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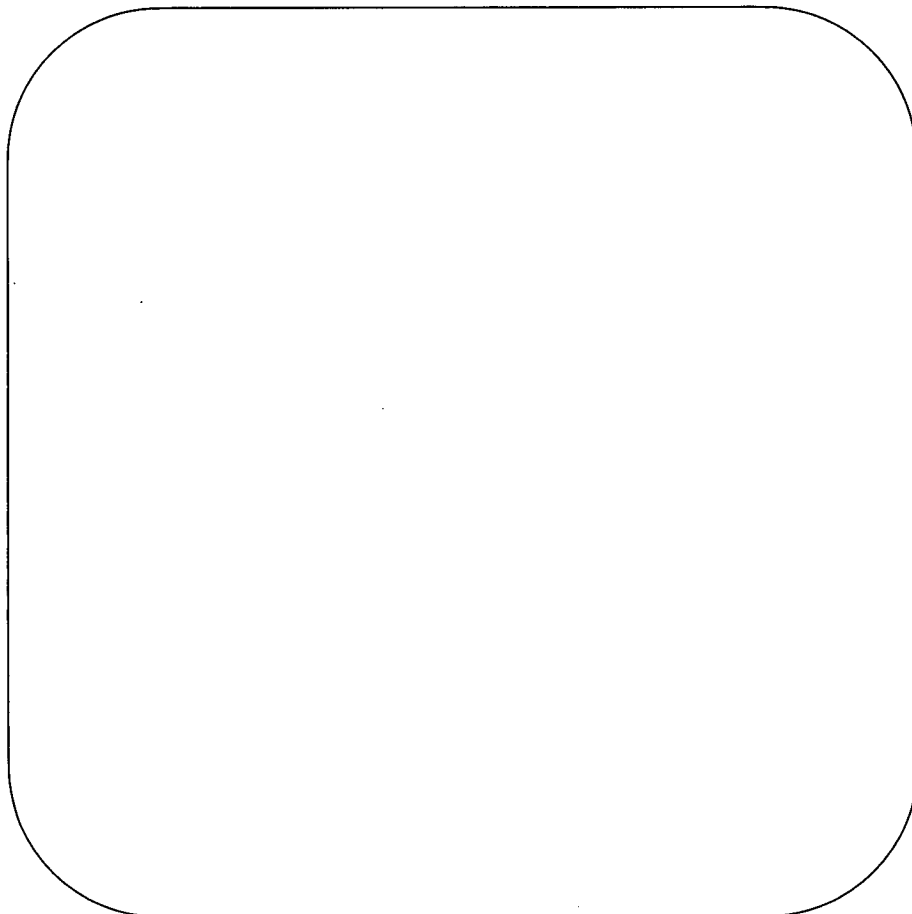
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Anexos:

- ☒ Comprobante de pago de la tasa de presentación de la solicitud por \$ 482.000.oo.
- ☐ Comprobante de pago por reivindicación de prioridad.
- ☐ Documento que acredita la existencia y representación legal cuando el solicitante sea persona jurídica.
- ☐ Poderes, si fuere el caso.
- ☐ Documento de cesión del inventor al solicitante o a su causante.
- ☐ Declaraciones.
- ☒ Copia de la solicitud internacional. (Resumen, descripción, reivindicaciones, dibujos)
- ☒ Traducción de la solicitud internacional al castellano. (Resumen, descripción, reivindicaciones, dibujos)
- ☐ Copia del examen preliminar efectuado por la IPEA.
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- ☐ De ser el caso, documento que acredite la licencia o autorización de uso de conocimientos tradicionales de las comunidades indígenas.
- ☐ De ser el caso, certificado de depósito del material biológico.
- ☐ Arte final 12x12
- ☐ De ser el caso, información sobre otras solicitudes de patente o títulos obtenidos en el extranjero por el mismo titular o su causante, relacionadas parcial o totalmente con la invención de esta solicitud.
- ☒ Otros: VER HOJA DE DOCUMENTOS ANEXOS

11

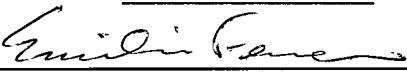
FIGURA CARACTERÍSTICA



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ning of each regular issue of the PCT Gazette.

(54) Title: PLANT VIRUS DESIGNATED TOMATO TORRADO VIRUS

(57) Abstract: The invention relates to the field of virology. The invention provides an isolated plant virus (ToTV) named Tomato
torrado virus (ToTV), and components thereof. The invention further relates to methods of producing a ToTV-resistant plant com-
prising the steps of identifying a ToTV-resistant donor plant, crossing said ToTV-resistant donor plant with a recipient plant, and
selecting from an offspring plant a resistant plant.

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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
5 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
10 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.

15 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
20 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
25 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.

5 SUMMARY OF THE INVENTION

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
10 *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).

15 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 phytosanitary precautions. At this moment no diagnostic tools are available. Consequently, there is a need for developing
20 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.

The invention provides in a first aspect a plant virus tentatively named Tomato torrado virus (ToTV), deposited with the Deutsche Sammlung von
25 Mikroorganismen und Zellkulturen (DSMZ) in Braunschweig, Germany, on 24 November 2004 under depositors reference number ToTV-E01 (DSM 16999).

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.

30 The first systemic symptoms consist of necrotic spots at the top of the

plant, starting at the base of the leaves of a leaflet. Necrotic spots expand and are surrounded by a light-green or yellow area (see Figure 1). Not all systemic infected leaves show symptoms, however, the virus can be detected in these leaves, for instance by electron microscopy. Fruits infected with ToTV exhibit necrotic rings. 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 (icosahedral) in shape with a diameter of approximately 28 nm (see Figure 2). Virus particles consist of at least three capsid proteins of approximately 23, 26 and 35 kDa (see Figure 3). Upon purification, the virus displays at least two visible bands in a cesium sulfate gradient. The upper visible band (virus top fraction) contains an RNA molecule of approximately 5.5 kb (more precisely 5.2 kb) and the lower visible band (bottom fraction) contains an RNA molecule of approximately 8 kb (more precisely 7.7 kb) (see Figure 4). The inoculation of both bands combined on tobacco plants results in an infection.

ToTV is mechanically transmissible to several *Nicotiana* species. A standard inoculation buffer (e.g. a 0.03 M phosphate buffer at pH 7.7) is suitable. For propagation of ToTV, *N. glutinosa*, *N. tabacum* and *N. benthamiana* are preferred. The tobacco species *N. hesperis* '67A' and *N. occidentalis* 'P1' are very sensitive to ToTV and show systemic symptoms after 3-4 days. These tobacco species become very necrotic in short time and are therefore more suitable for use as an indicator plant than as propagation host. *N. glutinosa* reacts with chlorotic local lesions and a systemic chlorosis and mild deformation of the leaves. *N. benthamiana* does not show local symptoms and reacts with systemic chlorosis and deformation of the leaves.

The invention further provides a virus comprising at least one nucleic acid sequence selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2 and sequences having a nucleotide sequence homology of at least 30, preferably at least 40, preferably at least 50%, preferably at least 60%, more preferably at least 70%, 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% thereto. Such viruses are also encompassed by the term ToTV as used herein.

In a preferred embodiment of a virus of the invention having the above referred sequence homology, said virus is associated with tomato diseases known under the names of "Torrado", "Marchitez" and/or "Chocolate spot disease", and/or said virus shows, based on numerical taxonomic analysis of taxonomic descriptors essentially as defined in Table 1, to be more closely related to the virus as defined in claim 1 than to any other viral isolate available in public collections, and said virus has as an essential characteristic that it is associated with a disease that causes necrotic lesions in tomato.

In another aspect, the invention provides an isolated or recombinant nucleic acid comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, sequences having a nucleotide sequence homology of at least 50%, preferably at least 70%, 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 SEQ ID NO:1 or SEQ ID NO:2, and their complementary strands and ToTV-specific fragments thereof. Such a nucleic acid may be obtained from a virus according to the invention.

In another aspect, the invention provides a polynucleotide capable of hybridizing under stringent conditions to the isolated or recombinant nucleic acid of the invention as described above.

In another aspect, the invention provides an isolated or recombinant polypeptide obtainable from a virus according to the invention, or a ToTV-

specific fragment thereof. In a preferred embodiment said polypeptide is selected from the group consisting of the 23, 26 and 35 kDa capsid proteins of ToTV and ToTV-specific fragments thereof.

In another aspect, the invention provides an antigen comprising a polypeptide or ToTV-specific fragment thereof according to the invention.

In another aspect, the invention provides an antibody specifically directed against an antigen according to the invention.

In another aspect, the invention provides a method for producing an antibody against ToTV comprising the steps of: a) providing a ToTV 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 an antibody obtainable by a method of producing an antibody against ToTV according to the invention.

In another aspect, the invention provides a method for identifying a viral isolate as a ToTV 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 ToTV 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 ToTV in a sample comprising determining in said sample the presence of a ToTV 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 ToTV-resistant plant comprising the steps of: a) exposing a plant or plant part to an infective dosage of ToTV, and b) identifying said plant as a ToTV-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) ToTV virus or ToTV genomic sequences are not present in said plant or plant part or the presence of ToTV 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 ToTV 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 ToTV-resistant plant comprising the steps of identifying a ToTV-resistant donor plant by performing either of the above methods for identifying a ToTV-resistant plant according to the invention, crossing said ToTV-resistant donor plant with a recipient plant (which recipient plant may be either ToTV-susceptible or ToTV-resistant, but is suitably a ToTV-resistant plant in case

the resistant phenotype is brought about by a recessive gene), and selecting from an offspring plant (e.g. an F₁, an F₂, and a selfed plant) a resistant plant by performing a method for identifying ToTV resistance in a plant as described above. In the instance that the resistance trait is a recessive trait, resistant
5 plants may be found among offspring plants of the selfings of the F₁ or F₂ or still further generations. In preferred embodiments of this aspect, said ToTV-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,
10 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 ToTV-resistant plant, preferably a tomato plant, eggplant plant, pepper plant, melon plant,
15 watermelon plant or cucumber plant, or part thereof, such as a seed, obtainable by a method of producing a ToTV-resistant plant according to the invention.

In another aspect, the invention provides a diagnostic kit for detecting the presence of ToTV in a sample or for identifying ToTV resistance in a plant
20 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.

25 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 ToTV, or parts of the ToTV viral genome, as an expression vector.

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

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

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DETAILED DESCRIPTION OF THE INVENTION

Definitions

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.

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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 ToTV 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 *Adenophorea*), but also larger animals such as for instance birds, rabbits and mice, fungi (i.e. phylum *Eumycota*, in particular fungi of the class *Phycomycota*), (parasitic) plants (including members of the family *Cuscutaceae*), pollen, seed, water, soil particles, and even human hands, equipment and shoes.

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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

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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 F1'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 ToTV-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.

5 "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
10 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".

15 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.

20 "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
25 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
30 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
5 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
10 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
15 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 ToTV 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
20 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
25 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 ToTV, an attenuated strain of ToTV may be used that is symptomless or that shows at least reduced symptom expression in an infected plant, relative to a virulent strain of ToTV. Methods of
30 producing attenuated virus may for instance include random mutagenesis of

the ToTV 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.

- 5 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.

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

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

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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

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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
5 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
10 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
15 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
20 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, PlotSimilarity, and FASTA in the Wisconsin Genetics Software
25 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%,
30 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
5 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)
10 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
15 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
20 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.
25 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.
30 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 0.1xSSC, 0.1% SDS, at about 65°C incubation/wash temperature, and moderately stringent conditions are incubations in solutions which contain approximately 1-2xSSC, 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 (T_m) 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
5 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 2×SSC at 40°C. Exemplary high stringency conditions include hybridization in 50%
10 formamide, 1M NaCl, 1% SDS at 37°C, and a wash in 0.1×SSC 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
15 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
20 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
25 "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
30 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
5 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
10 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
15 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
20 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
25 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
30 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)

5

Identification and taxonomy.

The invention provides an isolated virus comprising at least one nucleic acid sequence according to SEQ ID NO:1 and/or SEQ ID NO:2 and sequences having a nucleotide sequence homology of at least 30%, 35%, 40%, 45%, 50%,
10 55%, 60% or preferably 65% 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 ToTV virus isolate revealed no significant homologies with any of the known viral or non-viral sequences in
15 the GenBank and EMBL databases. Highest percentage homology found thus far was 46.5 % between the helicase motifs in ORF1 on SEQ ID NO:2 corresponding to ToTV RNA1 with helicase motifs of other plant viruses. (see Examples below).

It should be understood that homologies may be large when two
20 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
25 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 or SEQ ID NO:2, this may refer to the sequence homology between regions amongst common motifs, where homology is greatest, but also between the coding sequence of two
30 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 ToTV virus isolate 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 ToTV shows the highest level of homology with viruses from the genera *Sequivirus* and *Waikavirus* (*Sequiviridae*) and the genera *Cheravirus* and *Sadwavirus*. Viruses from these genera are distinguished on the basis of the number of viral RNAs - *Sequiviridae* have 1 RNA while Cheraviruses and Sadwaviruses have two RNAs - and the number of capsid proteins - Sadwaviruses have two CPs, Cheraviruses have three CPs. These criteria suggest that Tomato torrado virus is most likely to group within the genus *Cheravirus*. However, phylogenetic analyses using several different motifs from the putative RdRp and helicase proteins, position ToTV clearly separate from the genera *Cheravirus* and *Sadwavirus* and in fact suggest that it is more closely related to the *Sequiviridae*. Unfortunately, full sequences from only one Cheravirus (CRLV) and two Sadwaviruses (SDV and SMoV) are currently available. Preliminary data on vector transmission suggest that ToTV 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 most likely a novel genus.

Tabel 1: Taxonomic descriptors for the newly discovered ToTV 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
<i>A. Morphological properties of virions</i>
1. 28 nm in size
2. Spherical (icosahedral) in shape
3. Envelop absent
<i>B. Physical properties of the virions</i> (not investigated)
<i>C. Properties of the genome</i>
1. RNA type nucleic acid
2. Single stranded RNA
3. Linear RNA
4. Positive sense coding
5. At least 2 segments (RNA 2; SEQ ID NO:1 and RNA 1; SEQ ID NO:2)
6. 5.5 kb (more precisely 5.2 kb or 5389 nucleotides excluding the poly(A)tail) and 8 kb (more precisely 7.7 kb or 7793 nucleotides excluding the poly(A)tail)
7. 5' terminal cap unknown
8. 5' terminal covalently linked polypeptide unknown
9. Poly(A) tract present at both segments
10. Nucleotide sequence comparisons:
- RNA 1 contains an ORF1 with motifs typical of helicase (Hel), Protease and RNA-dependent RNA polymerase (RdRp). Level of homology with helicase and RdRp motifs of other viruses is described in detail in the Examples.
- RNA 2 contains two ORFs of which one has at its N-terminus a motif typical of putative movement protein (MP) and which contains at the C-terminus the sequences of the three capsid (coat) proteins (CP). The level of homology with capsid proteins of other viruses is described in detail in the Examples.
<i>D. Properties of the proteins</i>
1. 3 x CP; Hel; RdRp; Protease; putative MP
2. CPs: 35, 26 and 23 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 see Fig. 7
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 ToTV	Local symptoms / systemic symptoms
Chenopodium quinoa	- / -
Gomphrena globosa	- / -
Nicotiana benthamiana	- / c
Nicotiana clevelandii	- / c
Nicotiana glutinosa	- / c
Nicotiana glauca	nl / c, n, mf
Nicotiana glauca	nl / c, n, mf
Nicotiana glauca	- / - (la)
Nicotiana glauca	- / - (la)
Physalis floridana	nl / c, n, mf, do
c= chlorosis; n= necrosis; l= lesions; mf= malformation; do= die off; la= latent infection, -= no symptoms.	
2. Pathogenicity	
<u>Symptoms on natural host plant:</u> Necrotic lesions finalizing in burn-like, full necrosis of plant material and death of the plant; concentric rings of necrotic spots on fruits.	
<u>Association with disease:</u> Torrado; presumably also Chocolate spot; presumably also Marchitez	
3. Tissue tropism: 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 found by the present inventors that genomic sequences of the associated virus of a tomato disease in Central America (e.g. Mexico and Guatemala) by the name of "Chocolate spot disease", "Chocolate" or

- 5 "Marchitez" were identical to those of the herein described ToTV (see Example 2). Therefore, the causal agent associated with this disease is an aspect of the present invention.

As more viral sequences become available, both from non-ToTV viruses that may or may not be closely related thereto, or from viruses closely related
 10 to or essentially resembling the ToTV virus, phylogenetic analysis will prove a valuable way of determining the breadth of the taxon or clade of ToTV 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
5 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
10 novel isolate, the sequence of ToTV 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
15 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 ToTV 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
20 from reference strains (*e.g.* divergent isolates), test-strains (ToTV-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
25 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
30 (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
5 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
10 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 ToTV sequences as provided herein. Relatedness is generally expressed in terms of percentage sequence similarity, herein termed sequence
15 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
20 said virus are herein also provided. As a rule of thumb, a ToTV 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
25 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 ToTV (e.g. more than 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98%, or 99% sequence homology) are considered to
30 belong to the same virus. The ToTV virus of the present invention is therefore

a virus having at least 50 or 60% homology, preferably at least 70%, 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% homology to the protein or nucleic acid sequences provided herein and causes ToTV-induced disease-symptoms in *Solanaceae*, more particularly in *Solanum* and *Nicotiana*, which disease-symptoms may or may not be similar to those described herein. In *Cucurbitaceae* the virus appears not to cause visible symptoms although the virus is capable of propagation in the plants.

10 When one wishes to compare a separate virus isolate with the protein or nucleic acid sequences described herein, the invention provides an isolated virus (ToTV) identifiable as phylogenetically corresponding to ToTV 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
15 sequence homology of at least 60%, preferably at least 70%, 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
20 homology than these mentioned maximum values are considered as phylogenetically corresponding and thus taxonomically corresponding to ToTV 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
25 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 ToTV-viruses isolated from different sources.

 Also, the nucleotide or amino acid sequence of the ToTV virus or
30 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
5 sequence homology with the respective nucleotide or amino acid sequence of any non-ToTV virus or closest relative.

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

The invention provides an isolated virus (ToTV) identifiable as
10 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
15 sequences of SEQ ID NO:1 or SEQ ID NO:2, as listed herein for ToTV than it is related to a virus isolate of a non-ToTV 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 5.5 kb or 8 kb RNA fragments (respectively termed herein RNA2 and
20 RNA1) as described in the Example.

With the provision of the sequence information of this ToTV virus, the invention provides diagnostic means and methods to be employed in the detection of ToTV virus in a sample. Preferably, the detection of ToTV virus is performed with reagents that are most specific for ToTV virus. This by no
25 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 ToTV in plants, preferably in tomato plants, more preferably in plants of *S. lycopersicum*. Said method may for instance comprise determining in said
30

sample the presence of a ToTV virus or component thereof by reacting said sample with a ToTV 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.

5 The invention provides the partial nucleotide sequence of a novel isolated virus (herein also called ToTV virus) and ToTV virus-specific components or synthetic analogues thereof. Additional genomic sequences of the ToTV virus to those provided herein may be determined by sequencing methods known to the skilled person. Genomic sequence determination is well
10 within reach of the skilled person now that the present invention provides the ToTV 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
15 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
20 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 ToTV virus nucleic acid. Additional probes and primers capable of hybridising to a nucleic acid
25 sequence of the ToTV 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
30 nucleic acid according to the invention. To begin with, expression vectors such

as plasmid vectors containing (parts of) a double stranded sequence of the ToTV viral genome, viral vectors containing (parts of) the genome of ToTV (for example, but not limited to vaccinia virus, retroviruses, baculovirus), or ToTV virus containing (parts of) the genome of other viruses or other pathogens are
5 part of the present invention.

The expression vector may comprise a ToTV 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
10 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
15 expression vector according to the invention. Plasmid or viral vectors containing the nucleic acids encoding protein components of ToTV 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
20 other eukaryotic cells). Plasmid or viral vectors containing full-length or partial copies of the ToTV 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 ToTV.

25 The ToTV virus may be isolated from infected plants or other sources by any method available. Isolation may comprise purifying or partially purifying ToTV 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 ToTV may for instance be performed by using
30 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 ToTV. Such a mild purification method may for instance comprise homogenizing infected material of a plant (such as leaves) in a homogenization buffer (comprising for instance 0.1 M TRIS-HCl buffer at a pH of about 8, about 20 mM of sodium sulphite [Na₂SO₃], about 10 mM of sodium diethyldithiocarbamate [Na-DIECA] and about 5 mM of sodium ethylenediaminetetraacetate (Na-EDTA)), separating the debris by centrifugation (for instance for 30 min. at 49,000 g), placing the supernatant on a suitable sucrose cushion (*e.g.* 20%) and pelleting the viral particles (for instance by centrifuging for 1.5 h at 70,000 g). Thereafter, the pellet comprising the viral particles may be resuspended in a suitable buffer (*e.g.* in TRIS-HCl, pH 8), and the virus-containing fraction may be separated from the remainder of the suspension by using density centrifugation in a sucrose gradient (*e.g.* 10-40% sucrose in homogenization buffer centrifuged for 2 h at 110,000 g). The virus-containing fraction may be determined by using infection experiments. Further purification may occur by using density centrifugation in a cesium sulfate gradient (*e.g.* 10-40% cesium sulfate gradient in TRIS-HCl, pH 8, centrifuged for 16 h at 125,000 g). Enriched viral bands may then be collected from the gradient and further concentrated by centrifugation or dialysis (*e.g.* against 0.1 M TRIS-HCl, pH 8).

Infectivity of ToTV after purification may be checked by inoculation onto a sensitive plant (*e.g.* *N. hesperis* '67A').

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

Having prepared purified or partially purified ToTV 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 ToTV viral proteins, such as viral coat proteins, by either electrophoresis using for instance a sodium dodecylsulphate-polyacrylamide gel (SDS-PAGE) or by
5 affinity chromatography. Each of these methods will be described below.

Proteins of interest of ToTV 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
10 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
15 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.

20 Proteins of interest of ToTV may also be purified by affinity chromatography using an antibody (such as a monoclonal antibody) that specifically binds to a ToTV 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
25 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
30 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.

5 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).

10 With the teachings provided herein, the skilled person is capable of isolating a virus-specific protein of ToTV, 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
15 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

20 Antibodies, either monoclonal or polyclonal, can be generated to a purified or partially purified protein or peptide fragment of the ToTV 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
25 *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
30 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 1×10^5 - 1×10^7 M⁻¹.

A recombinant protein derived from the ToTV 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 ToTV protein that combines with any ToTV-specific antibody of a mammal exposed to or infected with ToTV virus. Preferred antigens of the invention include those that bring about the immune response in mammals exposed to ToTV, which therefore, typically are recognised most readily by antibodies of a mammal. Particularly preferred antigens include the capsid proteins of ToTV. 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 ToTV 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 ToTV antigen suitable to
5 produce an anti-ToTV antibody or fragment thereof. Of course ToTV 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
10 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 ToTV proteins. These antibodies are also useful for assaying for ToTV infection.

15 The present invention thus provides a ToTV-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
20 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 ToTV virus specific antibodies, whether *in vivo* (e.g. for providing diagnostic antibodies) or *in vitro* (e.g. by phage display technology or another
25 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 ToTV virus-specific functional fragment thereof, such as a capsid
30 protein according to the invention.

Methods for identifying a viral isolate as a ToTV virus

Apart from the detection of ToTV virus, which involves diagnostic methods, the present invention also relates to methods for identification, i.e. confirmation that the isolate is ToTV. 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 ToTV virus and non-ToTV 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 ToTV. An isolate having more than 50% sequence homology with SEQ ID NO:1 or SEQ ID NO:2 as provided herein is considered taxonomically corresponding to ToTV or belonging to the ToTV viral taxon. Such a virus is part of the present invention.

In order to identify a virus as a Tomato torrado virus (ToTV), 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 genus, of which the ToTV strain identified by the nucleic acid sequences of SEQ ID NOs: 1 and 2 provided herein, and deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH on 24 November 2004 under depositors reference number ToTV-E01 (DSM 16999) may be assigned as the type species. Thus, it is not essential that a sequence comparison is performed in order to assess that a virus is ToTV. Rather, a method of identifying a virus as a Tomato torrado virus (ToTV) 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 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 5.5 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 torrado, marchitez and/or chocolate spot.

The combination of taxonomic descriptors that results in a positive identification of a virus isolate as being a Tomato torrado virus (ToTV) is that combination which shows the isolate as being more closely related (based on numerically taxonomic methods well known to the skilled artisan) to ToTV as described herein, than to other viruses, and wherein said isolate preferably produces the disease symptoms in tomato typical of torrado 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 ToTV 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 ToTV viral taxon. Depending upon the phylogenetic relatedness, or distinctness with other

viral taxa, the ToTV 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 ToTV virus may be based on symptomatology, i.e. recognition of the virus by its disease-
5 symptoms.

However, in a preferred embodiment, antibodies of the invention are used in a method for identifying a viral isolate as a ToTV virus, provided cross-reactivity of such antibodies with related non-ToTV strains has been effectively ruled out. Such methods comprise the step of reacting said viral
10 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 ToTV virus or parts thereof (proteins, peptides). Preferably, infected cells or cell cultures are used to identify viral antigens using any suitable immunological method.
15 Specifically useful in this respect are antibodies raised against ToTV viral capsid proteins.

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

ToTV 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.

5 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 ToTV by nucleic acid amplification and/or probe
10 hybridization, the capsid protein of ToTV 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
15 sequence of the ToTV protein, nucleic acid hybridization probes and/or nucleic acid amplification primers may be designed and 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
20 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
25 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
30 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 Figs. 5 and 6 are the DNA equivalent of the viral RNA genome), a suitable detection method
5 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-
10 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
15 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
20 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.

25 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 ToTV genomic sequence or preferably at least capable of hybridizing under stringent conditions to the nucleic acid sequence of SEQ ID NO:1 or SEQ ID NO:2, or a
30 ToTV-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 MgCl₂). 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.

5 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.

10 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.

15 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
20 temperature of the final wash solution. For nucleic acid hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl (1984): $T_m = 81.5\text{ }^\circ\text{C} + 16.6 (\log M) + 0.41 (\% \text{ GC}) - 0.61 (\% \text{ form}) - 500/L$; where M is the molarity of monovalent cations, $\% \text{ GC}$ is the percentage of guanosine and cytosine nucleotides in the nucleic acid, $\% \text{ form}$ is the percentage of formamide in the
25 hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about $1\text{ }^\circ\text{C}$ for each 1 % of mismatching; thus, the hybridization and/or wash conditions can be adjusted to hybridize to sequences of the desired
30 identity. For example, if sequences with $> 90\%$ identity are sought, the T_m can

be decreased 10°C. Generally, stringent conditions are selected to be about 5 °C lower than the thermal melting point (T_m) 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 (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10 °C lower than the thermal melting point (T_m); 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 (T_m). Using the equation, hybridization and wash compositions, and desired T_m , 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 T_m 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 ToTV 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.

ToTV resistant plants

The invention further relates to a method for identifying a ToTV-resistant plant, or part thereof. There are various possibilities of identifying ToTV-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
5 an alternative embodiment only virus-detection means are used.

A first step of a method for identifying a ToTV-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 ToTV, the aim of which is to achieve an infection. The exposure may in many cases involve the establishment of
10 physical contact. An infective dosage may vary between plants and between ToTV-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.

15 Alternatively, infection may be achieved by, for instance:

- growing a healthy scion on a ToTV-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
20 coding region of the ToTV virus genome;
- the use of agro-infectious clones, such as *Agrobacterium tumefaciens* strains containing an expression vector harbouring a coding region of the ToTV virus genome.

In the context of the present invention, methods for exposing a plant or
25 plant part to an infective dosage of ToTV 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,
30 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.

5 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

10 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

15 leaves are preferably immediately washed with water.

A second step of a method for identifying a ToTV-resistant plant comprises identifying said plant as a ToTV-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

20 relative to a susceptible and/or sensitive control plant, and/or ii) ToTV virus or ToTV genomic sequences are not present in said plant or plant part or the presence of ToTV 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.

25 Determining the development of ToTV-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

30 severity of the symptoms is indicated.

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In addition to determining the development of ToTV-induced disease-symptoms or as an alternative thereto, depending on the type of ToTV-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
5 method may in principle be used. For instance, a method may be employed wherein a ToTV 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
10 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 ToTV-
15 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*):

- 20 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;
- 25 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.

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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.

10 In an alternative embodiment of a method for identifying a ToTV-resistant plant only virus-detection means are used. For instance, a ToTV-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 ToTV-resistant plant wherein step a) of exposing a plant or plant part to a infective dosage of ToTV, is performed passively (e.g. naturally). When such a method is performed it is preferred that a method for detecting the presence of ToTV in a sample according to the present invention is used wherein the presence of a ToTV 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 ToTV-resistant plant requires the use of either the virus of the present invention or a polynucleotide or antibody according to the present invention.

25 The invention further relates to a method of producing a ToTV-resistant plant, or part thereof. Once a ToTV-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 ToTV resistance, the transfer may
5 suitably occur by transferring complete chromosomes. Preferably, the ToTV-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,
10 such a susceptible parent will herein also be included in the term recipient plant.

In a method for producing a ToTV-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.

15 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
20 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 ToTV. For example, a protoplast from a ToTV-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
25 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.

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

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

Preferably, said ToTV-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 ToTV-susceptible plant, a ToTV sensitive plant or a ToTV 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 ToTV-resistant plant, or a part thereof, obtainable by a method of the invention.

As stated, a preferred embodiment of a method for producing a ToTV-resistant plant comprises the transfer by introgression of said resistance-conferring nucleic acid sequence from a ToTV-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 ToTV-resistance from the donor plant.

In one method, which is referred to as pedigree breeding, a donor plant that exhibits resistance to ToTV 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 F₁ 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 ToTV. 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 ToTV-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 ToTV-resistant plant, or a part thereof, obtainable by a method of the invention.

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

In one embodiment of a method for producing a ToTV-resistant plant, or part thereof, the present invention provides a method of producing a ToTV-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.

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

10 Determining the development of ToTV-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
15 reduction in severity of the symptoms is indicated.

 In a preferred embodiment, the presence of ToTV 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 ToTV virus or component thereof by reacting said plant or plant part with a polynucleotide according to the
20 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 ToTV virus infection by virological diagnosis.
25 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 ToTV infection comprising a ToTV virus, a ToTV virus-specific nucleic acid,
30 proteinaceous molecule or fragment thereof and/or an antibody according to

the invention, and preferably a means for detecting said ToTV virus, ToTV 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
5 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
10 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
15 fragment thereof can be identified as ToTV-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 ToTV viral
20 sequences and with known non-ToTV viral sequences (preferably the closest phylogenetic relative of ToTV is used) using for example phylogenetic analyses as provided herein. Depending on the degree of relationship with said ToTV or non-ToTV viral sequences, the component or synthetic analogue can be identified.

25 A kit for detecting a ToTV 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 ToTV, and preferably also includes a substantially purified ToTV protein or anti idiotypic antibody for use as a positive control.

30 Antiviral agents

The invention also provides methods to obtain an antiviral agent useful in the treatment of ToTV 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
5 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 ToTV-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
10 products, bacteria, fungus, insects and virus. Most of them are related to systemic acquired resistance (SAR). The present invention contemplates the use of the ToTV genome or a part thereof as an inductor of systemic acquired resistance in plants. The systemic acquired resistance may be directed to ToTV or to other diseases. In this aspect, ToTV, its genome, or resistance-conferring
15 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 ToTV infection in plants, and provides a pharmaceutical composition comprising an antiviral agent according to the invention, useful in
20 a method for the treatment or prevention of a ToTV 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
25 *Nicotiana* species can be infected with the ToTV virus, thereby showing disease-symptoms dissimilar to those found in tomato plants suffering from the ToTV 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 ToTV, or parts of the ToTV 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 ToTV 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 Isolation and characterization of ToTV from tomato plants.

Methods

Introduction

Samples of tomato plants from Spain were received for diagnostic research. Symptoms on tomato plants consisted of necrotic spots and chlorosis on the leaves and brown rings on the fruits. Serological tests (ELISA) pointed out the presence of *Pepino mosaic virus* (PepMV), but, considering the symptoms, it was likely that another, still undefined, agent was present.

Spherical virus particles were found in electron microscopic studies of infected leaf tissue.

Subsequently an infection test was conducted and multiple accessions of tomato were found to be susceptible for ToTV, several of which reacted with clear symptoms (necrosis of the leaves, beginning at the base of the individual leaflets (see Fig. 1).

5

Virus transmission and propagation

ToTV was isolated as described below from a diseased plant obtained from Spain. ToTV was mechanically transmissible to several *Nicotiana* species. A standard inoculation buffer (e.g. 0.03 M phosphate buffer, pH 7.7) proved suitable. Throughout this Example, ToTV was mechanically inoculated onto and propagated in *N. glutinosa* or *N. benthamiana*. Virus purification was carried out approximately 14 days after inoculation.

10

Virus purification

Several attempts were made to purify ToTV according standard protocols for e.g. nepoviruses or luteoviruses (with the aid of organic solvents). These protocols resulted always in loss of infectivity of the virus. Moreover, ToTV tended to aggregate when low temperatures were used in centrifugation steps (below 5°C).

15

Eventually a very mild purification method resulted in reasonable clean virus preparations on which further experiments could be carried out.

20

The following procedure was used to purify ToTV (all centrifugation steps were performed at 6°C). Infected leaves of *N. glutinosa* or *N. benthamiana* were homogenized in 5 parts of 0.1 M TRIS-HCl (pH 8) plus 20 mM Na₂SO₃, 10 mM Na-DIECA en 5 mM Na-EDTA (homogenization buffer) and the homogenate was centrifuged for 30 min. at 49,000 x g. The supernatant was placed on a 20% sucrose cushion and centrifuged for 1.5 h at 70,000 x g. The pellet was resuspended in 2 ml TRIS-HCl, pH 8, and the suspension was placed onto a sucrose gradient (10-40 % sucrose in homogenization buffer) and centrifuged for 2 h at 110,000 x g. Since a virus

25

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band was not visible, the gradient was aliquoted into discrete fractions and the presence of virus in each fraction was determined by inoculation of a portion of said fraction on leaves of *N. hesperis* '67A' as described herein and observing the occurrence of infection. The virus-containing fraction was placed onto a
5 10-40% cesium sulfate gradient (in TRIS-HCl, pH 8) and centrifuged for 16 h at 125,000 x g. The virus bands were collected and concentrated by centrifugation or dialyzed against 0.1 M TRIS-HCl, pH 8.

Infectivity of ToTV after each purification step was checked by inoculation onto *N. hesperis* '67A' (cesium sulfate gradient fractions were
10 dialyzed against TRIS-HCl, pH 8, prior to inoculation).

Electron microscopy

Virus suspensions were "mounted" on a grid coated with Formvar®, otherwise known as polyvinyl formal, stained with 2% uranylacetate and
15 examined in a Philips CM12 electron microscope.

PAGE analysis

Viral proteins were separated by 12% denaturing polyacrylamide gel electrophoresis (SDS-PAGE, Laemmli, 1970) and silver-stained.
20

Nucleic acid isolation and evaluation

Purified virus was concentrated by centrifugation (2 h at 115,000 g). Pellets were subjected to RNA extraction according to the Qiagen RNeasy MinElute Cleanup procedure (Qiagen, Hilden, Germany). RNA concentration
25 was determined in a UV- spectrophotometer (Beckman Coulter, Inc., Fullerton, USA).

RNA integrity was checked by agarose gel electrophoresis. After electrophoresis for 2 hrs at 60V, the RNA was stained using orthotoluidine blue.
30

cDNA synthesis and cloning

cDNA was synthesised using the Invitrogen Superscript Choice system (Invitrogen, Breda, The Netherlands) for cDNA synthesis according to the manufactures instructions. First strand cDNA was primed using either Oligo-
5 d(T) or random hexamer primers. After second strand synthesis, EcoRI adapters were ligated to facilitate cloning. Following phosphorylation of the EcoRI-adapted cDNA, unligated linkers were removed by column chromatography. The resulting cDNA was ligated in pBluescript II EcoRI pre-digested expression vector (Stratagene, La Jolla, USA) and the ligation mix
10 transformed to Top10 competent cells (Invitrogen).

The 5' terminal end of the ToTV sequence was determined using the 5'RACE System for Rapid Amplification of cDNA Ends (LifeTechnologies) using dCTP according to the manufacturer's instructions.

15 Analysis of cDNA

Upon transformation recombinant clones were analysed for the presence of inserts by PCR using both T3 and T7 specific primers. PCR products were analysed for size on a 1% agarose gel. Clones containing inserts of about 1500 nucleotides were used for further sequence analysis. Resulting sequence data
20 were analysed using the DNASTAR program package.

Determination of amino acid sequences of the three capsid proteins of ToTV.

Purified ToTV particles were loaded onto a denaturing PAGE gel and capsid proteins were separated. Separated capsid protein bands were isolated
25 from the gel, treated with trypsin and the digests were analyzed using a tandem mass spectrometer (MSMS) using essentially the method as described by Kinter & Sherman, 2000. This resulted in amino acid (AA) sequences of small peptides each of which showed homology with the amino acid sequence predicted from the RNA2 nucleotide sequence of ToTV. Comparison with other
30 virus sequence data was done with programs from the PHYLIP package.

Results

Virus transmission and propagation

For ToTV propagation *N. glutinosa* and *N. benthamina* could be used.

- 5 The tobacco species *N. hesperis* '67A' and *N. occidentalis* 'P1' are very susceptible to ToTV and showed symptoms after 3-4 days. These tobacco species became very necrotic in short time and were therefore considered more suitable as indicator plant than as propagation host. *N. glutinosa* and *N. benthamiana* react with chlorotic local lesions and a systemic chlorosis and
- 10 mild deformation of the leaves.

Virus purification

- Purification of virions from infected leaf tissue turned out to be rather difficult. ToTV cannot resist organic solvents and tends to aggregate when
- 15 centrifuged at low temperatures. The purification protocol used (see 1.2.) resulted in two visible virus-containing bands in the cesium sulfate gradient. The bands appeared in the bottom part of the gradient, indicating a rather high buoyant density in Cs_2SO_4 of equal to or greater than 1.4 g/cm^2 .

- The infectivity of ToTV is affected by cesium sulphate, but not
- 20 completely lost when the virus concentration in the starting material was high. Fractions of the cesium sulphate-gradient containing both virus bands were infectious. Infectivity of individual bands was not determined.

Electron microscopy

- 25 The two bands were examined by electron microscopy and both contained virus particles of about 28 nm in diameter (Fig. 2).

PAGE analysis

- Polyacryl Amide Gel Electrophoresis (PAGE) of purified virus fractions,
- 30 followed by silver-staining of the gel showed three capsid proteins (CP) of

approximately 23, 26 and 35 kDa (see Fig. 3, indicating purified virus top (TAgV-T) and bottom (TAgV-B) fractions).

Nucleic acid isolation and analysis of cDNA

5 RNA isolation of both virus bands together revealed two RNA's: approximately 5.5 kb and 8 kb (see Fig. 4). RNA isolation of the upper band resulted in a 5.5 kb RNA fragment only.

The 5.5 kb RNA from the top band was used as the template for cDNA synthesis and cloning. Sequence reactions were performed using forward and
10 reverse sequence primers (T3/T7 or M13F/M13R) on different clones. A 5' Rapid Amplification of c-DNA Ends (RACE) was performed to determine the exact 5'-end of the RNA. Analysis of the resulting sequence data, using the Lasergene® software package (DNASTAR, Inc., Madison, WI, USA), resulted in the complete sequence of RNA2 of the virus (SEQ ID NO:1; see Figure 5).
15 The size of the RNA is 5389 nucleotides excluding the poly A tail. On the RNA two open reading frames (ORF's) are found. ORF1 is located from nucleotide 182 to 742, is 561 nucleotides in length, and codes for a protein of 187 amino acids. No homologies were found for this sequence in the NCBI databases on protein and nucleotide level.
20 ORF2 stretches from nucleotide 702 to 4298, is 3597 nucleotides in length, and encodes a protein of 1199 amino acids. After a BLAST search in the NCBI database, low homologies were found with several viral polyproteins. For the found homologies, the accession numbers from the NCBI database are provided together with the virus name and the type of protein. Both the
25 nucleic acid sequences and derived amino acid sequences in all three reading frames were used in a BLAST analysis. MAPDRAW from Lasergene® software package was use to identify the ORFs.

ORF maps of both RNA's:

30 RNA1

RNA1 contains one ORF (ORF1) with motifs typical of helicase, RNA dependent RNA polymerase (RdRp). In addition a low level of amino acid (aa) sequence homology was observed with a protease co-factor (Pro-Co) of *Patchouli mild mosaic virus* (PatMMV) (NP647592.1), for the amino acid positions 106-338, with an identity of 22 %.

Typical Helicase motifs A (GKS), B (D), C (N) were identified at aa positions 398-400, 444 and 495 of the putative polypeptide.

For the helicase region the closest identities were found with *Rice tungro spherical virus* (RTSV; 42% identical in 140 aa overlap), *Maize chlorotic dwarf virus* (MCDV; 43% identical in 137 aa overlap), *Strawberry mottle virus* (SMoV; 42% identical in 135 aa overlap) and *Parsnip yellow fleck virus* (PYFV; 42% in 138aa overlap). In the putative VpG region no sequence similarity with other viruses was found.

The highest similarity in protease is found for aa 1000-1100, 25% identity is found with *Potato virus V* (PVV; NIa protease in 86 aa overlap).

The RdRp region between motifs I (KDE) to VII (FLSR) was found between aa 1303-1554 (Koonin 1991).

The closest identities of the polyprotein sequence were found with: *Rice tungro spherical virus* (RTSV) with 29% identity in a 751 aa overlap, *Maize chlorotic dwarf virus* (MCDV) with 28% identity in a 742 aa overlap, *Parsnip yellow fleck virus* (PYFV) with a 33% identity in 501 aa, *Apple latent spherical virus* (ALSV) with 32% identity in 472 aa, *Strawberry mottle virus* (SMoV) with 30% identity in 680 aa and *Cherry rasp leaf virus* (CRLV) with 33% in 465 aa

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Table 2. Overall levels of homology (in %) between the **RdRp** motifs in ORF1 on ToTV RNA1 with RdRp motifs of other plant viruses.

	NIMV	PYFV	RTSV	SDV	SMoV	ToTV	ALSV	CRLV	MCDV
NIMV	100	32.7	33.9	88.6	49.4	33.1	39.6	39.6	35.1
PYFV		100	43.7	33.2	32.0	35.2	36.4	36.0	42.9
RTSV			100	34.3	32.7	35.1	37.5	35.1	69.4
SDV				100	50.2	33.1	38.4	38.4	36.7
SMoV					100	35.7	40.6	38.5	38.5
ToTV						100	38.1	38.1	33.7
ALSV							100	79.5	35.5
CRLV								100	35.1
MCDV									100

5 *NIMV= Navel orange Infectious mottling virus (Sadwa); PYFV= Parsnip yellow fleck virus (Sequivirus); RTSV= Rice tungro sperical virus (Waikavirus); SDV= Satsumae dwarf virus (Sadwavirus); SMoV= Strawberry mottle virus (Sadwavirus); ToTV- Tomato torrado virus (genus proposed); ALSV= Apple latent spherical virus (Cheravirus); CRLV= Cherry rasp leaf virus (Cheravirus); MCDV= Maize chlorotic dwarf virus (Waikavirus)*

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Table 3. Overall levels of homology (in %) between the **helicase** motifs in ORF1 on ToTV RNA1 with helicase motifs of other plant viruses.

	SDV	SMoV	ToTV	ALSV	CRLV	MCDV	PYFV	RTSV
SDV	100	42.5	37.2	31.9	33.6	39.8	39.8	38.1
SMoV		100	42.5	31.0	31.9	32.7	33.6	32.7
ToTV			100	36.0	34.2	46.5	40.4	44.7
ALSV				100	86.7	34.5	31.0	30.1
CRLV					100	34.5	30.1	31.0
MCDV						100	39.8	69.0
PYFV							100	42.5
RTSV								100

PYFV= Parsnip yellow fleck virus (*Sequivirus*); RTSV= Rice tungro sperical virus (*Waikavirus*); SDV= Satsumae dwarf virus (*Sadwavirus*); SMoV= Strawberry mottle virus (*Sadwavirus*); ToTV- Tomato torrado virus (*genus proposed*); ALSV= Apple latent spherical virus (*Cheravirus*); CRLV= Cherry rasp leaf virus (*Cheravirus*); MCDV= Maize chlorotic dwarf virus (*Waikavirus*)

RNA2:

10 RNA2 contains two potential ORFs (Figure 7). ORF1 encodes a predicted protein of 187 aa with a molecular weight of 20 kDa. Sequence analysis revealed no homologies with any protein from the EMBL databases. It is not known whether this ORF codes for an actual protein.

15 The second ORF which partially overlaps with ORF1 starts with three ATG start codons in frame. It encodes a putative protein of 1198 aa with a predicted molecular weight of 134 kDa. Protein identification by mass spectrometry of the isolated coat proteins clearly maps the three coat protein cistrons at the C-terminus of RNA2-ORF2.

20 The N-terminal region of the RNA2-ORF2 polypeptide most likely codes for the putative movement protein (MP) since a motif LRVPML highly similar to the proposed movement protein consensus sequence LxxPxL (Mushegian,

1994) was found at aa position 262-267. No other sequence homologies were found in the N-terminus of the RNA2 ORF2.

ORF2 putatively encodes four proteins which are must be cleaved from the polyprotein precursor by proteolytic cleavage. However, no apparent
5 homologies with known polyprotein cleavage sites could be identified. The exact positions of these cleavage sites remain to be determined.

For CP1 polyprotein sequence we found only any homology with *Human parechovirus* (HPeV) with 21% identity in 103 aa.

The closest identities of the CP2 polyprotein sequence were found with:

10 *Rhopalosiphum padi virus* (RhPV) with 25% identity in 168aa, *Avian encephalomyelitis virus* (AEV) with 33% in 74 aa, *Black queen cell virus* (BQCV) with 43% identity in 37 aa and *Solenopsis invicta virus* (SINV-1) with 30% indentity in 51 aa. With the CP3 polyprotein sequence no homologies were found.

15

Untranslated regions (UTRs)

The 3'UTRs of RNA1 and RNA2 are 1210 nt and 1092 nt respectively. There is 98% identity in the final 988 nucleotides of both RNA's. To confirm that the 3'-regions of the UTRs of both RNAs are identical, RT-PCRs were
20 performed on total viral RNA with one reverse primer derived from the identical 3'-UTR region and two RNA specific forward primers. Resulting PCR products were sequenced.

From these results, the following was concluded: The virus isolated from
25 tomato plants and tentatively named Tomato torrado virus (ToTV) has spherical (icosahedral) particles of approximately 28 nm in diameter. Upon purification the virus displays at least two bands in a cesium sulphate-gradient. Both bands combined are infectious when inoculated on tobacco plants. Virus particles appear to consist of at least three capsid proteins of
30 approximately 23, 26 and 35 kDa.

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The cesium sulphate-gradient top fraction of the virus contains an RNA molecule of approximately 5.5 kb (RNA 2; SEQ ID NO:1) and the bottom fraction an RNA molecule of approximately 8 kb (RNA 1; SEQ ID NO:2).

cDNA synthesis and cloning, using the 5.5 kb RNA from the viral top fraction, and subsequent analysis of sequence information, resulted in the compilation of several contigs into SEQ ID NO:1. Two contigs clearly contained a poly-A tail suggesting the viral RNA has a poly-A tail. BLAST analysis of the nucleotide and derived amino acid sequences did not reveal any significant homology with any known virus from the EMBL database.

The information above indicates that ToTV is a new and so far undescribed virus. The information obtained so far does not yet allow a grouping of the virus in a particular virus family or genus.

For virus detection and identification purposes two RT-PCR-primer sets have been designed (Table 4) on the basis of the sequence of SEQ ID NO:1.

Table 4. RT-PCR primers for the detection of ToTV.

Primer set A:	length/ temp	Sequence	SEQ ID No.
forward primer	17-mer	5'-GAGAGCCGGCATTACACA-3'	SEQ ID NO:3
reverse primer	17-mer	5'-GCACAGCTTGGCGACAC-3'	SEQ ID NO:4
Product length	493 bp		
Optimal annealing temp.	54.8 °C		
Primer set B:			
forward primer	24-mer	5'-CCCATCATCACCTCCTCTTCGTA-3'	SEQ ID NO:5
reverse primer	22-mer	5'-TTCCAGTAATGATCCAACCAAT-3'	SEQ ID NO:6
Product length	585 bp		
Optimal annealing temp.	54.9 °C		

A suitable RT-PCR Protocol would comprise the following. Total RNA is isolated and purified from about 100 µg of infected leaf material using a RNA purification kit, such as for instance Qiagen RNA-Easy. Of this total RNA, an amount of about 1 µg of is used in a 50 µl reaction mixture of the Superscript One-Step RT-PCR reaction (Invitrogen), which reaction mixture further comprises 25 µl 2x reaction mix, 1 µl (100 ng) of both upper primer and down primer, 1 µl of RT/Taq mix and 22 µl of MilliQ water. In order to reverse transcribe the RNA into cDNA and amplify this cDNA the following RT-PCR programme may be used: Step 1: 30 min @ 50 °C (Reverse transcription reaction); Step 2: 3 min @ 94 °C (activation of Taq Polymerase); Step 3: 30 sec @ 94 °C; Step 4: 30 sec @ 55 °C; Step 5: 1 min @ 72 °C; Step 6: Repeat steps (3 to 5) 40x; Step 7: 10 min @ 72 °C; Step 8: 10 °C as long as needed. PCR products may be analysed on a 1% agarose gel in TAE or TBE buffer.

2.6. Determination of amino acid sequences of the three capsid proteins of ToTV.

Fragments of the largest coat protein (CP1: approximately 35 kDa) could be aligned with parts of an area in the ORF2 of RNA2 (AA 487-729). Fragments of the middle coat protein band (CP2: app. 26 kDa) could be aligned with an area of ORF2 of RNA2 between AA 730 and 983.

Fragments of the smallest coat protein (CP3: app. 23 kDa) could be aligned with the C-terminus of ORF2 of RNA2 (AA 984-1195). From these results it can be concluded that the coding sequences of the three capsid proteins are located on the ORF2 of RNA2 (5.5 kb) of ToTV, and thus that the isolated viral RNA molecules are part of the ToTV virus particles.

Example 2. Isolation and characterization of the causal agent of Marchitez.

In 2003, tomato plants grown in Central America (Mexico and Guatemala) with symptoms similar to symptoms of ToTV were found. The

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causal agent was suspected to be viral. The disease is locally known under the names "Chocolate", "Marchitez (virus)" or "Chocolate spot disease". Susceptible plants grown in the field become heavily infected from 2003 onwards.

The goal of the present investigation was to compare the sequence of the
5 so-far unknown virus causing the "Chocolate spot disease" to the sequence of ToTV as isolated in Example 1.

Methods

RNA was isolated from about 100 µg of "Chocolate spot disease" infected
10 leaf material using the Total RNA Isolation System (Promega SV 96). Two µl of the total RNA was used in a 50 µl reaction mixture of the Superscript One-Step RT-PCR reaction (Invitrogen), which reaction mixture further comprises 25 µl 2x reaction mix, 1 µl (100 ng) of both forward primer and reverse primer, 1 µl of RT/Taq mix and 22 µl of MilliQ water. In order to reverse transcribe the
15 RNA into cDNA and amplify this cDNA the following RT-PCR program was used: Step 1: 30 min @ 50 °C (reverse transcription reaction); Step 2: 3 min @ 94 °C (activation of Taq Polymerase); Step 3: 30 sec @ 94 °C; Step 4: 30 sec @ 55 °C; Step 5: 1 min @ 72 °C; Step 6: Repeat steps (3 to 5) 40x; Step 7: 10 min @ 72 °C; Step 8: 10 °C as long as needed. PCR products were analysed on a 1%
20 agarose gel in TAE or TBE buffer.

Different primer sets were used in the RT-PCR, which were known to anneal at different locations with the RNA1 or RNA2 of the ToTV genome.

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Table 5. RT-PCR primers based on the RNA-2 sequence of ToTV used for the characterization of the causal virus of Chocolate spot disease as used in Example 2 and as indicated in Figure 7.

Primer set P1048/1049:	Sequence	SEQ ID No.
forward primer	5'-CAAGCCATCACGGAACCTAC-3'	SEQ ID NO:7
reverse primer	5'-AGCATCTTCTTCCTCCGCT-3'	SEQ ID NO:8
Product length	From base 36 - 544 = 508 bases	
Primer set P1056/1057:		
forward primer	5'-TGCTGAGGTGCTATCACTGG-3'	SEQ ID NO:9
reverse primer	5'-CACACTTTCCACGATTTCCA-3'	SEQ ID NO:10
Product length	From base 2422 - 2955 = 523 bases	
Primer set P1060/1061:		
forward primer	5'-AAAGGAAAGAAGCAGCCACA-3'	SEQ ID NO:11
reverse primer	5'-GGAAATCTTGGTCAAGCCAG-3'	SEQ ID NO:12
Product length	From base 3599 - 4170 = 571 bases	
Primer set P1064/1065:		
forward primer	5'-GCAATGCCAGTGGTTCAGAG-3'	SEQ ID NO:13
reverse primer	5'-GGTCAAATGGATACTCGGGA-3'	SEQ ID NO:14
Product length	From base 4820 - 5327 = 507 bases	

5 Results

The primer sets used promoted the amplification of part of the ToTV sequence as expected, but the use of four different primer sets based on the RNA-2 sequence (P1048/1049, P1056/1057, P1060/1061 and P1064/1065) in the RT-PCR resulted also in amplification of part of the “Chocolate spot disease”

10 genome. The site of annealing of these primer sets is illustrated in Figure 1.

The RT-PCR-product of the four primer sets that amplified fragments of "Chocolate spot disease" were sequenced, and showed 100% identity with the corresponding parts of the sequence of the viral genome of the virus as described in Example 1.

LEGENDS TO THE FIGURES

Figure 1 shows a photograph of symptoms of the ToTV-disease on leaves of a tomato plant.

Figure 2 shows an electron micrograph of purified ToTV particles. Particles are about 28 nm in diameter.

Figure 3 shows the result of a silver-stained PAGE gel of purified ToTV virus top (TAgV-T) and bottom (TAgV-B) fractions indicating the three capsid proteins of approximately 23, 26 and 35 kDa.

Figure 4 shows the result of denaturing agarose gel electrophoresis. Sizes of the segments are indicated in kilobases (kb). The gel was stained using ortho-toluidine blue. M: molecular size standard. TAgV: 1 µg of isolated ToTV RNA.

Figure 5 shows the complete sequence of the ToTV RNA 2 molecule (SEQ ID NO:1).

Figure 6 shows the complete sequence of the ToTV RNA 1 molecule (SEQ ID NO:2)

Figure 7 shows the general structure of the ToTV virus indicating the site of annealing of the various primer sets as used in Example 2 to RNA 2.

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Claims

1. A plant virus named Tomato torrado virus (ToTV), deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH on 24 November 2004 under depositors reference number ToTV-E01 (DSM 16999).
2. A virus comprising at least one nucleic acid sequence selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2 and sequences having a nucleotide sequence homology of at least 50% thereto.
3. A virus according to claim 2, wherein said virus is associated with tomato diseases known under the names of "Torrado", "Marchitez" and/or "Chocolate spot disease", and/or wherein said virus, 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 than to any other virus, and wherein said virus is associated with a disease that causes necrotic lesions in tomato.
4. An isolated or recombinant nucleic acid or ToTV-specific fragment thereof comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, sequences having a nucleotide sequence homology of at least 50% to SEQ ID NO:1 or SEQ ID NO:2, and their complementary strands and ToTV-specific fragments thereof.
5. An expression vector comprising a nucleic acid according to claim 4.
6. A polynucleotide capable of hybridizing under stringent conditions to a nucleic acid according to claim 4.

7. An isolated or recombinant polypeptide obtainable from a virus according to any one of claims 1-3, or a ToTV-specific fragment thereof.
8. A polypeptide according to claim 7, wherein said polypeptide is selected from the group consisting of the 23, 26 and 35 kDa capsid proteins, the helicase, the RNA-dependent RNA polymerase and the putative movement protein (MP) of ToTV and ToTV-specific fragments thereof.
9. An antigen comprising a polypeptide according to claim 7 or 8.
10. An antibody specifically directed against the antigen according to claim 9.
11. A method for producing an antibody against ToTV comprising the steps of
 - a) providing a ToTV virus, 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.
12. A method according to claim 11, 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.
13. An antibody obtainable by a method according to claim 11 or 12.

14. A method for identifying a viral isolate as a ToTV virus comprising reacting said viral isolate or a component thereof with an antibody according to claim 10 or 13.
15. A method for identifying a viral isolate as a ToTV virus comprising reacting a viral isolate or a component thereof with a polynucleotide according to claim 6.
16. A method for detecting the presence of ToTV in a sample comprising determining in said sample the presence of a ToTV virus or component thereof by reacting said sample with a polynucleotide according to claim 6 or an antibody according to claim 10 or 13.
17. A method for identifying a ToTV-resistant plant, comprising the steps of:
- a) exposing a plant or plant part to an infective dosage of ToTV, and
 - b) identifying said plant as a ToTV-resistant plant 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
 - ToTV virus or ToTV genomic sequences are not present in said plant or plant part or the presence of ToTV virus is at least quantitatively reduced relative to a susceptible control plant.
18. A method according to claim 17, wherein in step b) the presence of ToTV in said plant or plant part is determined by performing a method according to claim 16.
19. A method of producing a ToTV-resistant plant comprising the steps of:

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- a) identifying a ToTV-resistant donor plant by performing a method according to claim 17 or 18,
- b) crossing said ToTV-resistant donor plant with a recipient plant, and
- c) selecting from offspring plants a resistant plant by performing a method according to claim 17 or 18.

20. Method according to claim 19, wherein said donor plant is a plant of the family *Solanaceae* or *Cucurbitaceae*.

21. Method according to claim 19, wherein said donor plant is a tomato plant, an eggplant plant, a pepper plant, a melon plant, a watermelon plant or a cucumber plant.

22. Method according to any one of claims 19-21, wherein said recipient plant is a plant of the family *Solanaceae* or *Cucurbitaceae*.

23. Method according to any one of claims 19-21, wherein said recipient plant is a tomato plant, an eggplant plant, a pepper plant, a melon plant, a watermelon plant or a cucumber plant.

24. Method according to any one of claims 19-21, wherein said recipient plant is a tomato plant of the species *Solanum lycopersicum*, more preferably an *S. lycopersicum* line that possess commercially desirable characteristics.

25. A ToTV-resistant plant, or part thereof, obtainable by a method according to any one of claims 19-24.

26. A diagnostic kit for performing a method according to claim 16, comprising at least one component selected from the group consisting of a virus according to any one of claims 1-3, a polynucleotide according to claim 6,

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a polypeptide according to claim 7 or 8, an antigen according to claim 9 and an antibody according to claim 10 or 13.

27. Use of a virus according to any one of claims 1-3, a polynucleotide according to claim 6, a polypeptide according to claim 7 or 8, an antigen according to claim 9 or an antibody according to claim 10 or 13 for the production of a diagnostic composition.

28. A diagnostic composition comprising a virus according to any one of claims 1-3, a polynucleotide according to claim 6, a polypeptide according to claim 7 or 8, an antigen according to claim 9 or an antibody according to claim 10 or 13.

29. Use of a virus according to any one of claims 1-3, its genome, or resistance-conferring parts thereof as antiviral agent.

30. Use of a virus according to any one of claims 1-3, or its genome, or parts thereof as expression vector.

31. Use of an attenuated form of a virus according to any one of claims 1-3, or its genome, or parts thereof, for premunition of a plant.

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Figure 1



Figure 2

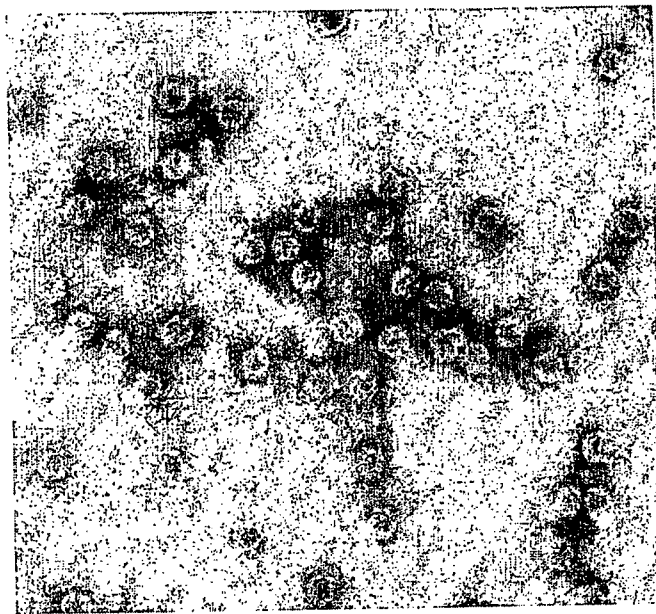
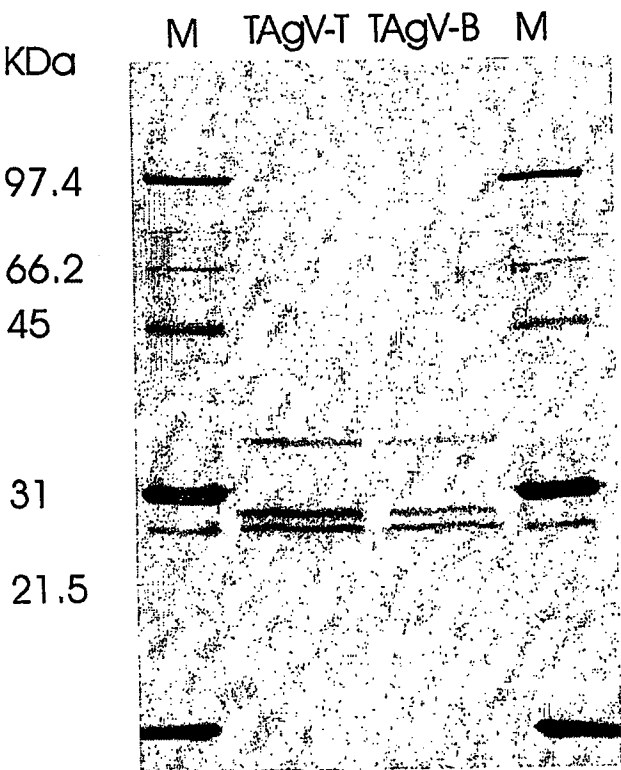


Figure 3



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Figure 4

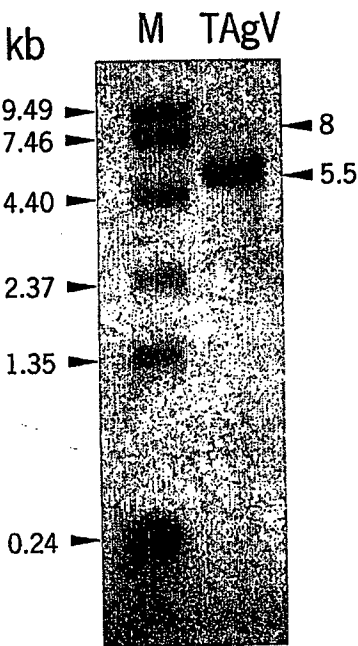


Figure 5

SEQ ID NO: 1

(RNA 2 complete sequence)

TTTAAAGAATAATTTTATACAATATTTATGTGATCCAAGCCATCACGGAACCTACGTCGTTCCCTA
AGATATTGTATCAATTATCTTTGCTGTTACCATTTTGGAGTGCACCGTCAACTGAAGGAACCTACGC
CGTTTCCAAGGACAGTGTATCTTTTGTATTATTTTGGTTACATCTCTATGTCATTTATTTCTCG
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Figure 5
(continued codings)

SEQ ID NO:1 (continued)

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Figure 6

SEQ ID NO:2

(RNA 1 complete sequence)

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Figure 6
(continued codings a)

SEQ ID NO:2 (continued)

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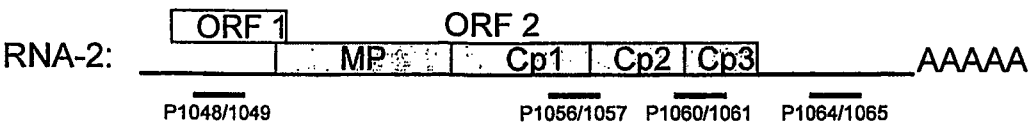
Figure 6
(continued codings b)

SEQ ID NO:2 (continued)

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Figure 7



VIRUS DE PLANTA DESIGNADO VIRUS TORRADO DEL TOMATE

CAMPO DE LA INVENCION

La presente invención está en el campo de las
5 enfermedades de las plantas. Más en particular, la invención
se refiere a un nuevo virus de planta aislado del tomate, a
los métodos para detectar dicho virus, a los métodos para
detectar las plantas resistentes y a los métodos para
producir plantas resistentes.

10

ANTECEDENTES DE LA INVENCION

El tomate *Solanum lycopersicum* (antiguamente
Lycopersicon esculentum) es susceptible a un gran número de
especies virales. Algunos de los virus del tomate más
15 prominente incluyen el virus del marchitamiento punteado del
tomate (TSWV; género *Tospovirus*); virus mosaico del pepino
(PepMV; género *Potexvirus*), y virus del rizo de la hoja
amarilla del tomate (TYLCV; género *Begomovirus*). El daño que
estas enfermedades infligen a la planta van desde la
20 decoloración de las hojas y lesiones necróticas, hasta
pérdida severa de la cosecha y muerte de la planta.

La habilidad para proporcionar plantas resistentes
es de la mayor importancia para los productores comerciales,
y para algunos de los virus económicamente más dañinos, han
25 sido producidas variedades de plantas resistentes. No

obstante, de cuando en cuando, surgen nuevos virus que pueden infligir daño considerable a las cosechas.

En 1996 fue reportado un nuevo virus del tomate que había infectado plantas de tomate en los Estados Unidos y en Italia desde 1993, y fue nombrado virus de la clorosis infecciosa del tomate (TICV; género *Crinivirus*; Duffus et al., 1996). Otro virus nuevo del tomate del mismo género fue reportado en 1998. Este virus mostró que había infectado plantas de tomate en los Estados Unidos desde 1989 y fue nombrado virus de la clorosis del tomate (ToCV; Wisler et al., 1998). Estos dos nuevos virus probaron ser difundidos por una mosca blanca, el insecto que es un vector de transmisión de enfermedades muy efectivo.

Se cree en general que la distribución geográfica de los virus conocidos se incrementará, y que nuevos virus continuarán apareciendo, parcialmente como resultado de la recombinación de diferentes virus para formar nuevas cepas y nuevos virus. El desarrollo de cultivos resistentes puede jugar un papel importante en el manejo exitoso de estas enfermedades.

BREVE DESCRIPCION DE LA INVENCION

Recientemente, fue descubierto un nuevo virus sobre las plantas del tomate en España, el cual provocaba síntomas que no pudieron ser atribuidos a ningún virus conocido. Las

plantas mostraron lesiones necróticas sobre las hojas y anillos cafés sobre las frutas, y mostraron crecimiento reducido. Las pruebas serológicas (ELISA) indicaron la presencia del virus mosaico del pepino (PepMV).

5 Investigaciones de microscopía electrónica revelaron más bien partículas en forma de varilla típicas de los potexvirus. No obstante, también fueron encontradas partículas virales de forma esférica en el tejido infectado de la hoja. Los inventores fueron capaces de separar el nuevo virus del

10 complejo con PepMV. El nuevo virus fue tentativamente nombrado virus torrado del tomate (ToTV).

Para ser capaces de rastrear su origen, de monitorizar su epidemiología y prevenir la posible dispersión de la enfermedad, es de mayor importancia ser capaces de

15 reconocer la enfermedad en una etapa temprana. Únicamente entonces pueden ser tomadas medidas suficientes para aislar las plantas e iniciar precauciones fitosanitarias. En este momento no están disponibles herramientas de diagnóstico. En consecuencia, existe una necesidad para desarrollar

20 herramientas de diagnóstico para esta enfermedad. Además, a la fecha no existen plantas conocidas que alberguen resistencia específica a este nuevo virus, mientras que exista una necesidad para desarrollar tales plantas resistentes.

25 La invención proporciona en un primer aspecto un

100

virus de planta tentativamente nombrado virus torrado del tomate (ToTV), depositado con la Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) en Braunschweig, Alemania, el 24 Noviembre de 2004 bajo el número de
5 referencia de los depositantes ToTV-E01 (DSM 16999).

El virus provoca síntomas de enfermedad en plantas de tomate así como en otras plantas, y puede provocar los síntomas por si mismo o en un complejo con otros virus o enfermedades.

10 Los primeros síntomas sistémicos consisten de manchas necróticas en la parte superior de la planta, comenzando en las base de las hojas de una hojuela. Las manchas necróticas se expanden y son rodeadas por un área verde claro o amarilla (ver Figura 1). No todas las hojas
15 infectadas sistémicas muestran síntomas, no obstante, el virus puede ser detectado en estas hojas, por ejemplo, mediante electroscopía electrónica. Las frutas infectadas con ToTV muestran anillos necróticos. El desarrollo de las plantas infectadas puede ser reducido en comparación a las
20 plantas no infectadas.

La descripción anterior se refiere a las plantas recién infectadas con el virus aislado, y necesariamente no reflejan los síntomas exactos encontrados en el campo. Factores tales como la raza o variedad de la planta, la etapa
25 de desarrollo, la presión adicional de la enfermedad, y

factores abióticos (por ejemplo, la temperatura y humedad relativa) eventualmente determinarán la expresión y características de los síntomas.

Las partículas virales son de forma esférica (icosahédricas) con un diámetro de aproximadamente 28 nm (ver Figura 2). Las partículas virales consisten de al menos tres proteínas de cápside de aproximadamente 23, 26 y 35 kDa (ver Figura 3). Después de la purificación, el virus muestra al menos dos bandas visibles en un gradiente de sulfato de cesio. La banda visible superior (fracción superior del virus) contiene una molécula de ARN (ácido ribonucleico) de aproximadamente 5.5 kb (más preferentemente 5.2 kb) y la banda visible inferior (fracción inferior) contiene una molécula de ARN de aproximadamente 8 kb (más precisamente 7.7 kb) (ver Figura 4). La inoculación de ambas bandas combinadas sobre las plantas del tabaco da como resultado una infección.

ToTV es mecánicamente transmisible a varias especies de *Nicotiana*. Un amortiguador de inoculación estándar (por ejemplo, un amortiguador de fosfato 0.03 M a pH 7.7) es adecuado. Para la propagación a ToTV, se prefieren *N. glutinosa*, *N. tabacum* y *N. benthamina*. La especie de tabaco *N. hesperis* "67A" y *N. occidentales* "P1" son muy sensibles a ToTV y muestran síntomas sistémicos después de 3-4 días. Estas especies de tabaco se vuelven muy necróticas

en corto tiempo y son por lo tanto adecuadas para el uso de planta indicadora que como hospedero de propagación. *N. glutinosa* reacciona con lesiones locales cloróticas y una clorosis sistémica y deformación leve de las hojas. *N. benthamiana* no muestra síntomas locales y reacciona con clorosis sistémica y deformación de las hojas.

La invención proporciona además un virus que comprende al menos una secuencia de ácido nucleico seleccionada del grupo que consiste de la SEQ ID No.: 1 y la SEQ ID No.: 2 y secuencias que tienen una homología de secuencia de nucleótidos de al menos 30, preferentemente al menos 40, preferentemente al menos 50%, preferentemente al menos 60%, más preferentemente al menos 70%, más preferentemente al menos 80%, todavía más preferentemente al menos 90%, todavía más preferentemente al menos 95%, todavía más preferentemente al menos 98%, y lo más preferentemente al menos 99% a ésta. Tales virus son también abarcados por el término ToTV como se utiliza en la presente.

En una modalidad preferida de un virus de la invención que tiene la homología de secuencia anteriormente referida, el virus está asociado con las enfermedades del tomate conocidas bajo los nombres "Torrado", "Marchitez" y/o "enfermedad de la mancha del chocolate", y/o el virus muestra, con base en el análisis taxonómico numérico de los descriptores taxonómicos esencialmente como se definen en la

tabla 1, que está más estrechamente relacionado al virus como se define en la reivindicación 1 que cualquier otro aislado viral disponible en colecciones públicas, y dicho virus tiene una característica esencial de que está asociado con una enfermedad que provoca lesiones necróticas en el tomate.

En otro aspecto más, la invención proporciona un ácido nucleico aislado o recombinante que comprende una secuencia de ácido nucleico seleccionada del grupo que consiste de la SEQ ID No.: 1, SEQ ID No.: 2, secuencias que tienen una homología de secuencia de nucleótido de al menos 50%, preferentemente al menos 70%, más preferentemente al menos 80%, todavía más preferentemente al menos 90%, todavía más preferentemente al menos 95%, todavía más preferentemente al menos 98%, y lo más preferentemente al menos 99% a la SEQ ID No: 1 o a la SEQ ID No:2, y sus hebras complementarias a fragmentos específicos de ToTV de las mismas. Tal ácido nucleico puede ser obtenido a partir de un virus de acuerdo a la invención.

En otro aspecto más, la invención proporciona un polinucleótido capaz de hibridarse bajo condiciones estrictas o severas al ácido nucleico aislado recombinante de la invención como se describe anteriormente.

En otro aspecto más, la presente invención proporciona un polipéptido aislado o recombinante obtenible a partir de un virus de acuerdo a la invención, a un fragmento

específico de ToTV del mismo. En una modalidad preferida el polipéptido se selecciona del grupo que consiste de las proteínas de cápside 23, 26 y 35 kDa de ToTV y fragmentos específicos de ToTV de las mismas.

5 En otro aspecto más, la invención proporciona un antígeno que comprende un polipéptido o Fragmento específico de ToTV del mismo de acuerdo a la invención.

 En otro aspecto más, la invención proporciona un anticuerpo específicamente dirigido contra un antígeno de
10 acuerdo a la invención.

 En otro aspecto más, la invención proporciona un método para producir un anticuerpo contra ToTV que comprende los pasos de: a) proporcionar un virus ToTV, o una (recombinante) proteína o fragmento peptídico de la misma; b)
15 la inmunización de un hospedero vertebrado apropiado con el virus, la proteína o el fragmento peptídico, y c) cosechar de la sangre (incluyendo el suero) o los esplenocitos del hospedero vertebrado, los anticuerpos contra el virus, la proteína o el fragmento peptídico. En una modalidad
20 preferida, el método comprende además los pasos de d) seleccionar un esplenocito productor de anticuerpo, e) fusionar dicho esplenocito a una línea celular de hibridoma inmortalizada y f) permitir que la fusión de hibridomas produzca anticuerpos monoclonales.

25 En otro aspecto más, la invención proporciona un

anticuerpo obtenible mediante un método para producir un anticuerpo contra ToTV de acuerdo a la invención.

En otro aspecto más, la invención proporciona un método para identificar un aislado viral como un virus ToTV, 5 que comprende hacer reaccionar el aislado viral o un componente del mismo con un anticuerpo de acuerdo a la invención.

En otro aspecto más, la invención proporciona un método para identificar un aislado viral como un virus ToTV 10 que comprende hacer reaccionar el aislado viral o un componente del mismo con un polinucleótido de acuerdo a la invención.

En otro aspecto más, la invención proporciona un método para detectar la presencia de ToTV en una muestra, que 15 comprende determinar en la muestra, la presencia de un virus ToTV o un componente del mismo, al hacer reaccionar dicha muestra con un polinucleótido o un anticuerpo de acuerdo a la invención.

En otro aspecto más, la invención proporciona un 20 método para identificar una planta resistente a ToTV que comprende los pasos de: a) exponer una planta o parte de la planta a una dosis infecciosa de ToTV, y b) identificar la planta como una planta resistente a ToTV cuando, después de la exposición, ya sea i) los síntomas de la enfermedad en la 25 planta o parte de la planta permanecen ausentes o son

retrasados en la expresión o son al menos reducidos en severidad con relación a una planta control susceptible, y/o ii) el virus ToTV o las secuencias genómicas de ToTV no están presentes en la planta o la parte de la planta o la presencia de virus ToTV es al menos cuantitativamente reducida en la planta con relación a una planta control susceptible. El paso a) incluye un periodo de incubación por una duración suficientemente larga para permitir el establecimiento de síntomas detectables de la enfermedad en plantas control susceptibles expuestas a una dosis infecciosa comparable del virus. Mediante la realización de este método todas las formas de resistencia, incluyendo la resistencia completa, la resistencia parcial, hipersensibilidad y tolerancia pueden ser identificadas en una planta. Con el fin de confirmar la tolerancia, la presencia (sistémica) del virus en la planta (células) debe ser confirmada. El paso b) puede involucrar la realización de un método para detectar la presencia de ToTV en una muestra de la planta o parte de la planta, de acuerdo a la presente invención, en donde se hace uso de un anticuerpo o polinucleótido de acuerdo a la invención en método estándares para ensayos de hibridación de nucleótidos o inmunoensayos, bien conocidos para las personas expertas en la técnicas. Alternativamente, el paso b) puede comprender la puesta en contacto de una parte de la planta expuesta, con una planta indicadora susceptible. De esta manera, se puede

detectar la presencia de una infección sistémica local en la planta expuesta, a través de una observación de la emergencia de la enfermedad en la planta indicadora, o incluso una planta indicadora contactada posteriormente, puesta en
5 contacto con la primera planta indicadora contactada.

En otro aspecto más, la invención proporciona un método para producir una planta resistente a ToTV, que comprende los pasos de identificar una planta donadora resistente a ToTV mediante la realización de cualquiera de
10 los métodos anteriores para identificar una planta resistente a ToTV de acuerdo a la invención, el cruce de la planta donadora resistente a ToTV con una planta recipiente (cuya planta recipiente puede ser ya susceptible a ToTV o resistente a ToTV, pero es adecuadamente una planta
15 resistente a ToTV en el caso en que el fenotipo resistente sea originado por un gen recesivo), y la selección a partir de una planta de progenie (por ejemplo, una F_1 , una F_2 , y una planta autopolinizada) una planta resistente mediante la realización de un método para identificar la resistencia a
20 ToTV en una planta como se describe anteriormente. En el caso en que el rasgo de resistencia sea un rasgo recesivo, las plantas resistentes pueden ser encontradas entre las plantas de progenie de las autopolinizaciones de las generaciones F_1 o F_2 o generaciones posteriores. En
25 modalidades preferidas de este aspecto, la planta donadora

resistente a ToTV y la planta recipiente son plantas de la familia *Solanaceae* o familia *Cucurbitaceae*. En otras modalidades preferidas de este aspecto, la planta recipiente es una planta de tomate, por ejemplo, berenjena, planta de
5 pimiento, planta de melón, planta de sandía o planta de pepino, más preferentemente, una planta de la especie *Solanum lycopersicum*, más preferentemente una línea de *S. lycopersicum* que posee características comercialmente deseables.

10 En otro aspecto más, la invención proporciona una planta resistente a ToTV, preferentemente una planta de tomate, una planta de berenjena, planta de pimiento, planta de melón, planta de sandía o planta de pepino, o una parte de las mismas, tal como una semilla, obtenible mediante un
15 método de producción de una planta resistente a ToTV de acuerdo a la invención.

En otro aspecto más, la invención proporciona un kit de diagnóstico para detectar la presencia de ToTV en una muestra, o para identificar la resistencia a ToTV en una
20 planta que comprende un virus, un polinucleótido, un polipéptido, un antígeno y/o un anticuerpo de acuerdo a la invención.

En otro aspecto más, la invención proporciona el uso de un virus, un polinucleótido, un polipéptido, un
25 antígeno o un anticuerpo de acuerdo a la invención, para la

producción de una composición de diagnóstico.

En otro aspecto más, la invención proporciona una composición de diagnóstico que comprende un virus, un polinucleótido, un polipéptido, un antígeno o un anticuerpo
5 de acuerdo a la invención.

En otro aspecto más, la invención proporciona el uso de ToTV, o partes del genoma viral de ToTV, como un vector de expresión.

En otro aspecto más, la invención proporciona el
10 uso de ToTV, o partes del genoma viral de ToTV, para producir resistencia en plantas, derivada de patógenos.

En otro aspecto más, la invención se refiere al uso de una forma atenuada de un virus ToTV, o su genoma, o parte del mismo, para la premunición de una planta.

15

DESCRIPCION DETALLADA DE LA INVENCION

Definiciones

Como se utiliza en la presente, el término "parte de planta" indica una parte de una planta, incluyendo las
20 células simples o tejidos celulares tales como células vegetales que están intactas en las plantas, grupos de células y cultivos de tejidos entre los cuales pueden ser regeneradas las plantas. Los ejemplos de partes de plantas incluyen, pero no están limitados a, células simples y
25 tejidos provenientes de polen, óvulos, hojas, embriones,

raíces, puntas de raíz, anteras, flores, frutos, brotes de los tallos y semillas; así como polen, óvulos, hojas, embriones, raíces, puntas de raíz, anteras, flores, frutos, tallos, brotes, retoños, reservas de raíz, semillas, 5 protoplastos, callos y similares.

El término "muestra" incluye una muestra proveniente de una planta, de una parte de planta o de un vector de expresión, o una muestra de suelo, de agua, o de aire.

10 El término "vector de transmisión" como se utiliza en la presente, se refiere al agente o sustancia que difunde la enfermedad. Los vectores de transmisión del ToTV en el campo pueden comprender, pero no están limitados a animales tales como artrópodos (en particular a aquellos de las clases 15 *Insecta* y *Arachnida*), Nemátodos (en particular aquellos de la clase adenoforeas), pero también animales superiores tales como por ejemplo, aves, conejos y ratones, hongos (por ejemplo, phylum *Eumycota*, en particular hongos de la clases *Phycomycota*), plantas (parasitarias) (incluyendo miembros de 20 la familia *Cuscutaceae*), polen, semillas, agua, partículas de suero, incluso manos humanas, kit y zapatos.

El término planta de "progenie" se refiere a cualquier planta resultante como progenie de una reproducción vegetativa o sexual de una o más plantas progenitoras o 25 descendientes de las mismas. Por ejemplo, una planta de

progenie puede ser obtenida mediante la clonación o autopolinización de una planta progenitora o mediante el cruce de dos plantas progenitoras e incluyen las autopolinizaciones así como las generaciones F1 o F2 o 5 generaciones posteriores. Una generación F1 es una progenie de primera generación producida a partir de los progenitores al menos uno de los cuales es utilizado por primera vez como donador de un rasgo, mientras que la progenie de la segunda generación (F2) o generaciones subsiguientes (F3, F4, etc.) 10 son especímenes producidos a partir de las autopolinizaciones de F1's, F2's, etc. Un F1 puede ser de este modo (y usualmente es) un híbrido que resulta de una cruce entre dos progenitores de crianza verdaderos (la crianza o producción verdadera es homocigota para un rasgo), mientras que un F2 15 puede ser (y usualmente es) una progenie que resulta de la autopolinización de dichos híbridos F1.

El término "resistente", como se utiliza en la presente, se refiere a una planta que es capaz de resistir la multiplicación en sus células y/o el movimiento (sistémico) 20 del virus hacia otras células y/o el desarrollo de los síntomas de la enfermedad después de la infección con el virus, cuyo virus es capaz de infectar y multiplicarse en variedades correspondientes no resistentes o susceptible de dicha planta. El término es utilizado para incluir formas 25 separadamente identificables de resistencia tales como

"resistencia completa", "inmunidad", "resistencia parcial ",
"hipersensibilidad" y "tolerancia".

"Resistencia completa" se refiere a una falla completa del virus para desarrollarse después de la
5 infección, y puede ser ya sea el resultado de la falla del virus para entrar a la célula (sin infección inicial) o puede ser el resultado de la falla del virus para multiplicarse en la célula e infectar células subsiguientes (sin infección subliminal, ni difusión). La presencia de resistencia
10 completa puede ser determinada por el establecimiento de la ausencia de partículas virales o ARN viral en las células de la planta, así como la ausencia de cualesquiera síntomas de la enfermedad en dicha planta, después de exposición de la planta a una dosis infecciosa del virus (por ejemplo, después
15 de la "infección"). Entre los productores, este fenotipo es a menudo denominado como "inmune". "Inmunidad" como se utiliza en la presente se refiere de este modo a una forma de resistencia caracterizada por ausencia de replicación viral incluso cuando el virus es activamente transferido hacia las
20 células mediante, por ejemplo, electroporación.

Una "dosis infecciosa" es definida como una dosis de partículas virales o ácido nucleico capaz de infectar una planta, cuya dosis puede variar entre las plantas y entre los aislados de ToTV probados. Teóricamente, una cantidad de
25 aproximadamente 1 a 10 hasta una cantidad de aproximadamente

500-5000 partículas virales del virus o de los ácidos nucleicos del mismo será suficiente. La infección de esta manera puede ser lograda mediante inoculación mecánica de las partículas virales purificadas o el ácido nucleico viral
5 sobre las plantas.

"Resistencia parcial" es denominada como la multiplicación reducida del virus en la célula, como el movimiento reducido (sistémico) del virus, y/o como el desarrollo reducido de los síntomas después de la infección.
10 La presencia de resistencia parcial puede ser determinada mediante el establecimiento de la presencia sistémica de bajo título de las partículas virales o el ARN viral en la planta y la presencia de síntomas de la enfermedad disminuidos o retardados en la planta, después de la exposición de la
15 planta a una dosis infecciosa del virus. Los títulos virales pueden ser determinados mediante el uso de un método de detección cuantitativo (por ejemplo, como un método de ELISA o una reacción en cadena de polimerasa de transcriptasa inversa, cuantitativa [RT-PCR], por sus siglas en inglés).
20 Entre los productores, este fenotipo es a menudo denominado como "resistente intermedio".

El término "hipersensible" se refiere a una forma de resistencia mediante la cual la infección permanece local y no se difunde sistémicamente, por ejemplo, debido a la
25 necrosis local del tejido infectado, o la falta de movimiento

sistémico más allá del tejido inoculado. Las plantas hipersensibles muestran síntomas de la enfermedad locales, pero severos, y la presencia local del virus puede ser establecida en tales plantas.

5 "Tolerante" se utiliza en la presente para indicar un fenotipo de una planta en donde los síntomas de la enfermedad permanecen ausentes después de la exposición de la planta a una dosis infecciosa del virus, mediante la cual la presencia de la infección viral sistémica o local, la
10 multiplicación del virus, al menos la presencia de las secuencias genómicas virales en las células de la planta y/o la integración genómica de las mismas, puede ser establecida. Las plantas tolerantes son por lo tanto resistentes para la expresión sintomática, pero son portadores asintomáticos del
15 virus. Algunas veces, las secuencias virales pueden estar presentes o incluso multiplicarse en plantas sin provocar síntomas de la enfermedad. Este fenómeno es también conocido como "infección latente". Algunos virus de ADN y ARN, pueden volverse no detectables después de una infección primaria
20 únicamente para reaparecer posteriormente y producir enfermedad aguda. En infecciones latentes, el virus puede existir en una forma oculta, no infecciosa, verdaderamente latente, posible como un genoma integrado o un agente episódico (de modo que las partículas virales no pueden ser
25 encontradas en el citoplasma, mientras que los protocolos de

PCT pueden indicar la presencia de secuencias de ADN viral) o como un agente infeccioso o que se replica continuamente. Un virus reactivado puede difundirse e iniciar una epidemia entre contactos susceptibles. La presencia de una "infección latente" es indistinguible de la presencia de un fenotipo "tolerante" en una planta.

El término "susceptible" es utilizado en la presente para referirse a una planta que no tiene resistencia al virus, dando como resultado la entrada del virus a las células de la planta y la multiplicación y dispersión sistémica del virus, dando como resultado síntomas de la enfermedad. El término "susceptible" es por lo tanto equivalente a "no resistente". Una planta susceptible muestra títulos virales normales en sus células después de la infección. La susceptibilidad puede ser de este modo determinada por el establecimiento de la presencia de títulos normales (por ejemplo, con relación a otras infecciones virales en plantas) de las partículas virales o del ARN viral en células de la planta, y la presencia de síntomas normales de la enfermedad (por ejemplo, con relación a los síntomas de la enfermedad como se describe en la presente para la planta de la cual fue primeramente aislado el ToTV) en la planta después de la exposición de la planta a una dosis infecciosa del virus.

El término "sensible" refleja la reacción

sintomática de una planta susceptible después de la infección del virus. La reacción o los síntomas pueden ser más o menos severas dependiendo del nivel de sensibilidad de la planta. Si la planta es dañada o incluso muerta por el virus, la
5 planta es calificada como "sensible".

Las plantas artificialmente inoculadas con cepas virales atenuadas son subsecuentemente protegidas de los virus virulentos estrechamente relacionados. Como virus protectores, las cepas leves de origen natural o una cepa
10 atenuada (un mutante leve artificialmente inducido) pueden ser utilizadas. Preferentemente, con el fin de lograr la premunición de una planta contra ToTV, puede ser utilizada una cepa atenuada de ToTV que sea asintomática o que muestre al menos expresión reducida de los síntomas en una planta
15 infectada, con relación a una cepa virulenta de ToTV. Los métodos de producción de virus atenuados pueden incluir por ejemplo la mutagénesis aleatoria del genoma de ToTV y la selección de cepas con atenuación sintomática. Se hace referencia expresa a los métodos para producir virus de
20 plantas atenuados como se describe en los artículo de Takeshita et al, 2001; Lu et al, 2001; Hagiwara, et al, 2002; e Hirata et al, 2003.

Como se utiliza en la presente, el término "tomate" significa cualquier planta, línea o
25 población de *Lycopersicon* o *Solanum* que incluye,

pero no está limitada a, aquellas presentadas en la lista siguiente. Recientemente, ha sido cambiada la nomenclatura del *Lycopersicon*.

La nueva nomenclatura para *Lycopersicon* es proporcionada en el siguiente listado (de: Peralta, Knapp y Spooner, monografía no publicada (ver: <http://www.sgn.cornell.edu> "Guide to revised *Solanum* nomenclature"))).

10	Nombre en la monografía del tomate (Peralta et al., en preparación para publicación en Systematic Botany Monographs) (Monografías Sistemáticas de Botánica)	Equivalente de <i>Lycopersicon</i>
	<i>Solanum juglandifolium</i> Dunal	<i>Lycopersicon juglandifolium</i> (Dunal) J.M.H. Shaw
15	<i>Solanum ochranthum</i> Dunal	<i>Lycopersicon ochranthum</i> (Dunal) J.M.H. Shaw
	<i>Solanum sitiens</i> I.M. Johnst.	<i>Lycopersicon sitiens</i> (I.M. Johnst.) J.M.H. Shaw
	<i>Solanum lycopersicoides</i> Dunal	<i>Lycopersicon lycopersicoides</i> (Dunal in DC.) A. Child ex J.M.H. Shaw
20	<i>Solanum pennellii</i> Correll	<i>Lycopersicon pennellii</i> (Correll) D'Arcy
	<i>Solanum habrochaites</i> S. Knapp & D.M.	<i>Lycopersicon hirsutum</i> Dunal
25	<i>Solanum "Nperuvianum"</i> para ser descrita por Peralta (4 razas geográficas: humifusum, lomas, Marathon, Chotano-Yamaluc)	Parte de <i>Lycopersicon peruvianum</i> (L.) Miller (incluye variedad de humifusum y razas Maratón)

5	<i>Solanum</i> 'Callejón de Huaylas' para ser descrita por Peralta	Parte de <i>Lycopersicon peruvianum</i> (L.) Miller (de Ancash, junto con Río Santa)
	<i>Solanum neorickii</i> D.M. Spooner, G.J. Anderson & R.K. Jansen	<i>Lycopersicon parviflorum</i> C.M. Rick, Kesiclti, Fobes & M. Holle
	<i>Solanum chmielewskii</i> (C.M. Rick, Kesicki, Fobes & M. Hollé) D.M. Spooner, G.J.	<i>Lycopersicon chmielewskii</i> C.M. Rick, Kesicld, Fobes & M. Hollé
	<i>Solanum corneliomuelleri</i> J.F. Macbr. (1 raza geográfica: Misti nr. Arequipa)	Parte de <i>Lycopersicon peruvianum</i> (L.) Miller; también conocida como <i>Lycopersicon glandulosum</i> C.F. Mull.
	<i>Solanum peruvianum</i> L.	<i>Lycopersicon peruvianum</i> (L.) Miller,
10	<i>Solanum ahilense</i> (Dunal) Reiche	<i>Lycopersicon ahilense</i> Dunal
	<i>Solanum cheesmaniae</i> (L. Riley) Fosberg	<i>Lycopersicon cheesmaniae</i> L. Riley (publicada como <i>cheesmanii</i>)
	<i>Solanum galapagense</i> S. Darwin & Peralta	Parte de <i>Lycopersicon cheesmaniae</i> L. Riley (previamente conocida como forma o variedad minor)
15	<i>Solanum lycopersicum</i> L.	<i>Lycopersicon esculentum</i> Millar
	<i>Solanum pimpinellifolium</i> L.	<i>Lycopersicon pimpinellifolium</i> (L.) Miller

Un "vector de expresión" es definido como una molécula de ácido nucleico que contiene un gen, usualmente un gen heterólogo, que es expresado en una célula hospedera.

20 Típicamente, este gen comprende una secuencia de codificación de la proteína. La expresión del gen es siempre colocada bajo el control de un promotor, y se dice que tal gen es "operablemente enlazado al" promotor. El término "heterólogo" se refiere a una molécula de ADN (ácido

25 desoxirribonucleico) o una población de moléculas de ADN que