7 or an antibody according to claim 12.

18. A method for identifying a plant that is resistant to a virus as defined to any one of claims 1-4, comprising the steps of:

a) exposing a plant or plant part to a dosage of viral particles or viral nucleic acid capable of infecting a plant, wherein infection is achieved by mechanical inoculation of purified virus particles or virus nucleic acid of a virus as defined to any one of claims 1-4, and

b) identifying said plant as a plant that is resistant to a virus as defined to any one of claims 1-4 when, after said exposure, either

- disease-symptoms in said plant or plant part remain absent or are delayed in expression or are at least reduced in severity or are localized relative to a susceptible control plant, and/or

- said virus or genomic sequences thereof are not present in said plant or plant part or the presence of said virus is at least quantitatively reduced relative to a susceptible control plant.

19. A method according to claim 18, wherein in step b) the presence of said virus in said plant or plant part is determined by performing a method according to claim 16.

20. A method of producing a plant that is resistant to a virus as defined to any one of claims 1-4 comprising the steps of:

a) identifying a donor plant that is resistant to a virus as defined to any one of claims 1-4 by performing a method according to claim 18 or 19,

b) crossing said donor plant with a recipient plant, optionally performing several backcross and/or selfing steps to provide offspring plants, and

c) selecting from offspring plants a resistant plant by performing a method according to claim 17 or 18.

21. Method according to claim 20, wherein said donor plant is a plant of the family *Solanaceae* or *Cucurbitaceae*.

22. Method according to claim 20, wherein said donor plant is a tomato plant, an eggplant plant, a pepper plant, a melon plant, a watermelon plant or a cucumber plant.

23. Method according to any one of claims 20-22 wherein said recipient plant is a plant of the family *Solanaceae* or *Cucurbitaceae*.

24. Method according to any one of claims 20-22, wherein said recipient plant is a tomato plant, an eggplant plant, a pepper plant, a melon plant, a watermelon plant or a cucumber plant.

25. Method according to any one of claims 20-22, wherein said recipient plant is a tomato plant of the species *Solanum lycopersicum*, more preferably an *S. lycopersicum* line that possess commercially desirable characteristics.

26. A diagnostic kit for performing a method according to claim 17, comprising at least one component selected from the group consisting of a virus according to any one of claims 1-4, a polynucleotide according to claim 7, a polypeptide according to claim 8 or 9, an antigen according to claim 10 or 11 and an antibody according to claim 12.

27. Use of a virus according to any one of claims 1-4, a polynucleotide according to claim 7, a polypeptide according to claim 8 or 9, an antigen

according to claim 10 or 11 or an antibody according to claim 12 for the production of a diagnostic composition.

28. A diagnostic composition comprising a virus according to any one of claims 1-4, a polynucleotide according to claim 7, a polypeptide according to claim 8 or 9, an antigen according to claim 10 or 11, or an antibody according to claim 11.

29. Use of a virus according to any one of claims 1-4, its genome, or resistance-conferring parts thereof as antiviral agent.

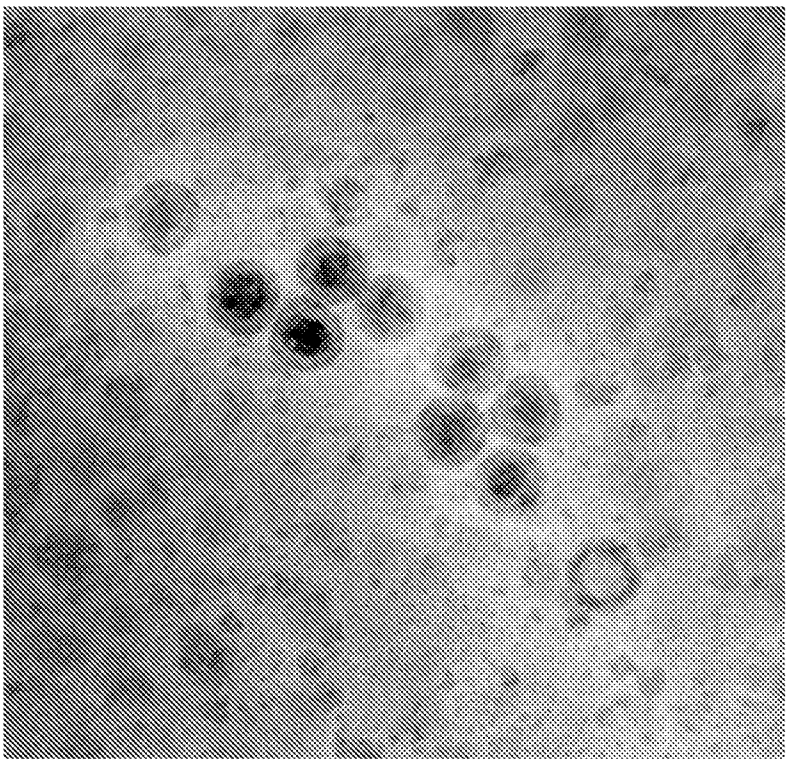
30. Use of a virus according to any one of claims 1-4, or its genome, or parts thereof as expression vector.

31. Use of an attenuated form of a virus according to any one of claims 1-4, or its genome, or parts thereof, for premunition of a plant.

Figure 1

Figure 2

A



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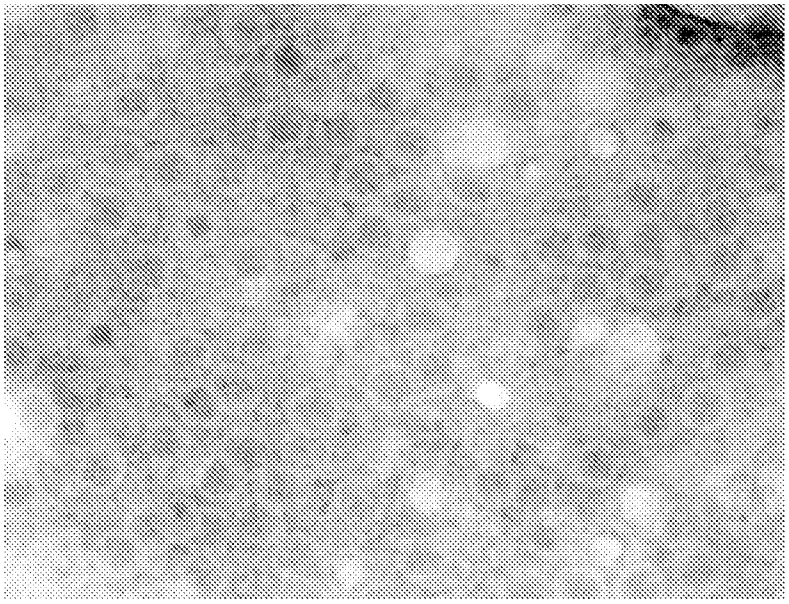

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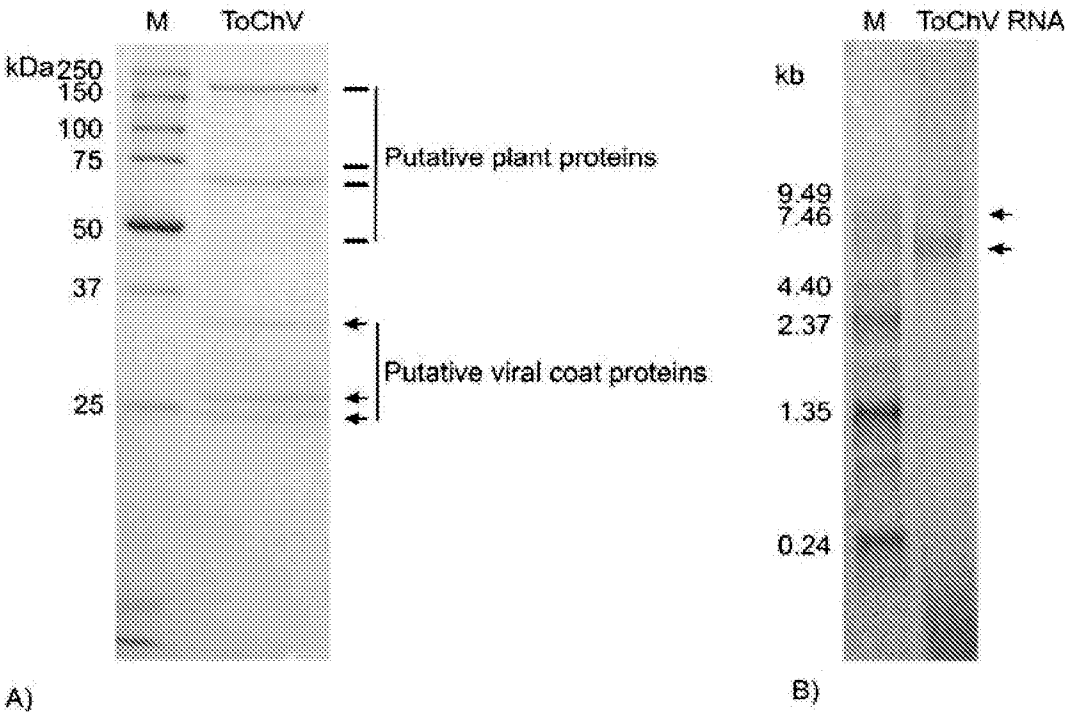
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Figure 4

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TAGCAAAGTGCAGAGTCCATGCTGAGATCTCAAGCAAGCTGTTCTCCACTGAATCAGGATTAGCAGGAGAGAA
GGTGGCACAAGTGAATGTCAATGTGGATGATGTTTTGGATTCCATTGATAGTGCTCTTCTAATTACAAAGCAA
GATGATGTGGAATTTCCTCCTGTTATCATCTCGGAGACTGACAAAGTCCTGTATTCACAGGAACTTACTGTTG
AGGCATTGAAGTTTGCTTATCAGGGTTCACTCAACAAGGAAAAGTATTTCCCACATGATCATCATAAGCAAGC
TTTGTTTGATGCTTTGAGTGCAGAAAACAAGGAGATTTTTGGAAGGTGGGTGTCTGATTTGATATATCGTGGA
GCTGACAGTGATCAATATCGCTGGGTGGTACAGAATATACCATCGGACTATTTGATGCATTTCAAGAGTTTTG
TTTATGCCACCACAGTTTGTGAGAAGAAGTTGGCAGTGCAGGTGGAGCTTGAAGATGGATTTGCACATAGATC
TCTTGATGCAGATGTTGACACTCTGATCTGTATAGAACAAATGCCACCTTTTGTGCAATTTCTGTACACAGCT
TTTGTGCGCTATTGGTGTGCCAAAGTTGGCAGTGCCAAGCAAGAATCCTGGGTTAAAGTGTGTTATAACACTA

Figure 4 (continued)

TTCTGGAAAAGATTCAAAAAGCGTGGTATGACTTACCATACATCTTGCGTTTGTTGATTAAGGCTGGTCTCAT
AATCATTGCTCTAAATGGGGCTTTTGGTGCAGTAACAGCTTTTGTTTCCTGCTGGCAAAGCAATTCATTCCCA
TCTGCAAGTGGCAAAGGGGGTGTAACAAACGAATCCAACAGTATTTCGAGCAAGAAAAACAAAGGCAACAAAC
AGTTGAAGAACTTATTGGTTGGTCAATCAAATGGAGATTGTGTCAAAGATTGGGCCAGCGAGGATGGGTTTGT
CAACCAGGCTCTTAAGAAAAACATGGTTGCACTGCGTTTGGGAGAAGGGGTGTACTTCAGAGGCACCTATGTT
TGTTCAGGGTGGATAATGACAGTTGCTCATGCATTTCATAATGTACGAGATGGAACTCCTTTTACCATTATCC
ATCCACATTCCAGGTCACCTGTTCAATTTAATGCACGAGAATCGCGTGTCATAGAGGGGCAGGATATTATCCT
TCTCAAGGTGGGCGATCCCGATGGCCCAAAACCTGACATACGCAAACATTTTCCATACAGGGATGATGTTTGT
TTTGCAAAGGGCACCCAGGGATTGTGTTGCAGGGCCATATCATCAAAGGATCCACGTGAAGGCAATCTAGAAT
TCTTCCGTATGCCTGTAATGATGTCAAAGGGTTATATGGTCAAGGTAGAGTATGAATTAGATTCAGCCAGTTT
TAAGCTGTGCGCAAATAATTCATATGAATACCACATCAATGGTGAGAATGGAGATTGTGGTACACTGTTGCTT
TTACCTAGTGTCAAAAACAAGCAACCATGTATTGTTGGCATTCATTGTGCCTCTTATGATGAGGATGCTGCTG
GACGTGGGTTTATAGCATCAAATGCTTCTGCAATTTTCAGAGAACAATTGGAAGATCTACCAACGGGGCCAGT
CAAAACAGCAATGGTAAGGTGCGAACTGCTCAAATCAGTTCGAAACCGTGAAATGCAGTTGTTTGACGAAAAT
CAAGTTTTTTATCTTGGTGATGTGCCTCAGGAATTGGCAGCCACAGTGCCTCACAAAACAACACTCAAGAAGA
GTCAGATCTTTGAGGCTTTTGGTCCAGCTGAAACGGCACCCTCTATCTTGTCAGTCAAGGACAAGAGGGGAAA
TGGATTTGATCCCTATGTTGCTGGCGTGATGAAATATAATGAGACAGCATATGGGTTCAACCAGGATGTTGCT
GAGTTGGCTTTTGAAACACTCAAGAGTTCAATTTTGCCAGTAATGCGCAAGCACAGGGTTCCAGGGGGAAAAC
CAACCATCAGAAGTGAAGATGTGGTCCTCAATGGAATTGATGGATTTGATTACTATGATGGAATGGAACTGAG
CACGTCTTGTGGCTATCCCTTCAACAAAATGGGCCTAGGACTTAACAAGAGGGAGTTTGTTGAACCCACTGGT
GAAGGGGATAGAGTTTTACTTAAACGGGATACCCCTTTGTTCACAGCATGGGAGGAGATGGATGTAGAGATAA
GGAAAGGAAATTTCTGTGAGTTGGTCACAACCCAGTGTGCCAAGGATGAACGTTTGCCCTTGGAAAAGATTTA
TGGGAAAAGGAAAACCAGATTGTTTGAGATATTGCCTTTTCATTATAATATGCTGGTTAGGAAGTACTTTCTT
GATTTTTCTGCAGCACTCATGGCACTTCACAACAGTTTGCCCTGTAAAGTTGGCATTGACCCTACTGGTTCTG
AATGGTCCTTATTAGCAAATGGGTTCAGGTCTGTTTCCAATGTAGGGTTTTCAGCAGATTACTCAAGCTTTGA
TGGGAGAGCCCCAATATTTGCATTTCAATGGTTCTGTGATTTGGTTGATATTTATTATGGTTCAGAGCCAGGA
AGTGAGGACTCAAATGCAAGGCATTCACTGTTGATGATGGCATCATGCCATTATACTCTCTGTGAAAACAAGT
TGTTCCGATTAGTTGGGGGGATGCCTTCTGGGTTTGCGCTCACTGTCATCTTCAACTCTCTGCTGAATGAATT
CTACATGAGATATGCATTCATCTCCTTGTTGAGGAGACCTCATATTGCAGCAAGGGCCATAGGTGTTAAACCC
AATGATTTCAATCAACTCTTCATTGCTGTGTATGGTGATGACAACCTGGTGGCAGTTCCACTGCATCTTCAAT
GGTATTCTTTGCCCAACATTGCTGCGGAGTTGGAGAATGTCAATGTCATAATCAAGAATGGCCTGGATAAATC
AATGGATGTGAATGATGTTGTTTTCCAAGATCTCTCTGAACTGACATTTTTGAGCCGGGGTTTCAAAAGGCAT
GCATTGGGATATCATTTGGCGCCTCTCAAGTGGGTGTCCATTGTTGAACCAATGTATTGGATTCGACCTGCAC
CAGGTTGCCCTGATTCTGCTGCCATGCTTGAAAATATTGAAACAGGAATCAGAGAAAGTTTTCACCATGGGCG
TGCGGTTTATGACAAGTTGATTCTTGATGTGCAGCAAGTCCTTGATGATAAAGGATTTGGAACAATTGTTTTT
CCATCATATATGGAAGTGGAACAGGAATGGATTGCCAAAGTAACAGGTGATTCATCTGCACTGTCTATCTGCG
AGATGGCAAAATCTAGCTTAACATTCACACCATTGGCTCCAGGTGAAACAGTTTCTGATTTCTCCATGGGTGT
CCATTGGTTTACACCAGACATAGGATTTTGTGCTGCTCGTACTGCTGCCAAGCTTGAATGGCCAGATGACCAG

Figure 4 (continued)

GTGGTCGTGAATTGTACAGGGGCAAAGAAAACAAAATGGGTGCGTGGTCCAGCAAGCTGGAAAGATTTTGAAG
GAAAAATGTGGCCATACACCATGACCGCAATTTTAGATGCACAAAGATATAAACTGTTACAAGGTGGTCATGT
GAGTAAGACCATTTTTGTGTGCGGAAATGGTTATGCAATTGGACCAGTATGTGCTGCACTGATGGCATTGGCA
ACACGTCAATATGCCATTGAGGATATCATAGTGCGGTTGCGTACTATTGCTGGAGTTCTCAATCTTAACACAT
ACCCGGGTGGTTGTGCTCAGTATCTGTTaAATTGTGTGCCTGGGATGGAAGGCAAGGTGGCACAAAGTGGTGC
AAGATATTATAGTAATTTCATGCACCAAGGTTTTGAATTGGGACAACTTAGGATTATCCATGGAGACATCTCA
AGAGAAATGGCTTTGAGGATGCCAAATACAGTTGGACCACAAGGAGGTTGGGGCAATCTCTCCACTTATAACG
TTGCATCATTTTTAGAGCAACTGCAGTCTGTTTATGAGAGTTTGGTCTTGAACAATGCAAGTTTAACAATCTA
CTTGAAGAAGATGACCATCCATGATGCTAATTTGATCTTGGATTTCATCAAGAAACAAGGAATTTTCCCAAGT
CAGTCAACAGTTTCACGTCTCAAGACATTTGTGGATATGGAATTGTTGTGTTACAAGACAAAAGAGTACAGTC
ATGTTGTGTTTAGCAAGTCTATGCTTTCAAGAGAGTGGAAACTCCATGGTTCCCAAAAAGCTGCATCTTTATC
AGCTGAGAGCTTATTTCCAGGCAAATTATCAGCCAGTGTTATGTCAGTTTTACTTGAGAAGTATAAGCAAAAC
ATGTCTTGCAAGTCAATTGAACTGGCTCTTAAGATATTTCTTCTTCAACATGAAGAAATAAATGAAAACATCT
TGCTCAAGTTTGAACATAGCTTTGGCAAACAAATCCCGGAGGCACTATTGGTCAAGGTGTTGTTTTGGTTGTC
AGAGAGTCAGCTTAAATGTAAACACGTTTGGTTTGATTTCTCACATCGCATTCAACACAGTGGCATTTATGTT
GAGAAAGAAGGCTTCAGTTTGGATCCTTATTCCATCAACATGAATGCAGCGGATGCAATCTTCTTCACTCTGA
ATCAACATTATGATCAAAGTGAAAGAAAATATAGGGTAGCAACTTCTTTGTCTTTGCGAGCCCTGTTGTTTGT
TCTAGTTTTGGGCTGGCACTTAGGTCAAGTGGAAAGTCCAGATTTGGAAAATAATTATGATTTCTTAACTTTT
AAAACTATTCTTACAGGTTTTAAGGCGCCTTGAGTCAGGCATCACGACTCAACTTGTGTATATAATATGTGTA
CTGTATGTAATATTATATATGATGGGTTTATTAGTTTTAGAAATTAACGGAGAGTACTGTTCTTTATTGAATG
GGAGTCCGGTCCACTTTGTGGATACCCGTGAATCTACATGGGTTAGAGATTTGCACGCCTCTCTTTAAATATG
GTTCGTGTACCCTGCTTGGCTAGAAAGCAATTGTTTGGTGTTGTCAACGCGCCGCCTCTGGCCAAGAAAACAC
CCCTTAGGCTTTGTTGGTAAAGTCAAGATGTGGAGAAGGAATGCCAGCAAGCATAAGCAGAGTCTCTTAGGGT
CGGGCTTAAGATGGCAGCCAGAATTAGAGCCTTTGAGTATGCTTGTGGGCATCTGGTTTTCACATCAACTCGT
TTGGAGTATAAACAACTCGTGTGATGGTGTAATCACGTAAAGCAATGACACCCGGGGTGTGCTTTCCCTAGTT
AACGTAAGTACAGGTTGTTCTCCTATAGGAACTAGAGCGTTGGTCTCAACTCAAAGAGAGACCCGTTTGAAAA
TCGATAAATTTCCGTAGCTTGCGTCAAGCTGCTGTTGTTAGGGGCAACAGTGAAGAATCATCATACCTCTTCT
TCCCTAGGTTCGTCCAGAGGTTTCAGAGTGATGGCTTCTTTGTCAAGAAGTAAAGAATGACACGTGTTGCGTC
GACAATGCACCGTATGGTTTGGTTAGCCATAAACATATATATTATATTAGATAGTTTTATCTGTGTGTTCTGA
TGATTTGAACTTACTTTCAATTATTAGTGGCGACAGGAGAGGTTTGTCCTTTTACTCTTCTTGCTATGTTGGA
CACAAAAAGATTTTCTTTCTTTTATTTT

Figure 5

AAAAGAATTTTTATATATATCTGTTATGTAACCCAAGCCGTTACGTGGACCAAGTCTCCGCTACCAGATATTT
ATTTATTTTCTTTTGAAGTTCACAGTGGTAATACTCTTCAAACTACGACTTTGGGGTATTCCACAATTGTTAC
AAATTCAAACTATCCAATTCCCTTCCTATCTTCACATGTCATTCATTTCCCGTTTGAATATTTCAGAGGAGGA
AAAAGCATTTCATTCACAGGTTAGTACAGCTGATTGGGTTTGCTCAGTTGACGTTGGTACCGGTCTTATTAAT
AGTAATCCTACTTTGGATTTTAAGGTTACACCCAAAACAGGAGGGTCAGTTTCAATCCTTTCTGTATCTTGGG
AGAATTCTTATCCACAACTCGTCCCAGGTCATTATCTGTTGAGGTCAGGTAATTGGCCAGTTAGCAACGTTAA
ACTTTCAGGACTATTGGTCCACAGATCAGTAAAGTTAGAAACAACCAGGAAATTGTTGGAGGCGCAAAAAGTT
CAAATTTTGAACCAAGTGCAGGCAGAAACTTCCACATCCACTAGTACCAAAGGAAAAGGAGTTGTTGATATAC
TCAAATCACAAGAGGAGGAGATATCAAAACTCAAAGAGGAGTTGTTAAGGAAAGAGAAGGAACTTTCTGAGAG
GGATTCAGAGATAGCCAAGCTCAAACTTCAAGTGTCAAATTTGCCAGGCAATAATGACATCTTTTCTGGATGG
GCGGAGACACCCCCCCAGTAACAAGGAGCAGGAAGCAGCATCAGAGCGTTTGATGTCACAAATCACAGCTGCT
GTCGAAGAAGGAAATAAAAACATGCTCAGAAAGCTGGGTATGGGATCATATGGATTGCTTCGAGGATCTCAAG
ACAAACAAGCAATGGAGTTATTTGATCCAAAGGATGTTGGGAAAATAACTTCTTTGTGGACCAGTTTTAAGCA
AAAATTTGTTGAGTCAAAGGAGCATGCCAACTTGTTTTTCCATCTATATGGGGTGTTATTTTTCATGGTGCCA
CATGTAACAGGCGGAGAGGGCAGAGTTAAGATATGTTTATGTTCAAGCAATGACCCAATGAATCCTGTGATCC
AGGAGAAGACCTTATCTTTGGCAGGTGGGGCACAAGCGGTCTTGATGAGTCCTAGCATCACATTGCCATTTCT
TGAGAGAGGACCCATGTTCTATTACACTCTAGAATGTCAAGGAACACAAGCCCAAATTCCTTGTTCAGTTGTG
GCAATCTGGAAGCAAAAAATTGACGCTCGCAGCGCAATTTACTCACAGCAGGAAACCATGTCATGGGCCATTG
AAGCTCTCAACAGACCACAATTTTTCCAGGATAGACAAGAGGCTGCACAATATATCGCGTCTGTTTATTCGAG
TGGGCAAAGCTCACAAATGGCCCTAGAGAATAGGGCCTTTGTTGGAGAACAGCTTGGGGATACGCGCATGAAT
GTAGTTAGTGAGAGTACAaTGTCAAGAAGTACTTCACTGCGAATCCCCACTCTGAGAGTTCAAAGCAAACGGT
TTCCTTCTCTAGAAATACCTAGTTCTGCTTTGTTGCAGCAACTAGAGACTCGAGAAGAAACTGTACAAGATGA
GGATGATGGTGGTTTGTTTCCCACAAAGAAAGCATGCAGTCCCAACTATAGTATAATCTGGGACAATCTGAAC
ATTGATACGTTCGTGGACATGGAATTTCCACCTGATTGGACTGATCGCACGCTTGCCCAACAAGTGCAATATG
TCATGTTTACAAAGGCCTCAATGGGTAATGTCATCATTCCAAATCACATCACAAAAAGGGAACTCCACAATAT
CAACCGTGAAACCATAACTCCAGATAACTATGAAAGCATATTGAAAGAATATGGTATCACCAAGGTTGGGAAC
TTGTCTCGAAGTGATGACTGGTATTCTTTGACTCTCAAACAAAGGGTTGTGGACCTTGTGCACCAACGAGATC
ATCAGTTGTATATGACAGGTGATTTTACCAATCCACTGCCGAGATTTGACTGCTATGATGGTTTGACTCTGGC
AGAAAGGCATATGAGAAGTATCAGAGCCAGCTTTGCTACATCTGGGAGCAGCCAAATTAGAGTAGCACAGACT
ATTGCTACTATTAAGAGTAGTGCTTCCAATGAAGGAAATGTCCAAGATTCATTTGTGTCTAGAACAGCAATGG
AGAGCACTAGTGGGGAAAAATCTATTCACATAGATACTTCCCATAGTGAAGAGGATGTAAAGCCAGCTGATGT
AATTTTTGACTTTGGCAATGATATGGAAGTTGGGGACGCATTGGAATTGGAAATGCAACAGCCAGTGTGTGTG
GCTAGTAATGAATATTTCAATGCGGGGGTTTTTGAATTTGTGTGGGAAAAGAGTGCCCATGTTGCTGAACAAG
TAATGGCAATAGCTTTGCCAGCGGCCTTGTTTTCCCAGAAGAAAGAGACATCAATGGGTGCCCAGATGCTTAA
GTATTATGATGCAGCACTCATAACCTACAAAATAATTTTGTATGTTTCTGGTGTTGGTGCCATATCAGGCCAG
CTTGCATTAGTGTGGGATGAATGCAATGTGCTAAATCGCAAGAAGGAATTTGTTAATATTGCAACACTCTATG

Figure 5 (continued)

CTAGCAAGCACACTTTGGTATCTGCGTCACAACAAAACAGTGCTGAGTTTATTTTCACACCAACCGGAATTGG
GAAGTTCGTGCCCCTTGATGAAGGTACCGGAGCTATGGATCTTGGAAGCGTGAGGGTTTTTGTTACCCATCCT
CTCTCCAGTGCAACTGAGTTGTCAGGTGTACCTTGTCATTTACACCTTCAATGTAAGGTTTTATCAACCAACA
TTCTGCAACCTCCGAGAGTAATAGCACAAACTCAGTATGGAATGAAAGCAGATCAGACATACTTCAAGAGTTT
CCCAACTAACCAGGTACTCCTTCATTATAATTGGAATACTACGGCTACCATGGGAACAACTCTAGTGAGTATC
TTTTCACCATCTGGCATTTATGAAAGTGATGGTACGTTGCAACCATCTTTACTTGGCAACATAGCTCGCAATT
GCAAATGGTGGACTGGAGTCTGTGTGTTTGAAATATGCATAGAAAAGACTCTCTTCCACTCTGGAAGTTTGGC
TATAGGGCTTGGCACCCTAAACACAAAGATGTCGAGTGCACATGACATATTCAATATGCCATATGTGGTCTGC
AATCTGGAAATGGGAAGAAAGTTCCAATTCAAATGTGCCATATCCAATTGGAATGGAAAGAACTTGTTGTCAA
CTGGCAGGAAAAGTTCGTTGCCGCGTCCAAATCATTACTCCCATTTGCGCCTTTTTGCCACAGTTATGAAACC
TCTTGTGTCAACCTCAGTTCACCTGGATTCAGTAGGGGTCACGGTACAATTGAAGAGAATAGAGGGTTTAACT
CTTGGAGGCACAGTGTCTGTCAAGCCTATTTATGGTCACTGGACTAAAGGCAAAAGTACCGTTGATTTTTTGT
TTTCAGAGATGAACTTGGAGCACAGAAAGGAAATTGAAAAACTCCGCAAGGTAAATGTGGAGGAATATGACAA
GAAAGGTAAAGATAAGCCAGGAAAAGAAGGACAGATTTCAGTTTCCATTCGTGAAAAATTCACTTATGGTGCG
GTGCAGTACTTGTGCATGGGTTGGAAAGATGAAGAGAGATTGCTGGTTTTGCCTTGTGCTCCATGGTCAATAC
GATTTGCACACACCAGTCCTGTCAAGGAGGCAATCACCTGTCCTTTTATAGATTGGTGCACCTCCTTTTGTTA
TTGGAGTGGTAGCTTACAATATTCCATTGTATTGCACCGCGTTCAAGCTTCAGCAAATGTTGGAGGGATATTG
AATGTGGCCTTAGATTCCTCAGGTTATCCTTTTCCAGCAGGTATGCATTCAGGGAGCTATGTTGTATCAGCTG
GAGGTGGTTCAAAGTGGGATTTTTCCTACGGTGTTTCTACTAACACCTTTTCTTTTGTAGTGTCAGATGATGA
GTTCTTTCCAAGGAGACACACCCGAATGCGTGAGTTTTCAAAGAACCAATCACGCATCATGTCCTTACAGGAT
CGTCTTGGAAATTTGATAATAAATCTTCCGCCAAAGGATCTCATAAGTTCCATTGAAATTCTTATTGCTCCAG
GTCCAGATTTTAAACTTGAGATCGCGCAGCCTCCATCTGCAAATCATGAAAAGTTCTTGGGCAACATGCAAAC
ACACACCTATCTCTATACCCCTGATTTCTCTGAATTAAGAGATTTCGGGGGTTGAGGACTAACTATAAGTTAT
AGGAGCCTTGGGTCAGGCATCACGACCCAACTTATTCAGATAATATGTGTACTGTAATTAATATTATTTGTGT
TTAGTTAGGTTAATAAGAAGACATAGTCCCAATTTACGGAGAGTAATGTTCTTAATTGAACAAGAGTCCGGCC
CACTTTATGGGTTCCCATGAATCTACATGGGCGGAGATTTGCACGCTTCTCCTTAAGTATGTTTCGTGTACCC
TGCTTGGATTGAAAGCCCTCTACACCAGAGGTTAAATGGTCTCGCGTGATGGTGTAATCGCGTTAAATGATGA
CACCCGGGGTGTGCTTTCCCTAGTAAACTCAAGTACAGGTTGTTTCCCTATAGAAACTATTCTCATGCAGAGG
CTAAATGCACCTGGTTGGTTGTCCAGGGTTTAAGCAACCCGTTTGAAATTCGATAATTTTCCGTAGCTTGCGT
CAAGCTGTCAGTATTAGGGGTACTGATGAAGTTGTAAACCACCTCTTCTTCCCTAGGTTCGTCCAGAGGTTTC
AAAGGTTTATCTTCTTTGGCAAGAAGCAAAGAATGCCACGTGTTGCGTCGACAAAGCACCTTTTGGTATGGTT
AACCAAGATTCAGCAAATAACGGAGAGTACTGTTCTTTATTGAATGGGAGTCCGGTCCACTTTGTGGATACCC
GTGAATCTACATGGGTTAGAGATTTGCACGCCTCTCTTTAAATATGGTTCGTGTACCCTGCTTGGCTAGAAAG
CAATTGTTTGGTGTTGTCAACGCGCCGCCTCTGGCCAAGAAAACACCCCTTAGGCTTTGTTGGTAAAGTCAAG
ATGTGGAGAAGGAATGCCAGCAAGCATAAGCAGAGTCTCTTAGGGTCGGGCTTAAGATGGCAGCCAGAATTAG
AGCCTTTGAGTATGCTTGTGGGCATCTGGTTTTCACATCAACTCGTTTGGAGTATAAACAACTCGTGTGATGG
TGTAATCACGTAAAGCAATGACACCCGGGGTGTGCTTTCCCTAGTTAACGTAAGTACAGGTTGTTCTCCTATA
GGAACTAGAGCGTTGGTCTCAACTCAAAGAGAGACCCGTTTGAAAATCGATAAATTTCCGTAGCTTGCGTCAA

Figure 5 (continued)

GCTGCTGTTGTTAGGGGCAACAGTGAAGAATCATCATACCTCTTCTTCCCTAGGTTCGTCCAGAGGTTTCAGA
GTGATGGCTTCTTTGTCAAGAAGTAAAGAATGACACGTGTTGCGTCGACAATGCACCGTATGGTTTGGTTAGC
CATAAACATATATATTATATTAGATAGTTTTATCTGTGTGTTCTGATGATTTGAACTTACTTTCAATTATTAG
TGGCGACAGGAGAGGTTTGTCCTTTTACTCTTCTTGCTATGTTGGACACAAAAAGATTTTCTTTCTTTTATTT
TAAAAAAAAAA

Figure 6

AAGGAAATAAAAACATGCTCAGGAAGCTGGGTATGGGATCATATGGATTGCTTCGAGGCTCCCAAGACAAACA
AGCAATGGAGTTGTTTGACCCAAAAGATGTTGGGAAAATAACTTCTTTGTGGACTAGTTTTAAACAAAAATTT
GTTGAGTCAAAGGAGCATGCCAACTTATTCTTTCATTTATATGGGGTGTTATTTTTTATGGTACCACATGTAA
CAGGAGGAGAAGGCAGGGTCAAAATATGTTTGTGCTCGAGCAATGATCCAATGAATCCTGTGGTCCAAGAGAA
GACTTTGTCTTTGGCAGGTGGGGCACAGGCAGTTTTAATGAGTCCCAGCATCACATTGCCATTTCTTGAAAGA
GGGCCTATGTTTTATTACACTCTGGAATGCCAAGGTACACAAGCTCAAATTCCTTGCTCAGTTGTGGCAATCT
GGAAGCAAAAAATCGATACTCGCAGTGCAATCTATTCACAACAAGAAACTATGTCGTGGGCTATTGAAGCTCT
CAATAGACCACAATTTTTCCAAGATAGACAAGAGGCTGCACAATATATTGCATCTGTTTATTCGAGCGGGCAA
AGTTCACAAATGGCCCTGGAGAATAGAGCCTTCGTTGGTGAGCAACTTGGGGATACACGCATGAATGTGGTTA
GTGAGAGTACAATGCCAAGAAGTACTTCATTGCGGGTTCCCACTCTAAGAGTGCAGAGTAAACGATTTTCTTC
ACTGGAAATACCCAACTCTGCTTTGTTGCAACAGCTTGAGACTAGAGAGGAAACTGTGCAAGATGAGGATGAT
GGCGGTTTGTTTCCCATGAAGAAAGCATGCAGTCCCAACCACAGCATAATTTGGGATAATTTGAATATTGACA
CATTTGTGGACATGGAATTTCCACCTGATTGGACCGAGCGTACACTTGCCCAGCAAGTGCAATATGTTATGTT
CACGAAGGCCTCAAAGGGTTGTGTCATTGTTCCAAATCACATTGCAAAAAGGGAATCCCACAACATCAACCGT
GAGAcCATAACTCCAGACAATTACGAAAGTATATTGAAAGGATATGGCATCACCAAAGTTGGAAGTTTGTCTC
AGAGTAATGACTGGTATTCTTTAACTCTCAAACAAAGGGTCATAAACCTTGTGCACCAACGTGATCATCAATT
GTACATGGCAGGTGATTTTACCAATCCATTGCCCAGATTTGATTGTTATGATGGTTTGACTTTGGCAGAAAGA
CACATGAGAAACATCAGAGCCAGTTTTGCCACATCTGAGAGCAGCCAAATTAGAATGGCACAGACTACTGTTA
CTGCTAAAAGTGGTGTTTCCAATGAAAGAAATGTCCAGGATTCCTTTGTGTCCAAAACAGCAATGGAAAGCAC
TAGCGGGGAAAAGTCTATTCATATAGATACTTCCCACAGTGAAGAGGATGTGAAACCAGCCGATGTCATTTTT
GACTTCGGTAATGACATGGAGATTGGGGATGCATTGGAATTAGAAATGCAACAGCCAGTGTGTGTGGCTAGTA
ATGAATTTTTCAATGCAGGAGTCTTTGAGTTTGTGTGGGAAAAGAGTGCCCATGTCGCTGAACAAGTGATGGC
AATAGCTCTGCCAGCAGCCTTATTTTCCCAAAAGAAGGAGACATCAATGGGTGCTCAGATGCTTAAGTATTAT
GATGCAGCGCTCATAACTTATAAAATAATTTTATACGTTTCTGGTGTTGGTGCCATATCAGGTCAGCTTGCAC
TGGTGTGGGATGAATGCAATGTGTTGAATCGCAAGAAGGAATTTGTTAACATTGCAACGCTCTATGCTAGCAA
GCACACTCTGGTGTCTGCATCCCAACAGAACAGTGCTGAGTTTGTTTTTACGCCAACTGGAATTGGGAAATTT
GTACCTCTTGATGAAGGTACTGGAGCCATGGATCTTGGAAGTGTGAGGGTTTTTGTTACCCATCCCTTGTCCA
GTGCGACTGAGCTAACAGGTGTACCTTGTCATCTGCATCTACAGTGCAAGGTTTTGTCAACTAACATTTTGCA
GCCTCCAAGAGTGATAGCACAAACGCAGTATGGAATGAAGGCAGATCAGACATACTTCAAAAGTTTTCCAACT
AATCAGGTACTCCTCCATTATAACTGGAATACCACGGCTGTCATGGGAACAACTTTGGTGAGCATCTTTTCAC
CGTCTGGAATTTATGAAAGTGATGGCACACTACAACCATCCTTACTTGGCAACATAGCTCGCAATTGCAAGTG
GTGGACTGGTGTATGTGTGTTTGAGATATGCATTGAAAAGACCCTTTTCCATTCTGGAAGTTTGGCTATAGGA
CTTGGCACCCTGAACACAAAGATGTCAAGCGCACATGACATATTTAATATGCCATATGTGGTTTGCAATCTAG
AAATGGGAAGAAAGTTCCAATTCAAATGTGCCATTTCCAATTGGAATGGAAAGAACTTGTTATCAACCGGTAG
GAAAAGTTCTTTGCCGCGGCCAAACCACTATTCTCATTTGCGCCTTTTTGCCACAGTTATGAAACCTCTTGTG
TCAACTTCAGTTCATCTGGATTCAGTAGGGGTCACGGTGCAGTTGAAGAGGATAGAGGGTTTGACTCTTGGAG

Figure 6 (continued)

GCACAGTGTCTGTCAAACCTATTTATGGTCATTGGACAAAGGGCAAGAGCACCGTTGATTTCCTATTTTCGGA
AATGAACTTAGAACATAGGAAGGAGATTGAGAAACTCCGCAGGGTGAATGTGGAAGAGTATGACAAGAAAGGC
AAAGACAAGCCAGGAAAAGAAGGGCAAGTTTCAGTTTCCATTCGTGAAAAATTCACCTATGGTGCTGTACAAT
ATTTGTGCATGGGCTGGAAAGATGAAGAAAGATTATTGGTTTTGCCCTGTGCTCCATGGTCCATACGATTTGC
ACACACCAGTCCCGTTAAGGAGGCAATTACTTGTCCCTTTATAGACTGGTGCACTTCTTTTTGTTATTGGAGT
GGCAGTTTGCAATACTCCATTGTGTTGCATCGTGTTCAAGCTTCAGCAGATGTTGGAGGGATATTGAACGTGG
CCCTAGACTCCTCAGGTTATCCTTTTCCTGCAGGCATGCACGCAGGGAGTTACGTTGTATCAGCTGGAGGTGG
TTCAAAGTGGGACTTTTCTTACGGTGTTTCTACCAACACCTTTTCTTTTGTAGTGTCAGATGATGAGTTCTTC
CCAAGGAGACATACTCGTATGCGCGAGTTTTCAAAGAGCCAATCACGTATCATGTCCTTGCAGGATCGACTTG
GAAACTTGATAATAAATCTTCCACCAAAGGGCCTTATAAGTTCCATTGAGATTCTCATTGCTCCAGGTCCAGA
TTTTAAACTTGAAATTGCACAGCCTCCATCTGCCAATCATGAGAAGTTCTTGGGCAACATGCAAACACACACC
TATCTTTACACCCCAGATTTTTCTGAATTGAGAGATTTTGGGGGTTGAGAACTAACTATAAGTTATAGGAGCC
TTGGGTCAGGCATCACGACCCAACTTATTCAGATAATATGTGTACTGTAATTAATATTATCTGTGTTTAGTTA
GGTTAGTGAAGAATCATGCCCTTAACTTGCGGAGAGTAATGTTCTTAATTGAGCAAGAGTCCGGCCCACTCTA
TGGGTTCCCATGAATCTACATGGGTGGAGATTTGCACGCTTCTCCTTAAATGTGTTTCGTGTACCCTGCTTGG
ATTGAAAGCCCTCTACACCAGAGGTTAAACGGTCTCGCGTGATGGTTAACGGAGAGTACTGTTCTTTATTGAA
TGGGAGTCCGGTCCACTTTGTGGATACCCGTGAATCTACATGGGTTAGAGATTTGCACGCCTCTCTTTAAATA
TGGTTCGTGTACCCTGtCTTGGCTAGAAAGCAATTGTTTGGTGTTGTCAACGCGCCGCCTCTGGCCAAGAAAA
CACCCCTTAGGCTTTGTTGGTAAAGTCAAGATGTGGAGAAGGAATGCCAGCAAGCATAAGCAGAGTCTCTTAG
GGTCGGGCTTAAGATGGCAGCCAGAATTAGAGCCTTTGAGTATGCTTGTGGGCATCTGGTTTTCACATCAACT
CGTTTGGAGTATAAACAACTCGTGTGATGGTGTAATCACGTAAAGCAATGACACCCGGGGTGTGCTTTCCCTA
GTTAACGTAAGTACAGGTTGTTCTCCTATAGGAACTAGAGCGTTGGTCTCAACTCAAAGAGAGACCCGTTTGA
AAATCGATAAATTTCCGTAGCTTGCGTCAAGCTGCTGTTGTTAGGGGCAACAGTGAAGAATCATCATACCTCT
TCTTCCCTAGGTTCGTCCAGAGGTTTCAGAGTGATGGCTTCTTTGTCAAGAAGTAAAGAATGACACGTGTTGC
GTCGACAATGCACCGTATGGTTTGGTTAGCCATAAACATATATATTATATTAGATAGTTTTATCTGTGTGTTC
TGATGATTTGAACTTACTTTCAATTATTAGTGGCGACAGGAGAGGTTTGTCCTTTTACTCTTCTTGCTATGTT
GGACACAAAAAGATTTTCTTTCTTTTATTTTAAAAAAAAAA

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2009/050753

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/08 A01H1/04 C12Nl5/82 C12N7/00
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K AOlH C12N

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal , WPI Data, BIOSIS, FSTA, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	WO 2006/085749 A (RUITER SEEDS R & D B V DE [NL]; VAN DEN HEUVEL JOHANNES FRANCI [NL]; M) 17 August 2006 (2006-08-17) cited in the application	2-12, 14-19, 26-31
Y	Claims 1-31 SEQ ID NO 2	1
X	WO 2007/139386 A (RUITER SEEDS R & D B V DE [NL]; MARIS PAULUS CORNELIS [NL]; DE HAAN AN) 6 December 2007 (2007-12-06)	18,19
Y	Claims 1-29	1

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[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents

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

"E[1] earlier document but published on or after the international filing date

'L" document which may throw doubts on prionty claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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

[1]P" document published prior to the international filing date but later than the priority date claimed

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

"X[1] document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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

'&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 March 2010	08/04/2010

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P B 5818 Patentlaan 2 NL - 228O HV RiJSWiJk Tel (+31-70) 340-2040, Fax (+31-70) 340-3016	Obel , Nicol ai

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2009/050753

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VERBEEK M ET AL: "Tomato marchitez virus , a new plant picoma-l ike virus from tomato rel ated t o tomato torrado virus" ARCHIVES OF VIROLOGY ; OFFICIAL JOURNAL OF THE VIROLOGY DIVISIONOF THE INTERNATIONAL UNION OF MICROBIOLOGICAL SOCIETIES, SPRINGER-VERLAG, VI , vol . 153, no. 1, 29 October 2007 (2007-10-29) , pages 127-134, XP019592473 ISSN: 1432-8798	3,4,8-10
Y	Abstract Figure 2a Tabl e 1	1
X	VERBEEK M ET AL: "Identification and characteri sati on of tomato torrado virus , a new pl ant picorna-l ike virus from tomato" ARCHIVES OF VIROLOGY ; OFFICIAL JOURNAL OF THE VIROLOGY DIVISIONOF THE INTERNATIONAL UNION OF MICROBIOLOGICAL SOCIETIES, SPRINGER-VERLAG, VI , vol . 152, no. 5, 18 January 2007 (2007-01-18) , pages 881-890, XP019519437 ISSN: 1432-8798	3,4,8-10
Y	Abstract Figure 3	1
X	TURINA M ET AL: "A severe di sease of tomato i n the Cul i acan area (Sinaloa, Mexico) i s caused by a new picorna-l ike viral species" PLANT DISEASE, AMERICAN PHYTOPATHOLOGICAL SOCIETY, ST. PAUL, MN, US , vol . 91, no. 8, 1 August 2007 (2007-08-01) , pages 932-941 , XP009102894 ISSN: 0191-2917	8-12 , 14-17
Y	Abstract Page 936 col 1 l i ne 28 - col 3 l i ne 19 Figure 1 Table	1
X, P	WO 2008/150158 A (RUITER SEEDS R & D B V DE [NL] ; VAN DEN HEUVEL JOHANNES FRANCISCUS JOH) 11 December 2008 (2008-12-11) Cl aims 1-30	3,8-12, 14-19, 26-31

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INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2009/050753

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MEJIA ET AL: "EVALUATION OF TOMATO GERMPLASM FOR RESISTANCE TO GEMINIVIRUSES IN SANARATE. GT - CHOCOLATE SPOT DISEASE OF TOMATO IN GUATEMALA:" INTERNET CITATION, [Online] XP002384317 Retrieved from the Internet: URL :http ://www .pi antpath .wisc .edu/Gemi nivi rusResistantTomatoes/CDR/Mar03/ ChocSpot.htm> [retrieved on 2006-06-08] the whole document	1-16,18, 26-30
A	WO 98/44803 A (CORNELL RES FOUNDATION INC [US]) 15 October 1998 (1998-10-15) the whole document	1-12, 14-16, 18,26-30

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NL2009/050753

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 13, 20-25
because they relate to subject matter not required to be searched by this Authority, namely:

Claim 13 describes subject falling within the provision of Rule 39.1 (iv) as step c) comprises the harvesting of blood or splenocytes. Claims 20-25 describe an essentially biological process for the production of plants, Rule 39.1(iT) PCT.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/NL2009/050753

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WO 2006085749 A	17-08-2006	AU 2006213170 A1	17-08-2006
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