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(54) Title: METHOD FOR PRODUCING A PLANT HAVING ENHANCED DISEASE RESISTANCE TO NEMATODES

(57) Abstract: The present disclosure provides a method for producing a plant having enhanced disease resistance to nematodes by introducing into said plant a Cf-2 gene as well as an Rcr3 gene encoding an RCR3 protein comprising certain amino acid modifications at certain positions as compared to a *Solanum lycopersicum* RCR3 protein. Alternatively, the endogenous RCR3 protein of said plant may be modified, e.g., using targeted mutagenesis.

Title: Method for producing a plant having enhanced disease resistance to nematodes

Field of the invention

The present invention is in the field of agriculture, in particular in the generation of nematode resistant plants.

Background of the invention

Dominant disease resistance genes encode highly specific immune receptors that offer plants protection against strains of pathogens carrying matching effector genes. This phenomenon is captured in the classical "gene-for-gene" model of recognition specificity in disease resistance genes that forms the basis of current disease resistance breeding in all major crops. Plant pathogens deliver effector proteins into the intercellular spaces and the cytoplasm of plant cells as virulence factors that target host molecules to enhance the disease susceptibility of the plant. Plant immune receptors can activate innate immune signaling upon direct recognition of unique non-self signatures in these pathogen-derived effector molecules. As opposed to directly recognizing effectors, most immune receptors in plants are thought to monitor the 'health' status of other host molecules as they react to disease-signaling perturbations in the virulence targets of pathogen effectors.

The relative contribution of the two immune receptor mechanisms - direct versus indirect recognition - to the disease resistance spectrum of plants is largely unknown. However, indirect recognition holds the promise to maximize the limited recognition repertoire of the innate immune system of plants. Unlike vertebrates, plants cannot adapt to novel pathogen-derived molecules by rapidly generating immune receptors with matching recognition specificities. Despite this lack of an adaptive immune system, plants are resistant to a myriad of potentially dangerous microbes, suggesting that they have evolved specific strategies to make more efficiently use of their innate immune receptor repertoire. Here, we demonstrate that 'guarding' key nodes in disease-signaling networks targeted by different pathogens enable plants to broaden the disease resistance spectrum of single immune receptors to multiple and unrelated pathogens.

Tomato plants carrying the Cf-2 receptor are resistant to strains of the leaf mold fungus *Cladosporium fulvum* secreting the effector protein Avr2. Cf-2 is thought to guard the apoplastic papain-like cysteine protease Rcr3, which is required for Cf-2-mediated resistance in tomato. The specific inhibition of Rcr3 by Avr2 activates Cf-2 function in immune signaling cascades resulting in effector-triggered immunity to the fungus. Tomato

secretes at least seven papain-like cysteine proteases into the apoplast, at least two of which are inhibited by Avr2 (i.e. Rcr3 and Pip1).

Despite the increasing body of information about disease resistance pathways, there is still a need to identify genes and proteins that can be used to create plants with durable, broad range disease resistance. It is an object of the invention to provide such nucleic acids, proteins and methods for creating plants, especially plants belonging to the family *Solanaceae,* with enhanced disease resistance.

Summary of the invention

In a first aspect, the present invention provides for a method for producing a plant having enhanced disease resistance to one or more nematodes compared to a control plant, said method comprising the steps of:

(a) introducing a Cf-2 gene into a plant or plant cell;

(b) introducing an Rcr3 gene encoding a RCR3 protein that binds to and/or is inhibited by Gr-VAP1 into a plant or plant cell; and

(c) regenerating a plant,

wherein at least one of steps (a) and (b) is carried out by means of transformation and/or transfection.

In an embodiment, said Rcr3 gene is the *Solanum pimpinellifolium* allele of the Rcr3 gene.

In an embodiment, both steps (a) and (b) are carried out by means of transformation and/or transfection.

In a second aspect, the present invention pertains to a method for producing a plant having enhanced disease resistance to one or more nematodes compared to a control plant, said method comprising the steps of:

(a) introducing a Cf-2 gene into a plant or plant cell by means of transformation and/or transfection;

(b) modifying the Rcr3 gene of the plant or plant cell such that it encodes a modified RCR3 protein comprising one or more of the following amino acids at the position of an endogenous RCR3 protein as compared to the amino acid position of RCR3 protein from *Solanum lycopersicum* (SEQ ID NO: 1): 197L, 222E, 229R, 241K, 284Q, 319F, and/or deletion of the amino acid corresponding to K122, and

(c) regenerating the plant.

In a third aspect, the invention is concerned with a method for producing a plant having enhanced disease resistance to one or more nematodes compared to a control plant, said method comprising the steps of:

(a) providing a plant or plant cell comprising a Cf-2 gene;

(b) modifying the Rcr3 gene of the plant or plant cell such that it encodes a modified RCR3 protein comprising one or more of the following amino acids at the position of an endogenous RCR3 protein as compared to the amino acid position of RCR3 protein from *Solanum lycopersicum* (SEQ ID NO:1): 197L, 222E, 229R, 241K, 284Q, 319F, and/or deletion of the amino acid corresponding to K122, and

(c) regenerating the plant.

In a further aspect, the invention is directed to a method for producing a plant having enhanced disease resistance to one or more nematodes compared to a control plant, said method comprising the steps of:

a) introgressing a Cf-2 gene into a plant or plant cell;

b) modifying the Rcr3 gene of the plant or plant cell such that it encodes a modified RCR3 protein comprising one or more of the following amino acids at the position of an endogenous RCR3 protein as compared to the amino acid position of RCR3 protein from *Solanum lycopersicum* (SEQ ID NO:1): 197L, 222E, 229R, 241K, 284Q, 319F, and/or deletion of the amino acid corresponding to K122, and

c) regenerating the plant.

In an embodiment, the endogenous Rcr3 gene of said plant or plant cell is modified using targeted mutagenesis.

The invention is also related to a method for producing a plant having enhanced disease resistance to one or more nematodes compared to a control plant, said method comprising the steps of:

(a) introducing a chimeric gene comprising a nematode-inducible promoter, operably linked to a nucleotide sequence encoding a Cf-2 protein into a plant or plant cell;

(b) introducing a chimeric gene comprising a nematode-inducible promoter, operably linked to a nucleotide sequence encoding a RCR3 protein that binds to and/or is inhibited by Gr-VAP1, such as a *Solanum pimpinellifolium* RCR3 protein or a variant thereof, into a plant or plant cell; and

(c) regenerating a plant.

In an embodiment, said Cf-2 gene is derived from *Solanum pimpinellifolium.*

The methods of the present invention may further comprise step:

(d) screening the regenerated plant, or a plant derived therefrom by selfing or crossing, for resistance to one or more plant pathogens and identifying a plant comprising enhanced resistance to one or more of said plant pathogens.

The nematode may be selected from a species selected from the group consisting of *Meloidogyne incognita, M. javanica, M. arenaria, M. hapla, M. mayaguensis, Globodera*

rostochiensis, G. pallida, G. Mexicana, M. graminicola, M. chitwoodi, M. fallax, Heterodera schachtii, H. zeae, and *H. glycines.*

Said plant or plant cell may be a dicotyledonous plant or a plant cell derived from a dicotyledonous plant, in which case said dicotyledonous plant preferably belongs to the family of Solanaceae, preferably to the genus of *Solanum,* preferably to the species *Solanum lycopersicum.*

In another aspect, the present invention is directed to a plant, plant cell, seed or fruit, obtainable or obtained by any method of the invention.

In a further aspect, the present invention is directed to the use of a modified Rcr3 gene of a *Solanum lycopersicum* plant or plant cell, said modified Rcr3 gene encoding a modified RCR3 protein comprising one or more of the following mutations with respect to an endogenous *Solanum lycopersicum* RCR3 protein (SEQ ID NO:1): 197L, 222E, 229R, 241K, 284Q, 319F, and/or deletion of K122, and a Cf-2 gene, for the generation of a plant having enhanced disease resistance to one or more nematodes compared to a control plant.

The plant or plant cell may be a *Solanum lycopersicum* plant or plant cell.

Said Cf-2 gene may be derived from *Solanum pimpinellifolium.*

The invention is further concerned with a plant, preferably a *Solanum lycopersicum* plant, or hybrid plant, plant cell, seed or fruit, obtainable or obtained by a method comprising the steps of:

a) preparing a first hybrid plant comprising a Cf-2 gene, preferably a *Solanum pimpinellifolium* Cf-2 gene, by crossing a plant comprising said Cf-2 gene, preferably a *S. pimpinellifolium* plant comprising said Cf-2 gene, with a sexually compatible plant, preferably a *Solanum lycopersicum* plant and selecting first hybrid plants comprising said Cf-2 gene, and

b) modifying an Rcr3 gene of the plant, preferably using targeted mutagenesis, such that it encodes a modified RCR3 protein comprising one or more of the following amino acids at the position of an endogenous RCR3 protein as compared to the amino acid position of RCR3 protein from *Solanum lycopersicum* (SEQ ID NO:1): 197L, 222E, 229R, 241K, 284Q, 319F, and/or deletion of the amino acid corresponding to K122.

Additionally, the invention deals with a plant, plant cell, seed or fruit, obtainable or obtained by a method comprising the steps of:

a) introgressing a Cf-2 gene, preferably a *Solanum pimpinellifolium* Cf-2 gene into a plant, preferably a *Solanum lycopersicum* plant, and

b) modifying an Rcr3 gene of the plant, preferably using targeted mutagenesis, such that it encodes a modified RCR3 protein comprising one or more of the following

amino acids at the position of an endogenous RCR3 protein as compared to the amino acid position of RCR3 protein from *Solanum lycopersicum* (SEQ ID NO:1): 197L, 222E, 229R, 241K, 284Q, 319F, and/or deletion of the amino acid corresponding to K122.

Said method may further comprise the step of regenerating the plant.

The method may further comprise the step of screening the regenerated plant, or a plant derived therefrom by selfing or crossing, for resistance to one or more nematode species and identifying a plant comprising enhanced resistance to one or more of said nematode species.

In an aspect, the present invention provides a nematode-resistant plant, preferably a *Solanum lycopersicum* plant, comprising an introgression comprising a Cf-2 gene, preferably an introgression from a *Solanum pimpinellifolium* plant comprising said Cf-2 gene, said plant further comprising a modified endogenous Rcr3 gene, wherein said endogenous Rcr3 gene is modified such that it encodes a modified RCR3 protein comprising one or more of the following amino acids at the position of an endogenous RCR3 protein as compared to the amino acid position of RCR3 protein from *Solanum lycopersicum* (SEQ ID NO:1): 197L, 222E, 229R, 241K, 284Q, 319F, and/or deletion of the amino acid corresponding to K122.

In a further aspect, the invention provides a nematode-resistant plant, preferably a Solanum lycopersicum plant, comprising an introgression comprising a Cf-2 gene, preferably an introgression from a Solanum pimpinellifolium plant comprising said Cf-2 gene, said plant further comprising an Rcr3 gene, wherein said Rcr3 gene encodes a RCR3 protein comprising one or more of the following amino acids at the position of the RCR3 protein from Solanum lycopersicum (SEQ ID NO:1): 197L, 222E, 229R, 241K, 284Q, 319F, and/or deletion of the amino acid corresponding to K122.

Additionally, the invention provides a nematode-resistant plant, preferably a *Solanum lycopersicum,* plant, comprising an introgression comprising a Cf-2 gene, preferably an introgression from a *Solanum pimpinellifolium* plant comprising said Cf-2 gene, said plant further comprising a modified Rcr3 gene, wherein said Rcr3 gene is modified such that it encodes a modified RCR3 protein that binds to and/or is inhibited by Gr-VAP1.

The plant may be selected from a *Solanum lycopersicum, Solanum peruvianum, Solanum cornelliomuelleri, Solanum chilense, Solanum pennellii, Solanum habrochaites* and *Solanum chmielewski* plant.

Figures

The invention is illustrated by means of the following Figures, showing:

Fig. 1. *G. rostochiensis* effector Gr-VAP1 interacts with the apoplastic papain-like cysteine protease Rcr3pim of tomato. Coimmunoprecipitation of Rcr3^{pim} (Rcr3^{pim}-His-HA in 1A) and Rcr3^{lyc} (Rcr3^{lyc}-His-HA in 1B), with and without prior treatment with the protease inhibitor E-64, by either Gr-VAP1 of *G. rostochiensis* (His-FLAG-Gr-VAP1), Avr2 of *C. fulvum* (His-FLAG-Avr2), or a bacterial alkaline phosphatase (FLAG-BAP). Rcr3^{pim} and Rcr3^{lyc} were detected in the immunoprecipitate (IP) with anti-HA serum using different exposure times (t). (C) Bimolecular fluorescence complementation in *Nicotiana benthamiana* leaf mesophyll cells following the transient co-expression of Rcr3^{pim} fused to the carboxyl terminal fragment of the yellow fluorescent protein YFP (Rcr3^{pim}-YFPc) with either Gr-VAP1 including its signal peptide fused to the amino terminal fragment of YFP (Gr-VAP1-YFPn) or the signal peptide of the pathogenesis-related protein PR1a fused to the amino terminal fragment of YFP (PR1aSP-YFPn). (M, cell membrane; N, perinuclear endoplasmic reticulum and C, cytoplasmic strands).

Fig 2. Gr-VAP1 inhibits binding of the protease activity probe DCG-04 to plant apoplastic papain-like cysteine proteases. DCG-04 protease activity profiling on apoplastic fluids of agroinfiltrated *Nicotiana benthamiana* leaves transiently overexpressing apoplastic cysteine proteases of *Solanum pimpinellifolium* (Rcr3^{pim}), S. *lycopersicum* (Rcr3^{lyc}, Pip1^{lyc}, CatB^{lyc}, Cyp3^{lyc}, and C14^{lyc}) and S. *tuberosum* (C14^{tub}). The apoplastic fluids isolated from agroinfiltrated leaf areas were first incubated for 30 min with either Gr-VAP1 or Avr2. Fluorescent DCG-04 (2 μM) was subsequently added to bind to the remaining available active sites in the cysteine proteases. Bound DCG-04 was visualized on western blots in a fluorescence scanner. Treatments with egg white cystatin, apoplastic fluids from agroinfiltrations with the empty binary expression vector (Empty vector), and with buffer alone (Buffer) were included in the DCG-04 activity profiles as controls.

Fig. 3. The immune receptor Cf-2 and Rcr3^{pim} confers resistance to *Globodera rostochiensis* in tomato. **(A)** Nematode susceptibility of five tomato genotypes with (Cf-2) and without the Cf-2 gene (Cf-0). Cf-0 and Cf-2 were either combined with the Rcr3^{pim} allele of *Solanum pimpinellifolium,* the Rrc3^{lyc} allele of S. *lycopersicum,* or with the Rcr3^{pim} null mutant allele rcr3-3. Nematode susceptibility is expressed as the number of *G. rostochiensis* individuals developing into cysts per plant at 21 days post inoculation. Bars indicate standard error over at least 10 replicates. Different characters indicate statistical differences as determined with an ANOVA (P-values <0.001). **(B and C)** Resistance to *G. rostochiensis* in Cf-2/Rcr3^{pim} tomato plants involves a hypersensitive response type of cell death (HR) in the feeding site of the nematode at the 7 days post inoculation. The arrows indicate the typical local cell wall dissolution inside the feeding sites of G. *rostochiensis.* Scale bars: 5 μm.

Fig. 4. Gr-VAP1 elicits a Cf-2-mediated and Rcr3^{pim}-dependent hypersensitive response in tomato leaves. **(A)** Transient co-expression of Gr-VAP1 (V), Avr2 (A), or the corresponding empty binary vector (E) in leaves of the tomato genotypes Cf-2/Rcr3^{pim}, Cf-2/rcr3-3, Cf-0/Rcr3^{pim}, and Cf-0/Rcr3^{lyc}. **(B)** Transient co-expression of Gr-VAP1 (V), Avr2 (A), or the empty binary vector (E) with or without Rcr3^{pim} (R) in leaves of the tomato genotypes Cf-2/rcr3-3 and Cf-0/rcr3-3. Leaves were harvested 5 days post infiltration.

General definitions

"HR" refers to the hypersensitive response, i.e. local plant cell death, seen as either microscopic lesions (as described by Rivas and Thomas, 2005, Ann Rev Phytopath 43: 395-436) and/or macroscopic lesions. Hypersensitive cell death is usually associated with other plant responses, such as production of reactive oxygen species and the activation of defense related genes in cells surrounding the HR lesion.

"Solanaceae" refers herein to plant genera, species, and varieties thereof, belonging to the family *Solanaceae.* These include species belonging to the genus *Solanum* (including *Solanum lycopersicum,* which used to be known as *Lycopersicon esculentum), Nicotiana, Capsicum, Petunia* and other genera.

"Disease resistance" refers herein to various levels of disease resistance or tolerance of a plant, including moderate resistance and high resistance or complete resistance to one or more nematodes. It can be measured and optionally quantified by comparison of disease caused symptoms (such as frequency and/or size of HR lesions, etc.) relative to those seen in susceptible control plants when grown under identical disease pressure. Such disease bioassays can be carried out using known methods. Disease resistance can also be indirectly measured as higher yield of resistant plants compared to susceptible plants when grown under disease pressure.

"Enhanced disease resistance" refers to any statistically significant increase in disease resistance of a plant or plant tissue compared to a suitable control. Both a qualitative increase (e.g. from susceptible to resistant) and a quantitative increase are encompassed herein. Also encompassed is both a reduction of disease incidence (percentage of plants becoming infected) and/or of disease severity. Preferably, a plant having enhanced disease resistance to at least one nematode is a plant comprising at least 1%, 2%, 5%, 10%, 15%, 20%, 30%, 50%, 70%, 80%, 90%, or even 100% higher levels of resistance to the nematode than the control plant, using appropriate bioassays and/or field assays for assessing disease resistance.

"Nematode caused symptoms" include any symptoms of disease, such as feeding sites, galls, retarded growth, plus opportunity of secondary infection of other pathogens, withdrawal of nutrients, etc.

The term "nucleic acid sequence" (or nucleic acid molecule) refers to a DNA or RNA molecule in single or double stranded form, particularly a DNA encoding a protein or protein fragment according to the invention. An "isolated nucleic acid sequence" refers to a nucleic acid sequence which is no longer in the natural environment from which it was isolated, e.g. the nucleic acid sequence in a bacterial host cell or in the plant nuclear or plastid genome.

The terms "protein" or "polypeptide" are used interchangeably and refer to molecules consisting of a chain of amino acids, without reference to a specific mode of action, size, 3 dimensional structure or origin. A "fragment" or "portion" of a protein may thus still be referred to as a "protein". An "isolated protein" is used to refer to a protein which is no longer in its natural environment, for example *in vitro* or in a recombinant bacterial or plant host cell.

"Functional", in relation to Rcr3 and Cf-2 proteins (or variants, such as orthologs or mutants, and fragments), refers to the capability to modify the level of disease resistance by modifying the expression level of the Rcr3- and/or Cf-2-encoding gene (e.g. by overexpression or silencing) in a plant. For example, the functionality of a putative RCR3 protein obtained from plant species X can be tested by various methods. If the protein is functional, silencing of the *Rcr3* gene encoding the protein in plant species X also comprising a Cf-2 gene, using e.g. VIGS or gene silencing vectors, will lead to a reduction of nematode resistance, as shown in the Examples (Fig. 4) for tomato using a non-functional allele of Rcr3. Also, complementation with a functional Rcr3 protein will be capable of restoring nematode resistance.

The term "endogenous" as used in the context of the present invention in combination with protein or gene means that said protein or gene originates from the plant in which it is still contained. Often an endogenous gene will be present in its normal genetic context in the plant.

The term "gene" means a DNA sequence comprising a region (transcribed region), which is transcribed into an RNA molecule (e.g. an mRNA) in a cell, operably linked to suitable regulatory regions (e.g. a promoter). A gene may thus comprise several operably linked sequences, such as a promoter, a 5' leader sequence comprising e.g. sequences involved in translation initiation, a (protein) coding region (cDNA or genomic DNA) and a 3'non-translated sequence comprising e.g. transcription termination sites.

A "chimeric gene" (or recombinant gene) refers to any gene, which is not normally found in nature in a species, in particular a gene in which one or more parts of the nucleic acid sequence are present that are not associated with each other in nature. For example the promoter is not associated in nature with part or all of the transcribed region or with

another regulatory region. The term "chimeric gene" is understood to include expression constructs in which a promoter or transcription regulatory sequence is operably linked to one or more coding sequences or to an antisense (reverse complement of the sense strand) or inverted repeat sequence (sense and antisense, whereby the RNA transcript forms double stranded RNA upon transcription).

A "3' UTR" or "3' non-translated sequence" (also often referred to as 3' untranslated region, or 3'end) refers to the nucleic acid sequence found downstream of the coding sequence of a gene, which comprises for example a transcription termination site and (in most, but not all eukaryotic mRNAs) a polyadenylation signal (such as e.g. AAUAAA or variants thereof). After termination of transcription, the mRNA transcript may be cleaved downstream of the polyadenylation signal and a poly(A) tail may be added, which is involved in the transport of the mRNA to the cytoplasm (where translation takes place).

"Expression of a gene" refers to the process wherein a DNA region, which is operably linked to appropriate regulatory regions, particularly a promoter, is transcribed into an RNA, which is biologically active, i.e. which is capable of being translated into a biologically active protein or peptide (or active peptide fragment) or which is active itself (e.g. in posttranscriptional gene silencing or RNAi). An active protein in certain embodiments refers to a protein being constitutively active. The coding sequence is preferably in sense-orientation and encodes a desired, biologically active protein or peptide, or an active peptide fragment. In gene silencing approaches, the DNA sequence is preferably present in the form of an antisense DNA or an inverted repeat DNA, comprising a short sequence of the target gene in antisense or in sense and antisense orientation. "Ectopic expression" refers to expression in a tissue in which the gene is normally not expressed.

A "transcription regulatory sequence" is herein defined as a nucleic acid sequence that is capable of regulating the rate of transcription of a (coding) sequence operably linked to the transcription regulatory sequence. A transcription regulatory sequence as herein defined will thus comprise all of the sequence elements necessary for initiation of transcription (promoter elements), for maintaining and for regulating transcription, including e.g. attenuators or enhancers. Although mostly the upstream (5') transcription regulatory sequences of a coding sequence are referred to, regulatory sequences found downstream (3') of a coding sequence are also encompassed by this definition.

As used herein, the term "promoter" refers to a nucleic acid fragment that functions to control the transcription of one or more genes, located upstream with respect to the direction of transcription of the transcription initiation site of the gene, and is structurally identified by the presence of a binding site for DNA-dependent RNA polymerase, transcription initiation sites and any other DNA sequences, including, but not limited to transcription factor binding sites, repressor and activator protein binding sites, and any other sequences of nucleotides

known to one of skill in the art to act directly or indirectly to regulate the amount of transcription from the promoter. A "constitutive" promoter is a promoter that is active in most tissues under most physiological and developmental conditions. An "inducible" promoter is a promoter that is physiologically (e.g. by external application of certain compounds) or developmental^ regulated. A "tissue specific" promoter is only active in specific types of tissues or cells. A "promoter active in plants or plant cells" refers to the general capability of the promoter to drive transcription within a plant or plant cell. It does not make any implications about the spatiotemporal activity of the promoter.

As used herein, the term "operably linked" refers to a linkage of polynucleotide elements in a functional relationship. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter, or rather a transcription regulatory sequence, is operably linked to a coding sequence if it affects the transcription of the coding sequence.

The term "selectable marker" is a term familiar to one of ordinary skill in the art and is used herein to describe any genetic entity which, when expressed, can be used to select for a cell or cells containing the selectable marker. Selectable marker gene products confer for example antibiotic resistance, or more preferably, herbicide resistance or another selectable trait such as a phenotypic trait (e.g. a change in pigmentation) or a nutritional requirements. The term "reporter" is mainly used to refer to visible markers, such as green fluorescent protein (GFP), eGFP, luciferase, GUS and the like.

The term "ortholog" of a gene or protein refers herein to the homologous gene or protein found in another species, which has the same function as the gene or protein, but (usually) diverged in sequence from the time point on when the species harbouring the genes diverged (i.e. the genes evolved from a common ancestor by speciation). Orthologs of the tomato *Rcr3* gene may thus be identified in other plant species based on both sequence comparisons (e.g. based on percentages sequence identity over the entire sequence or over specific domains) and functional analysis.

The terms "homologous" (herein also referred to as "endogenous") and "heterologous" refer to the relationship between a nucleic acid or amino acid sequence and its host cell or organism. A homologous or endogenous sequence is thus naturally found in the host species (e.g. a tomato plant transformed with a tomato gene), while a heterologous sequence is not naturally found in the host cell (e.g. a tomato plant transformed with a sequence from potato plants).

"Stringent hybridisation conditions" can be used to identify nucleotide sequences, which are substantially identical to a given nucleotide sequence. Stringent conditions are sequence dependent and will be different in different circumstances. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the

specific sequences at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridises to a perfectly matched probe. Typically stringent conditions will be chosen in which the salt concentration is about 0.02 molar at pH 7 and the temperature is at least 60°C. Lowering the salt concentration and/or increasing the temperature increases stringency. Stringent conditions for RNA-DNA hybridisations (Northern blots using a probe of e.g. 100nt) are for example those which include at least one wash in 0.2X SSC at 63°C for 20 min, or equivalent conditions. Stringent conditions for DNA-DNA hybridisation (Southern blots using a probe of e.g. 100nt) are for example those which include at least one wash (usually 2) in 0.2X SSC at a temperature of at least 50°C, usually about 55°C, for 20 min, or equivalent conditions. See also Sambrook *et al.* (1989) and Sambrook and Russell (2001).

"Sequence identity" and "sequence similarity" can be determined by alignment of two peptide or two nucleotide sequences using global or local alignment algorithms. Sequences may then be referred to as "substantially identical" or "essentially similar" when they (when optimally aligned by for example the programs GAP or BESTFIT using default parameters) share at least a certain minimal percentage of sequence identity (as defined below). GAP uses the Needleman and Wunsch global alignment algorithm to align two sequences over their entire length, maximizing the number of matches and minimises the number of gaps. Generally, the GAP default parameters are used, with a gap creation penalty = 50 (nucleotides) / 8 (proteins) and gap extension penalty = 3 (nucleotides) / 2 (proteins). For nucleotides the default scoring matrix used is nwsgapdna and for proteins the default scoring matrix is Blosum62 (Henikoff & Henikoff, 1992, PNAS 89, 915-919). Sequence alignments and scores for percentage sequence identity may be determined using computer programs, such as the GCG Wisconsin Package, Version 10.3, available from Accelrys Inc., 9685 Scranton Road, San Diego, CA 92121-3752 USA, or EmbossWin version 2.10.0 (using the program "needle"). Alternatively percent similarity or identity may be determined by searching against databases, using algorithms such as FASTA, BLAST, etc.

As used herein, the term "plant" includes plant cells, plant tissues or organs, plant protoplasts, plant cell tissue cultures from which plants can be regenerated, plant calli, plant cell clumps, and plant cells that are intact in plants, or parts of plants, such as embryos, pollen, ovules, fruit (e.g. harvested tomatoes), flowers, leaves, seeds, roots, root tips and the like.

The term "Rcr3 gene" refers to a nucleic acid sequence, e.g., a gene, encoding an RCR3 protein. RCR3 proteins are cysteine proteases. They belong to the family of papain-like cysteine proteases with members that occur in many organisms, including plants. The gene family is largely duplicated in *Solanum* species. Horger and colleagues (Plos Genetics 2012 8: e1002813; http://www.ncbi.nlm.nih.gov/pubmed/22829777) describe more than 50

natural variants of Rcr3 in wild tomato *(Solanum)* species. For example, amino acid sequences of RCR3 proteins of *Solanum peruvianum, Solanum corneliomuelleri, Solanum chilense, Solanum chmielewskii, Solanum pennellii,* and *Solanum habrochaites* can be accessed in publicly available databases. Although mostly studied for pathogen defenses in tomato, genes encoding proteins with similar cysteine protease functionality as Rcr3 can also be found in all other plant species (e.g. Arabidopsis, potato and maize). The skilled person will readily be capable of determining whether an amino acid sequence is an RCR3 protein. The term "$Rcr3^{pim}$" refers to the Rcr3 allele from *Solanum pimpinellifolium,* whereas the term "$Rcr3^{lyc}$" refers to the *Solanum lycopersicum* Rcr3 allele.

Tomato *(Solanum lycopersicum)* Cf genes confer resistance to several pathogens, including fungi and nematodes. Cf-2 is a member of a large family of genes that encode trans-membrane receptor-like proteins (RLPs) with extracellular leucine-rich repeats (LRRs) that are found in tomato and other (crop) plants. R-genes mediate recognition of pathogens through direct binding of pathogen-derive molecules, or indirectly through recognition of pathogen-induced changes of host processes. The skilled person will be capable of identifying a Cf-2 gene based on the publicly available Cf-2 nucleotide sequences.

In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded.

In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one of the element is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one". It is further understood that, when referring to "sequences" herein, generally the actual physical molecules with a certain sequence of subunits (e.g. amino acids) are referred to.

Detailed description of the invention

In a first aspect, the present invention relates to a method for producing a plant having enhanced disease resistance to one or more nematodes compared to a control plant, said method comprising the steps of:

(a) introducing a Cf-2 gene into a plant or plant cell;

(b) introducing into a plant or plant cell; and

(c) regenerating a plant,

wherein at least one of steps (a) and (b) is carried out by means of transformation and/or transfection.

The plant may be any plant, but is preferably a dicotyledonous plant, preferably belonging to the family of Solanaceae, preferably to the genus of Solanum, including the species *Solanum lycopersicum.*

The control plant as referred to herein is a plant of the same species and preferably same genetic background as the plant having enhanced disease resistance to nematodes, which control plant does not comprise the Cf-2 gene and/or the Rcr3 gene having a leucine at position 197, a glutamic acid at position 222, an arginine at position 229, a lysine at position 241 , a glutamine at position 284, and/or a deletion of K122 (at the positions corresponding to those indicated in the amino acid sequence of SEQ ID NO:1).

In a first step, a Cf-2 gene is introduced into said plant. A "Cf-2 gene" as used herein is a gene encoding the amino acid sequence as set forth in SEQ ID NO 3 or 4, or a fragment or variant thereof. Variants of Cf-2 include, for example, proteins having at least 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 98, 99%, or more, amino acid sequence identity (preferably over the entire length) to SEQ ID NO: 3 and/or 4. Amino acid sequence identity is preferably determined by pairwise alignment using the Needleman and Wunsch algorithm and GAP default parameters as defined above. Said Cf-2 gene (e.g., the *Solanum pimpinellifolium* Cf-2 gene as depicted in SEQ ID NOs: 2 and 3) may be introgressed or may be transgenically introduced using, for example, transformation or transfection.

In step (b), an Rcr3 gene encoding a RCR3 protein that binds to and/or is inhibited by Gr-VAP1, such as preferably the *Solanum pimpinellifolium* allele of the Rcr3 gene, is introduced into a plant or plant cell. The *Solanum pimpinellifolium* allele of the Rcr3 gene (also referred to as "$Rcr3^{pimp}$" encodes a protein having the amino acid sequence of SEQ ID NO:2). Particularly, the *Solanum pimpinellifolium* allele of the Rcr3 gene comprises all of a leucine at position 197, a glutamic acid at position 222, an arginine at position 229, a lysine at position 241, a glutamine at position 284, and/or a deletion of K122 (at the positions corresponding to those indicated in the amino acid sequence of SEQ ID NO:1). Said *Solanum pimpinellifolium* allele of the Rcr3 gene (i.e., any gene encoding the RCR3 protein of *Solanum pimpinellifolium* as depicted in SEQ ID NO:2) may be introgressed or may be introduced using transformation or transfection.

At least one of steps (a) and (b) is preferably carried out using transformation and/or transfection. In an embodiment, both steps (a) and (b) are carried out using transformation and/or transfection.

In a second aspect, the present invention relates to a method for producing a plant having enhanced disease resistance to one or more nematodes compared to a control plant, said method comprising the steps of:

(a) introducing a Cf-2 gene into a plant or plant cell;

(b) modifying the Rcr3 gene of the plant or plant cell such that it encodes a modified RCR3 protein comprising one or more of the following amino acids at the position of an endogenous RCR3 protein as compared to the amino acid position of RCR3 protein from *Solanum lycopersicum* (SEQ ID NO: 1): 197L, 222E, 229R, 241K, 284Q, 319F, and/or deletion of the amino acid corresponding to K122, and

(c) regenerating the plant.

The Cf-2 gene (e.g., the *Solanum pimpinellifolium* Cf-2 gene as depicted in SEQ ID NOs: 2 and 3) may be introgressed or may be transgenically introduced using, for example, transformation or transfection.

The Cf-2 gene may be introduced into a plant or plant cell in which the Rcr3 gene is not (yet) modified, or may be introduced into a plant or plant cell in which the Rcr3 gene is modified, i.e., step (a) may be carried out prior to step (b), or may be carried out after step (b).

Alternatively, in step (a) a plant may be provided that comprises a Cf-2 gene.

In an embodiment, an endogenous Rcr3 gene of a plant or plant cell may be modified such that it encodes a modified endogenous RCR3 protein comprising a leucine at position 197, a glutamic acid at position 222, an arginine at position 229, a lysine at position 241, a glutamine at position 284, a phenylalanine at position 319 and/or a deletion of K122 (at the positions corresponding to those indicated in the amino acid sequence of SEQ ID NO: 1, i.e. the amino acid sequence of *Solanum lycopersicum* RCR3 protein). The corresponding amino acid as compared to the amino acid sequence of *Solanum lycopersicum* RCR3 protein in the amino acid sequence of an endogenous RCR3 protein from a plant species may be determined by alignment of the amino acid sequence of said endogenous RCR3 protein to the amino acid sequence of the *Solanum lycopersicum* RCR3 allele (as shown in SEQ ID NO: 1), for example by pairwise alignment using the Needleman and Wunsch algorithm and GAP default parameters as defined above. A "Rcr3 gene" as used herein encompasses a gene encoding the amino acid sequence of SEQ ID NO: 1 (RCR3 protein), or encoding a fragment or variant of this amino acid sequence. Variants of RCR3 protein include, for example, proteins having at least 70, 75, 80, 85, 90, 95, 98, 99%, or more, amino acid sequence identity (over the entire length) to the amino acid sequence of SEQ ID NO: 1, said variants further exhibiting papain-like protease activity. Amino acid sequence identity is preferably determined by pairwise alignment using the Needleman and Wunsch algorithm and GAP default parameters as defined above. In an embodiment, said endogenous Rcr3 gene is not the *Solanum pimpinellifolium* Rcr3 gene (encoding an amino acid sequence of SEQ ID NO:2). In another embodiment, said endogenous Rcr3 gene does not encode an RCR3 protein comprising one or more of a leucine at position 197, a glutamic

acid at position 222, an arginine at position 229, a lysine at position 241, a glutamine at position 284, a phenylalanine at position 319 and/or a deletion of K122 (at the positions corresponding to those indicated in the amino acid sequence of SEQ ID NO:1). In the context of the present invention, the positions corresponding to those indicated in the amino acid sequence of SEQ ID NO:1 are determined by pairwise alignment, preferably using the Needleman and Wunsch algorithm and GAP default parameters as indicated above. In an embodiment, the plant is a *Solanum lycopersicum* plant, and said endogenous Rcr3 gene is the *Solanum lycopersicum* Rcr3 gene. The *Solanum lycopersicum* Rcr3 gene encodes an RCR3 protein comprising a phenylalanine at position 197, a glutamine at position 222, a glutamine at position 229, a glutamine at position 241, an arginine at position 284, and a tyrosine at position 319. It has an additional lysine residue at position 122 as compared to the S. *pimpinellifolium* Rcr3 allele (the amino acid sequence encoded for by the latter is shown in SEQ ID NO:2). Thus, in case the plant is a *Solanum lycopersicum* plant, step (b) may comprise the step of modifying the Rcr3 gene of a *Solanum lycopersicum* plant or plant cell such that it encodes a modified RCR3 protein comprising one or more of the following mutations with respect to an endogenous *Solanum lycopersicum* RCR3 protein: deletion of K122, F197L, Q222E, Q229R, Q241K, R284Q, and/or Y319F.

In an embodiment, the methods of the present invention further comprise step (d): screening the regenerated plant, or a plant derived therefrom by selfing or crossing, for resistance to one or more nematodes and identifying a plant comprising enhanced resistance to one or more of said nematodes.

RCR3 protein

It is an embodiment of the invention to modify an endogenous *Rcr3* gene using targeted mutagenesis methods. Targeted mutagenesis methods include, without limitation, those employing zinc finger nucleases, or targeted mutagenesis methods employing mutagenic oligonucleotides containing chemically modified nucleotides for enhancing mutagenesis with sequence complementarity to the *Rcr3* gene , into plant protoplasts (e.g., KeyBase® or TALENs).

Alternatively, mutagenesis systems such as TILLING (Targeting Induced Local Lesions IN Genomics; McCallum *et al.,* 2000, Nat Biotech 18:455, and McCallum *et al.* 2000, Plant Physiol. 123, 439-442, both incorporated herein by reference) may be used to generate plant lines which provide an Rcr3 gene encoding a RCR3 protein having one or more of the above mutations and/or having enhanced disease resistance to one or more nematodes. TILLING uses traditional chemical mutagenesis (e.g. EMS mutagenesis) followed by high-throughput screening for mutations. Thus, plants, seeds and tissues comprising an *Rcr3* gene having one or more of the desired mutations may be obtained.

The method comprises in one embodiment the steps of mutagenizing plant seeds (e.g. EMS mutagenesis), pooling of plant individuals or DNA, PCR amplification of a region of interest, heteroduplex formation and high-throughput detection, identification of the mutant plant, sequencing of the mutant PCR product. It is understood that other mutagenesis and selection methods may equally be used to generate such mutant plants. Seeds may for example be radiated or chemically treated and the plants screened for a modified phenotype, such as enhanced disease resistance and/or HR lesions.

In another embodiment of the invention, the modified *Rcr3* gene is derived from natural populations of the species or related species that comprise polymorphisms or variations in DNA sequence at the *Rcr3* orthologous coding sequence. Mutations at the *Rcr3* gene target can be screened for using a ECOTILLING approach (Henikoff et al 2004, *supra).* In this method natural polymorphisms in breeding lines or related species are screened for by the above described TILLING methodology, in which individual or pools of plants are used for PCR amplification of the *Rcr3* target, heteroduplex formation and high-throughput analysis. This can be followed up by selecting of individual plants having the required mutation(s) that can be used subsequently in a breeding program to incorporate the desired F?cr3-orthologous allele to develop the cultivar with desired trait, or that may be introduced using recombinant DNA techniques.

Mutant plants can be distinguished from non-mutants by molecular methods, such as the mutation(s) present in the DNA, and by the modified phenotypic characteristics (enhanced disease resistance to one or more nematodes).

The mutants may be homozygous or heterozygous for the mutation.

The *Cf-2* and/or *Rcr3* nucleic acid sequence may be inserted in a plant cell genome so that the inserted coding sequence is downstream (i.e. 3') of, and under the control of, a promoter which can direct the expression in the plant cell. This is preferably accomplished by inserting a gene, optionally a chimeric gene, in the plant cell genome, particularly in the nuclear or plastid (e. g. chloroplast) genome.

As the constitutive production of the RCR3 protein may lead to the induction of cell death (e.g. microscopic lesions and/or macroscopic lesions), it is in one embodiment preferred to use a promoter whose activity is inducible for expressing the RCR3 protein. Examples of inducible promoters are wound-inducible promoters, such as the MPI promoter described by Cordera *et al.* (1994, The Plant Journal 6, 141), which is induced by wounding (such as caused by insect or physical wounding), or the COMPTII promoter (WO0056897) or the PR1 promoter described in US6031 151. Alternatively the promoter may be inducible by a chemical, such as dexamethasone as described by Aoyama and Chua (1997, Plant

Journal 11: 605-612) and in US6063985 or by tetracycline (TOPFREE or TOP 10 promoter, see Gatz, 1997, Annu Rev Plant Physiol Plant Mol Biol. 48: 89-108 and Love et al. 2000, Plant J. 21: 579-88). Other inducible promoters are for example inducible by a change in temperature, such as the heat shock promoter described in US 5,447, 858, by anaerobic conditions (e.g. the maize ADH1S promoter), by light (US6455760), by nematodes or by senescence (SAG12 and SAG13, see US5689042). Obviously, there are a range of other promoters available.

In one embodiment preferably, a nematode inducible promoter is used, as thereby the RCR3 protein (or variant or fragment) will only be produced following nematode attack of the plant tissue. Especially, promoters of genes which are upregulated quickly after nematode attack are desired. Promoters inducible by a particular plant nematode may also be identified using known methods, such as cDNA-AFLP®.

Preferably, the promoter is inducible by a number of nematodes, i.e. it is inducible by a broad range of nematodes of the host plant. For each particular host plant species, a different promoter may be most suitable. For example, when tomato is used as a host, the promoter is preferably induced upon at least one, but preferably more than one nematode that causes disease on tomato.

Detailed descriptions of plant nematodes, the disease symptoms caused by them and their life cycles can be found for each plant species. For example, nematodes that cause disease on tomato are described in "Compendium of Tomato Diseases" , Editors Jones, Jones, Stall and Zitter, ISBN 0-89054-120-5, APS Press (http:/www.shopapspress/org). Nematodes that cause disease on potato are described in "Compendium of Potato Disease", 2nd edition, Editors Stevenson, Franc and Weingartner, APS Press, ISBN 0-89054-275-9.

Nematodes affecting tomato include, for example, *Meloidogyne incognita, M. javanica, M. arenaria, M. hapla, M. mayaguensis, Globodera rostochiensis, G. pallida,* and *G. mexicana,* as well as other *Globodera* species.

Nematodes affecting potato include, for example, *Globodera rostochiensis, G. pallida* and many other *Globodera species;* but also RKN species, such as *M. chitwoodi* are serious pathogens on potato.

See also http://www.apsnet.org/online/common/toc.asp for plant diseases of various plant species. For example, a nematode affecting soybean is soybean cyst nematode, and a nematode affecting sugar beet is beet cyst nematode. In one embodiment the promoter is preferably inducible by one or more of the above nematodes.

Alternatively, a host plant may comprise various *Rcr3* and/or *Cf-2* transgenes, each under control of a different nematode inducible promoter, to ensure that Rcr3 and/or Cf-2

protein is produced following attack by a variety of nematodes. For example, for transformation of tomato, one promoter may be inducible by *Meloidogyne incognita* and one by *M. mayaguensis.*

The word "inducible" does not necessarily require that the promoter is completely inactive in the absence of the inducer stimulus. A low level non-specific activity may be present, as long as this does not result in severe yield or quality penalty of the plants. Inducible, thus, preferably refers to an increase in activity of the promoter, resulting in an increase in transcription of the downstream *Rcr3* and/or *Cf-2* coding region following contact with the inducer.

In another embodiment constitutive promoters may be used, such as the strong constitutive 35S promoters or enhanced 35S promoters (the "35S promoters") of the cauliflower mosaic virus (CaMV) of isolates CM 1841 (Gardner *et al.,* 1981, Nucleic Acids Research 9, 2871-2887), CabbB-S (Franck *et al.,* 1980, Cell 21, 285-294) and CabbB-JI (Hull and Howell, 1987, Virology 86,482-493); the 35S promoter described by Odell *et al.* (1985, Nature 313, 810-812) or in US5164316, promoters from the ubiquitin family (e.g. the maize ubiquitin promoter of Christensen *et al.,* 1992, Plant Mol. Biol. 18,675-689, EP 0 342 926, see also Cornejo *et al.* 1993, Plant Mol. Biol. 23, 567-581), the gos2 promoter (de Pater *et al.,* 1992 Plant J. 2, 834-844), the emu promoter (Last *et al.,* 1990, Theor. Appl. Genet. 81,581-588), Arabidopsis actin promoters such as the promoter described by An *et al.* (1996, Plant J. 10, 107.), rice actin promoters such as the promoter described by Zhang *et al.*(1991, The Plant Cell 3, 1155-1 165) and the promoter described in US 5,641,876 or the rice actin 2 promoter as described in WO070067; promoters of the Cassava vein mosaic virus (WO 97/48819, Verdaguer ef *al.* 1998, Plant Mol. Biol. 37,1055-1067), the pPLEX series of promoters from Subterranean Clover Stunt Virus (WO 96/06932, particularly the S7 promoter), a alcohol dehydrogenase promoter, e.g., pAdhlS (GenBank accession numbers X04049, X00581), and the TR1' promoter and the TR2' promoter (the "TRT promoter" and "TR2' promoter", respectively) which drive the expression of the 1' and 2' genes, respectively, of the T-DNA (Velten *et al.,* 1984, EMBO J 3, 2723-2730), the Figwort Mosaic Virus promoter described in US6051753 and in EP426641, histone gene promoters, such as the Ph4a748 promoter from Arabidopsis (PMB 8: 179-191), or others.

Alternatively, a promoter can be utilized which is not constitutive but rather is specific for one or more tissues or organs of the plant (tissue preferred / tissue specific, including developmental^ regulated promoters), for example leaf preferred, epidermis preferred, root preferred, flower tissue e.g. tapetum or anther preferred, seed preferred, pod preferred, etc.), whereby the *Rcr3* and/or *Cf-2* gene is expressed only in cells of the specific tissue(s) or organ(s) and/or only during a certain developmental stage. For example, the *Rcr3* and/or

Cf-2 gene(s) can be selectively expressed in the roots of a plant by placing the coding sequence under the control of a root-specific promoter.

In one embodiment the promoter of the endogenous *Rcr3* and/or *Cf-2* gene is used. For example, the promoter of the tomato *Rcr3* gene may be isolated and operably linked to the coding region encoding RCR3 protein of SEQ ID NO:1. The *Rcr3* promoter (the upstream transcription regulatory region of the gene encoding the amino acid sequence of SEQ ID NO: 1) can be isolated from tomato plants using known methods, such as TAIL-PCR (Liu *et al.* 1995, Genomics 25(3):674-81; Liu *et al.* 2005, Methods Mol Biol. 286:341-8), Linker-PCR, or Inverse PCR (IPCR).

The Rcr3 and/or Cf-2 coding sequence is preferably inserted into the plant genome so that the coding sequence is upstream (i.e. 5') of suitable 3' end nontranslated region ("3'end" or 3'UTR). Suitable 3'ends include those of the CaMV 35S gene ("3' 35S"), the nopaline synthase gene ("3' nos") (Depicker *et al.,* 1982 J. Molec. Appl. Genetics 1, 561-573.), the octopine synthase gene ("3'ocs") (Gielen *et al.,* 1984, EMBO J 3, 835-845) and the T-DNA gene 7 ("3' gene *7")* (Velten and Schell, 1985, Nucleic Acids Research 13, 6981-6998), which act as 3'-untranslated DNA sequences in transformed plant cells, and others. In one embodiment the 3'UTR of the tomato *Rcr3* gene is used.

Introduction of the T-DNA vector into *Agrobacterium* can be carried out using known methods, such as electroporation or triparental mating.

A Rcr3 and/or Cf-2 encoding nucleic acid sequence can optionally be inserted in the plant genome as a hybrid gene sequence whereby the *Rcr3* and/or *Cf-2* sequence is linked in-frame to a (US 5,254, 799; Vaeck *et al.,* 1987, Nature 328, 33-37) gene encoding a selectable or scorable marker, such as for example the neo (or nptll) gene (EP 0 242 236) encoding kanamycin resistance, so that the plant expresses a fusion protein which is easily detectable.

In an aspect, the present invention provides for a plant, plant cell, seed or fruit, obtainable by any of the methods taught herein. In an embodiment, said plant is a genetically modified plant. The plant may be a transgenic plant. In another embodiment, said plant may be considered non-transgenic. In an embodiment, said plant is a *Solanum lycopersicum* plant.

The present invention also provides a plant, preferably a *Solanum lycopersicum* plant, or hybrid plant, plant cell, seed or fruit, obtainable or obtained by a method comprising the steps of: a) preparing a first hybrid plant comprising a Cf-2 gene, preferably a *Solanum pimpinellifolium* Cf-2 gene, by crossing a plant comprising said Cf-2 gene, preferably a S. *pimpinellifolium* plant comprising said Cf-2 gene, with a sexually compatible plant, preferably a *Solanum lycopersicum* plant and selecting first hybrid plants comprising said Cf-2 gene,

and b) modifying an Rcr3 gene of the plant, preferably using targeted mutagenesis, such that it encodes a modified RCR3 protein comprising one or more of the following amino acids at the position of an endogenous RCR3 protein as compared to the amino acid position of RCR3 protein from *Solanum lycopersicum* (SEQ ID NO:1): 197L, 222E, 229R, 241K, 284Q, 319F, and/or deletion of the amino acid corresponding to K122.

Further, the present invention provides a plant, plant cell, seed or fruit, obtainable or obtained by a method comprising the steps of: a) introgressing a Cf-2 gene, preferably a *Solanum pimpinellifolium* Cf-2 gene into a plant, preferably a *Solanum lycopersicum* plant, and b) modifying an Rcr3 gene of the plant, preferably using targeted mutagenesis, such that it encodes a modified RCR3 protein comprising one or more of the following amino acids at the position of an endogenous RCR3 protein as compared to the amino acid position of RCR3 protein from *Solanum lycopersicum* (SEQ ID NO:1): 197L, 222E, 229R, 241K, 284Q, 319F, and/or deletion of the amino acid corresponding to K122.

Said method may further comprise the step of regenerating the plant. Said method may further comprise the step of screening the regenerated plant, or a plant derived therefrom by selfing or crossing, for resistance to one or more nematode species and identifying a plant comprising enhanced resistance to one or more of said nematode species.

Additionally, the invention provides a nematode-resistant plant, preferably a *Solanum lycopersicum* plant, comprising an introgression comprising a Cf-2 gene, preferably an introgression from a *Solanum pimpinellifolium* plant comprising said Cf-2 gene, said plant further comprising a modified endogenous Rcr3 gene, wherein said endogenous Rcr3 gene is modified such that it encodes a modified RCR3 protein comprising one or more of the following amino acids at the position of an endogenous RCR3 protein as compared to the amino acid position of RCR3 protein from *Solanum lycopersicum* (SEQ ID NO:1): 197L, 222E, 229R, 241K, 284Q, 319F, and/or deletion of the amino acid corresponding to K122. Moreover, the present invention provides a nematode-resistant plant, preferably a *Solanum lycopersicum* plant, comprising an introgression comprising a Cf-2 gene, preferably an introgression from a *Solanum pimpinellifolium* plant comprising said Cf-2 gene, said plant further comprising an Rcr3 gene, wherein said Rcr3 gene encodes a RCR3 protein comprising one or more of the following amino acids at the position of the RCR3 protein from *Solanum lycopersicum* (SEQ ID NO:1): 197L, 222E, 229R, 241K, 284Q, 319F, and/or deletion of the amino acid corresponding to K122.

The invention also provides a nematode-resistant plant, preferably a *Solanum lycopersicum,* plant, comprising an introgression comprising a Cf-2 gene, preferably an introgression from a *Solanum pimpinellifolium* plant comprising said Cf-2 gene, said plant

further comprising a modified Rcr3 gene, wherein said Rcr3 gene is modified such that it encodes a modified RCR3 protein that binds to and/or is inhibited by Gr-VAP1.

The plant may be selected from a *Solanum lycopersicum, Solanum peruvianum, Solanum cornelliomuelleri, Solanum chilense, Solanum pennellii, Solanum habrochaites* and *Solanum chmielewski* plant.

In another aspect, the present invention pertains to use of a modified Rcr3 gene of a *Solanum lycopersicum* plant or plant cell, said modified Rcr3 gene encoding a modified RCR3 protein comprising one or more of the following mutations with respect to an endogenous *Solanum lycopersicum* RCR3 protein: F197L, Q222E, Q229R, Q241K, R284Q, Y319F, and/or deletion of K122 (at the positions corresponding to those indicated in the amino acid sequence of SEQ ID NO:1), and a Cf-2 gene, such as a S. *pimpinellifolium* Cf-2 gene, for the generation of nematode resistant *Solanum lycopersicum* plants.

In an aspect, the present invention provides for a method for producing a plant having enhanced disease resistance to one or more nematodes compared to a control plant, said method comprising the steps of: (a) introducing a chimeric gene comprising a nematode-inducible promoter, operably linked to a nucleotide sequence encoding a Cf-2 protein such as shown, for example, in SEQ ID NO:3 or SEQ ID NO:4, or a variant thereof having at least 70, 75, 80, 85, 90, 95, 96, 97, 98, or 99% sequence identity, preferably over the entire length, to the Cf-2 protein, such as shown, for example, in SEQ ID NO:3 or SEQ ID NO:4, into a plant or plant cell; (b) introducing a chimeric gene comprising a nematode-inducible promoter, operably linked to a nucleotide sequence encoding a *Solanum lycopersicum* RCR3 protein, such as shown, for example, in SEQ ID NO:1, or a variant thereof having at least 70, 75, 80, 85, 90, 95, 96, 97, 98, or 99% sequence identity, preferably over the entire length, to the *Solanum lycopersicum* RCR3 protein, such as shown, for example, in SEQ ID NO:1 , into a plant or plant cell; and (c) regenerating a plant.

Preferably, said variant of the *Solanum lycopersicum* RCR3 protein comprises at least a phenylalanine at position 197, a glutamine at position 222, a glutamine at position 229, a glutamine at position 241, an arginine at position 284, a tyrosine at position 319, and a lysine at position 122 at the positions corresponding to those indicated in the amino acid sequence of SEQ ID NO: 1.

Preferably, said variant of the *Solanum lycopersicum* RCR3 protein is auto-active, i.e. causes necrosis if a Cf-2 protein is also expressed.

SEQUENCE LISTING

SEQ ID NO:1: amino acid sequence of RCR3 protein of *Solanum Lycopersicum.*

SEQ ID NO:2: amino acid sequence of RCR3 protein of *Solanum pimpinellifolium.*

SEQ ID NO:3: amino acid sequence of Cf-2.1 protein of *Solanum pimpinellifolium* (GenBank accession no. AAC15779)

SEQ ID NO:4: amino acid sequence of Cf-2.2 protein of *Solanum pimpinellifolium* (GenBank accession no. AAC15780).

Examples

Materials and methods

Identification of full-length Gr-VAP1 cDNA. Messenger RNA was extracted from five stages of *Globodera rostochiensis* pathotype Ro1 for a cDNA-AFLP analysis (Qin *et al. Mol. Plant-Microbe Interact.* **13,** 830-836 (2000)). The primary cDNA templates synthesized from five mRNA pools were digested using the restriction enzymes *Nco*I and *Taq*I. Oligonucleotide primers annealing to the *Nco*I and *Taq*I adapter sequences were used to specifically amplify transcript-derived fragments flanked by these restriction enzymes (Popeijus *et al. Nature* **406,** 36-37 (2000)). Specific transcript-derived-fragments, including transcript-derived fragment NC4, were excised from acrylamide gels. Following reamplification using the original primers of the cDNA-AFLP analysis, NC4 was cloned into the TOPO-pCR4 plasmid (Invitrogen, Carlsbad, CA, USA) and sequenced (Genbank accession 1465499). Expressed sequence tags of G. *rostochiensis* matching NC4 were identified in the non-redundant database of Genbank with BLASTN (i.e. Genbank accessions AJ536826, AF343567, and AF374388).

To resolve the 5'- and 3'-ends of the transcript up- and downstream of the NC4 fragment and matching expressed sequence tags, we used the rapid amplification of cDNA ends system (Invitrogen). For the 5'-end, first strand cDNA was synthesized using oligo-dT primers from 1.5 mg of total RNA isolated from 0.5 g of grinded G. *rostochiensis* pre-parasitic second stage juveniles using Trizol (Invitrogen). Nested PCR products, generated with the gene specific primer Gr-VAP1-RaceRv (Table S1) and adaptor primers from the Generacer system, were cloned into the pGEM-T vector (Promega, Madison, WI, USA), and sequenced. For the 3'-end, a nested PCR was performed with the gene specific primer Gr-VAP1-RaceFw (Table 1) and adaptor primers included in the GeneRacer system (Invitrogen). PCR products were cloned into the pGEM-T vector (Promega), and sequenced. The resulting sequences were assembled into a full-length cDNA sequence, hereafter named Gr-VAP1 (Genbank accession 1466491).

Gr-VAP1* expression in *G. rostochiensis. Whole mount *in situ* hybridization was performed on preparasitic second stage juveniles of *G. rostochiensis* pathotype R01 to localize *Gr-*

VAP1 transcription in nematodes (Qin *et al. Mol. Plant-Microbe Interact.* **13,** 830-836 (2000); Smant *et al. Proc. Natl. Acad. Sci. U.S.A.* **95,** 4906 (1998)). Briefly, the NC4 fragment was subcloned into pCR2.1-TOPO vector (Invitrogen). Sense and antisense probes were generated by asymmetric PCR in the presence of DIG-dUTP (Roche, Basel, Switzerland) using N+0 and T+0 primers (Table 1). The DIG-labelled probes were purified in a G50 Mini Quick Spin DNA column (Roche). Alkaline phosphatase activity was detected by the addition of X-phosphate and NBT (Roche), and hybridization of the probes in infective juveniles was examined with an inverted microscope (Leica Microsystems, Wetzlar, Germany).

Semi-quantitative reverse transcription PCR was used to study the expression of *Gr-VAP1* at different time points post inoculation (Rehman *et al. Mol. Plant-Microbe Interact.* **22,** 330-340 (2009)). Messenger RNA extraction and cDNA synthesis was conducted on parasitic second, third, and fourth stage juveniles and the adult males and females isolated from roots of susceptible potato *(Solanum tuberosum* cultivar Bintje) at 13, 19, 23, 27, and 34 days post inoculation respectively. *Gr-VAP1* expression in each sample was examined with a gene specific fragment of 146 base pairs PCR-amplified in 26 cycles with primers Gr-VAP1-RTFw and Gr-VAP1-RTRv (Table 1). The constitutively expressed cAMP-dependent protein kinase *(Gr-cAMP;* Genbank accession BM343563) was PCR amplified with the primers cAMP-RTFw and cAMP-RTRv (Table 1) as a reference. Reactions without reverse transcriptase were included to test for contaminating genomic DNA of the nematodes, while non-infected potato roots were included to check for non-specific amplification of host-derived cDNA.

Identification of Gr-VAP1 in *G. rostochiensis* stylet secretions. To collect stylet secretions of *G. rostochiensis* about 100,000 freshly hatched preparasitic second juveniles were either incubated for 24 h in 2 ml filter sterile potato root diffusates with 4 mM 5-methoxy-N, N-dimethyltryptamine-hydrogen-oxalate or in tap water. Two ml of collected secretions was freeze-dried, dissolved in 50 µl 0.1% (w/v) RapiGest SF Surfactant (Waters, Milford, MA, USA), 5 mM DTT (Sigma-Aldrich, St. Louis, Mo, USA) in 0.1 M ammonium bicarbonate, and incubated at 60°C for 1 h. Alkylation was performed by incubation with 15 mM iodoacetamide (GE Healthcare, Chalfont St. Giles, UK) for 30 min at room temperature (in the dark). Proteolytic digestion was initiated by adding 1 µl of modified porcine trypsin (0.1 µg/µl; Sequence grade modified; Promega) and incubated overnight at 37°C. After adding trifluoroacetic acid (Fluka) to a final concentration of 0.5% (v/v), samples were centrifuged at 15,000 g for 10 min, and the supernatant was applied to a SupelClean™ LC-18 1 ml SPE column (Sigam-Aldrich) equilibrated with 0.1% TFA. Bound peptides were eluted with 84% acetonitrile (HPLC Supra-gradient, Biosolve) containing 0.1% formic acid

(Merck, Whitehouse Station, NJ, USA), dried down by vacuum centrifugation, dissolved in 40 □! 0.1% formic acid and further analyzed with mass spectrometry. The tryptic peptide samples were separated using a nanoAcquity UPLC system (Waters) using a BEH C_{18} column (75 µm x 25 cm, Waters) and a 65 min linear gradient from 3 to 40% acetonitrile (in 0.1% formic acid) at 200 nl/min. The eluting peptides were on-line injected into a Synapt Q-TOF MS instrument (Waters) using a nanospray device coupled to the second dimension column output. The Synapt MS was operated in positive mode with [Glu^1] fibrinopeptide B (1 pmol/µl; Sigma) as reference (lock mass) and sampled every 30 s. Accurate liquid chromatography-mass spectrometry (LC-MS) data were collected with the Synapt operating in MS/MS mode for data-dependent acquisition. LC-MS/MS was performed by peptide fragmentation on the three most intense multiple charged ions that were detected in the MS survey scan (0.6 s) over a 300-1400 m/z range and a dynamic exclusion window of 60 s with an automatically adjusted collision energy based on the observed precursor m/z and charge state. LC-MS/MS data were processed using ProteinLynx Global Server software (PLGS version 2.4, Waters) and the resulting list of masses containing all the fragment information was analyzed for matching proteins using a custom-build non-redundant database including all currently known (predicted) proteins of *G. rosiochiensis.*

Yeast-two-hybrid screening of tomato root cDNA with Gr-VAP1. The Matchmaker System 3 (Clontech, Mountain View, CA, USA) was used to screen a tomato root cDNA library *(Solanum lycopersicum* cultivar CGR161) in yeast with Gr-VAP1 as bait (Rehman *et al. Mol. Plant-Microbe Interact.* **22,** 330-340 (2009)). The region encoding Gr-VAP1 without its signal peptide for secretion was PCR-amplified using the primers Gr-VAP1-Y2HFw and Gr-VAP1-Y2HRv (Table 1) from cDNA and subcloned in-frame into the EcoRI/BamHI site of the bait vector pGBKT7-BD for expression as a GAL4 fusion protein in yeast *(Saccharomyces cerevisiae* strain AH109). Yeast cells were co-transformed with the bait vector (pGBKT7-BD::Gr-VAP1) and the tomato root cDNA-library cloned in pACT2 using the lithium acetate method. The yeast transformants were plated on high-stringency medium SD/-Ade/-His/-Leu/-Trp/X-a-gal to select for a-galactosidase activation. Yeast colonies were repeatedly incubated for 4 days at 30°C, while restreaking the positive colonies onto fresh medium for two times. After three rounds on selective medium tomato root library plasmids were isolated from positive yeast colonies using a lyticase-based method, followed by Wizard Plus Miniprep DNA Purification System (Promega). The isolated yeast plasmids were shuttled into *E. coli* Top10 (Invitrogen), and retransformed into yeast cells together with pGBKT7-LaminC encoding for human lamin C to test for transactivation. Library inserts of positive retested interactors were sequenced and further analyzed.

In vitro co-immunoprecipitation of Rcr3^{pim} and Gr-VAP1. Gr-VAP1 was PCR amplified from cDNA using the primers Gr-VAP1-PICFw and Gr-VAP1-PICRv (Table 1) and cloned into pPIC-9His using SmaI and EcoRI restriction sites to generate His-FLAG-tagged Gr-VAP1 for heterologous expression in *Pichia pastoris* strain GS115 (Invitrogen) (Rooney *et al. Science* **308,** 1783-1786 (2005)). Culture supernatant containing His-FLAG-Gr-VAP1 recombinant protein was first concentrated by centrifugation on a 10 kDa filter (Millipore, Billerica, MA, USA) and dialyzed overnight at 4°C against phosphate-buffered saline (pH 7.4). Recombinant His-FLAG-Avr2 and Rcr3-His-HA were produced as described in (Rooney et al., *supra).* His-FLAG-Gr-VAP1 and His-FLAG-Avr2 (Rooney et **al.,** *supra)* were each incubated with 40 µl of α-FLAG M2 agarose beads (Sigma) in Co-IP buffer (50 mM sodium acetate, 10 mM L-cysteine, pH 5.0) to bind Gr-VAP1 and Avr2 to the beads respectively. For some of the samples Rcr3^{pim}-His-HA was pre-incubated in Co-IP buffer with E-64 (40 µM; Sigma-Aldrich) for 30 min at room temperature to block the active site in Rcr3^{pim}. Next, agarose beads with Avr2, Gr-VAP1 , and agarose beads alone, were incubated overnight at 4°C on a rotary shaker with Rcr3^{pim}-His-HA. The agarose beads were subsequently collected by centrifugation at 3,000 g and washed several times in 1 ml Co-IP buffer. The bound proteins were eluted by boiling the beads 5 min at 95°C, and subsequently analyzed on 12% SDS-PAGE gel and western blot using a-Rcr3 (Rooney et al., *supra)* and a-FLAG serum (Sigma).

Bimolecular fluorescence complementation with Rcr3^{pim} and Gr-VAP1. The region encoding Gr-VAP1 including its native signal peptide for secretion was PCR amplified from cDNA, using the primers Gr-VAP1-GWFw and Gr-VAP1-GWRv (Table 1), and subsequently cloned (Gateway, Invitrogen) into pDH51-GW-YFPc (Zhong et al. *Transgenic Res.* **17,** 985-989 (2008)) to produce a Gr-VAP1-YFPc fusion protein in plants. Similarly, the region encoding the signal peptide for secretion of PR1a of *Nicotiana tabacum* (Genbank accession X06930) was PCR amplified from cDNA using the primers PR1aSP-GWFw and PR1aSP-GwRv (Table 1), and subsequently cloned into pDH51-GW-YFPc to produce a PR1aSP-YFPc fusion protein in plants. Rcr3^{pim} was PCR amplified from genomic DNA, using the primers Rcr3^{pim}-GWFw and Rcr3^{pim}-GWRv (Table 1), and subsequently cloned into pDH51-GW-YFPn (Zhong et al. *Transgenic Res.* **17,** 985-989 (2008)) to produce Rcr3^{pim}-YFPn fusion proteins in plants. All binary constructs were transferred to *Agrobacterium tumefaciens* GV3101 for agroinfiltration in leaves of 5 weeks old *N. benthamiana* plants (Bos *et al. Plant J.* **48,** 165-176 (2006)). Three days post infiltration the infiltrated leaf areas were cut into pieces and mounted in perfluorodecalin to minimize reflections by air trapped in intercellular spaces in mesophyll tissue (Littlejohn et al. *New Phytol.* **186,** 1018-1025

(2010)). Yellow fluorescent protein (YFP) in the samples was excited with 15% of a 30 mW Argon laser line at 514 nm in a confocal laser-scanning microscope using a 63x objective (NA 1.4 Plan-Apo lens; Zeiss LSM 510 with Axiovert 200M). YFP emission signaling bimolecular fluorescence complementation was specifically detected through a band pass filter 530-600 using a META detector (Zeiss, Jena, Germany).

Activity profiling of papain-like cysteine proteases in plants. The papain-like cysteine proteases *Rcr3^{pim} of S. pimpinellifolium, Pip1^{lyc}, Rcr3^{lyc}, CatB^{lyc}, Cyp3^{lyc},* and *C14^{lyc}* of S. *lycopersicum* and *C14^{tub}* of S. *tuberosum* were transiently overexpressed in the apoplastic fluids of *N. benthamiana* following agroinfiltration (Shabab *et al. Plant Cell* **20,** 1169-1 183 (2008)). For fluorescent activity profiling, 5 to 25 µl of apoplastic fluids containing the proteins Rcr3^{pim}, PIP1^{lyc}, Rcr3^{lyc}, CatB^{lyc}, Cyp3^{lyc}, C14^{lyc}, and C14^{tub} were preincubated with either 100 nM of *Pichia pastoris* produced Avr2, 100 nM cystatin from chicken egg-white (Sigma-Aldrich), and 300 nM of *N. benthamiana* produced Gr-VAP1 in 50 mM sodium acetate pH 5.5, and 100 µM DTT. The remaining non-inhibited proteases were labeled for 5 h with 1 µM of fluorescent DCG-04-TMR (Greenbaum *et al. Mol. Cell. Proteom.* **1,** 60-68 (2002)). Fluorescent proteins in 12% Bis-Tris gels (Invitrogen) were detected using a fluorescent imager scanner (Molecular Imager FX, Bio-Rad, Hercules, CA, USA) and images were acquired using the Quantity One software (version 4.6.7, Bio-rad Laboratories).

Nematode infection assays in tomato. The S. *lycopersicum* cultivar MoneyMaker carrying no resistance genes to *Cladosporium fulvum* and *Globodera rostochiensis (Cf-0/Rcr3!^{lyc})* was crossed either with a MoneyMaker line carrying an introgression including the *Cf-2* resistance and *Rcr3* allele from S. *pimpinellifolium (Cf-2/Rcr3^{pim})* or with an *EMS* mutant of *Cf-2/Rcr3^{pim}* carrying a translational stop mutation in *Rcr3^{pim} (Cf-2/rcr3-3;* (Dixon et al. *Proc. Natl. Acad. Sci. U.S.A.* **97,** 8807 (2000)). The F2 progenies of the two crosses were screened using allele-specific primers, resulting in *Cf-0* plants carrying *Rcr3^{pim}* and *rcr3-3.* Seeds of the five different genotypes (i.e. *Cf-2/Rcr3^{pim}, Cf-0/Rcr3^{pim}, Cf-0/Rcr3^{lyc}, Cf-2/rcr3-3,* and *Cf-0/rcr3-3)* were surface sterilized and grown in square plates (1 seed per plate) on Gamborg B5 medium including vitamins and minerals (Duchefa, Haarlem, The Netherlands), and 2% sucrose (pH 6.2). After 3 weeks, the seedlings were inoculated with 200 surface-sterilized freshly-hatched second stage juveniles of *G. rostochiensis* pathotype Ro1 per plate. After incubating the plates for three weeks at 24°C with 16 h light, the number of nematodes inside the roots stained with acid fuchsin were counted using a dissection microscope (Chen et al. *Mol. Plant-Microbe Interact.* **18,** 621-625 (2005)). The number of nematodes per plant was statistically analyzed in an ANOVA test in VassarStats

(http://faculty.vassar.edu/lowry/VassarStats.html). The nematode infection tests were repeated four times with 10 replicates per plant genotype.

So far, Cf-2 and Rcr3 have only been studied in leaves of tomato plants. Reverse transcription PCR was used to investigate expression of *Cf-2* and *Rcr3* in nematode-infected roots of the tomato genotypes *Cf-2/Rcr3^{pim}, Cf-0/Rcr3^{pim}, Cf-O/Rcd*^{c}, Cf-2/rcr3-3,* and *Cf-0/rcr3-3* (Swiecicka *et al. Mol. Plant Pathol.* **10,** 487 (2009)). *Rcr3, Cf-2,* and actin as a control were PCR amplified using primer combinations RC1 and RC4 (Shabab *et al. Plant Cell* **20,** 1169-1 183 (2008)), Cf-2Fw and Cf-2Rv (Table 1), and AC1 and AC2 (Kruger *et al., Science* **296,** 744-747 (2002)), respectively. The PCR products were quantified on agarose gels with ethidium bromide and KodakI D 3.6 software (Eastman, Rochester, NY, USA).

Microscopy of nematode-infected to tomato roots. Seeds of the tomato genotypes Cf-*O/Rcr*^^{c} and *Cf-2/Rcr3^{p,m}* were surface-sterilized in 4% (w/v) sodium hypochlorite for 10 min. The seeds were subsequently rinsed four times with sterile water and transferred into 1.5% (w/v) water agar for germination. Germinating seeds having 1.0-1.5 cm long roots were transferred to plates containing Murashige and Skoog (1962) medium supplemented with Gamborg's B5 vitamins, 2% (w/v) sucrose and 0.6% (w/v) agar and incubated for two weeks at 18°C with 16h light. Next, each seedling was inoculated with 200 surface-sterilized freshly-hatched second stage juveniles of *G. rostochiensis* pathotype Ro1. The aerial parts of the plants were removed after inoculation and the plates with roots were incubated in the dark at 18°C. Samples of roots containing syncytia were collected at 3, 7, 14 and 21 days after root invasion, fixed in glutaraldehyde and paraformaldehyde, and embedded in Epoxy resin (Golinowski et al. *Protoplasma* **194,** 103-1 16 (1996)). Serial semi-thin (3 µm) and ultrathin (70 nm) sections were examined under an Olympus AX70 "Provis" light microscope (Olympus, Tokyo, Japan) and a FEI M268D "Morgagni" transmission electron microscope (Fei, Hillsboro, OR, USA), respectively.

Transient expression of *Gr-VAP1* in tomato leaves

Gr-VAP1 and *RcrS^{pim}* including their native signal peptide for secretion, and *Avr2* carrying the signal peptide of PR1a (Genbank accession X06930), were subcloned into the binary vector pSOL2086 (kindly provided by Patrick Smit, Laboratory of Phytopathology, Wageningen University). Briefly, *Gr-VAP1* was PCR amplified from a cDNA clone using primers Gr-VAP1-GWFw and Gr-VAP1_STOP-GWRv (Table 1). *PR1aSP-Avr2* was PCR amplified from a cDNA clone (provided by Peter van Esse, Laboratory of Phytopathology, Wageningen University) using the primers PR1aSP-GWFw and Avr2_STOP-GwRv (Table 1). *Rcr3^{pim}* was PCR amplified from a genomic clone using the primers Rcr3^{pim}-GWFw and

Rcr3^{pim}-GWRv (Table 1). All PCR products were first sub-cloned into pENTR-D TOPO (Gateway, Invitrogen), checked by sequencing, and transferred further to the pSOL2086 destination vector. All binary constructs were introduced into *Agrobacterium tumefaciens* 1D1249 and infiltrated into leaves of 7 week-old plants of the tomato genotypes *Cf-2/rcr3^{pim}* and *Cf-2/Rcr3-3^{pim}*. The agroinfiltrated plants were kept in a growth chamber at 25°C with 16 h light, and visually inspected for the cell death symptoms in the infiltrated leaf area for 7 days after infiltration.

Table 1. Oligonucleotides used for PCR.

Name	**Oligonucleotide sequence** (5'- **to 3'-end)**
Gr-VAP1-RaceRv	CGTTGAGCGGTAGTTGTTGTGGC
Gr-VAP1-RaceFw	GCCACAACAACTACCGCTCAACG
N+0	GACTGCGTACTCATGG
T+0	GACGATGAGTCCTGAACCGA
Gr-VAP1-RTFw	GCATTGGGCATTGGAGTC
Gr-VAP1-RTRv	TTTGTAGACGACCTGGTTC
cAMP-RTFw	ATCAGCCCATTCAAATCTAC G
cAMP-RTRv	TTCTTCAGCAAGTCCTTCAAC
Gr-VAP1-Y2HFw	CCGGAATTCCTTTC TGCGTCCAGCC
Gr-VAP1-Y2HRv	CGCGGATCCC CAAAACGCACAGTCCG
Gr-VAP1-PICFw	GACTACAAGGACGACGATGACAAGCTTTCTGCGTCCAGCCGCGTGTCG
Gr-VAP1-PICRv	CGCGAATTCTAC TTATGG CAAAAC GCACAGTCCGCTGGT
Gr-VAP1-GWFw	CACCATG GCGTTTG CCCCAAC AAT
Gr-VAP1-GWRv	TGGCAAAACGCACAGTCCGCTGGT
PR1 aSP-GWFw	CACCATG GGATTTGTTCTC TTTTCA
PR1 aSP-GWRv	ATTTTGGGCACGGCAAGAGTGGGATA
Rcr3^{pim}-GWFw	CACCATGGCTATGAAAGTTGATTTG
Rcr3^{pim}-GWRv	TGCTATGTTTGGATAAGAAGACATC
RC1	TGGCTGTTTAGTTACGGCTTG
RC4	ACGAGCTGTGGATGTCACGTC
Cf-2Fw	GATCTCATTGCGATCCGTATA
Cf-2Rv	ATAG CCCATCAGAGCTGC TTTCC
AC1	ATGGCAGACGGTGAGGATATTCA
AC2	GCCTTTG CAATCCACATCTGTTG
Gr-VAP1_STOP-GWRv	TTATGGCAAAACGCACAGTCCGCTGG
Avr2_STOP-GWRv	TCAAC CGCAAAG ACCAAAACA GCA
Rcr3^{pim}stop-GWRv	CTATGCTATGTTTGGATAAGAAGA

Results

The Cf-2 immune receptor specifically guards the S. *pimpinellifolium* allele of Rcr3 ($Rcr3^{pim}$). We first examined whether affinity-tagged Gr-VAP1 binds to $Rcr3^{pim}$ in an *in vitro* pull-down assay, including Avr2 as a positive control (Fig. 1A). $Rcr3^{pim}$-His-HA was captured by His-FLAG-Gr-VAP1 bound to anti-FLAG antibodies on beads, while anti-FLAG beads alone captured no $Rcr3^{pim}$-His-HA. The presence of the cysteine protease inhibitor E-64 in a subset of the samples reduced the binding of $Rcr3^{pim}$ to Gr-VAP1, indicating that this binding depends on the availability of the active site of $Rcr3^{pim}$ (Fig. 1A). We subsequently tested if $Rcr3^{lyc}$ was also bound by Gr-VAP1. In contrast to the positive control (His-FLAG-AVR2), Figure 1B clearly shows that $Rcr3^{lyc}$-His-HA is not captured with affinity-tagged Gr-VAP1. Therefore, we conclude that, in contrast to $Rcr3^{pim}$, Gr-VAP1 does not bind to the $Rcr3^{lyc}$ allele. Next, we tested with bimolecular fluorescence complementation the physical interaction between Gr-VAP1 and $Rcr3^{pim}$ carrying their native signal peptides for secretion in *Nicotiana benthamiana* mesophyll cells (Fig. 1C). Transient co-expression of Gr-VAP1 fused to an N-terminal segment of Yellow Fluorescent Protein (Gr-VAP1-YFPn) and $Rcr3^{pim}$ fused to the complementary C-terminal segment of YFP ($Rcr3^{pim}$-YFPc) resulted in specific YFP fluorescence in the perinuclear endoplasmic reticulum, in cytoplasmic strands, and in the extracellular matrix of transformed mesophyll cells. By contrast, co-expression of $Rcr3^{pim}$-YFPc and YFPn fused to the signal peptide of the pathogenesis-related protein PR1a of *N. benthamiana* did not yield fluorescence (Fig. 1C). Based on these results, we conclude that Gr-VAP1 physically interacts with $Rcr3^{pim}$ *in vitro* and in plant cells.

The *C. fulvum* effector Avr2 both binds to and inhibits $Rcr3^{pim}$. To examine possible Gr-VAP1-mediated inhibition of $Rcr3^{pim}$, we used fluorescent DCG-04 protease activity profiling on apoplastic fluids of agroinfiltrated *N. benthamiana* leaves transiently expressing $Rcr3^{pim}$-His (Fig. 2). Fluorescent DCG-04 is an analogue of E-64 that covalently binds to the catalytic residues of papain-like cysteine proteases in an activity-dependent manner. Similar to both Avr2 and cystatin, pre-incubating $Rcr3^{pim}$ with Gr-VAP1 strongly reduced the DCG-04 labeling of $Rcr3^{pim}$, indicating that Gr-VAP1 inhibits $Rcr3^{pim}$ protease activity. By contrast, DCG-04 labeling of the S. *lycopersicum* allele of Rcr3 ($Rcr3^{lyc}$), which differs in 6 amino acids from $Rcr3^{pim}$, was reduced by Avr2 and cystatin, but not by Gr-VAP1 (Fig. 2).

To further investigate whether Gr-VAP1 inhibits other apoplastic papain-like proteases of tomato, we conducted DCG-04 activity profiling on apoplastic fluids of agroinfiltrated *N. benthamiana* leaves separately expressing $Pip1^{lyc}$, $CatB^{lyc}$, $C14^{lyc}$, and $Cyp3^{lyc}$ of S. *lycopersicum* (Fig. 2). In contrast to Avr2 and cystatin, pre-incubation of $Pip1^{lyc}$ with Gr-VAP1 did not reduce DCG-04 labeling. DCG-04 labeling of $CatB^{lyc}$ and $C14^{lyc}$ was only reduced by cystatin, while DCG-04-labeling of $Cyp3^{lyc}$ was only weakly reduced by Avr2. Potato is a host for *G. rostochiensis* and we therefore also included C14 of potato

(Solanum tuberosum; C14^{tub}) in this experiment. Remarkably, both Gr-VAP1 and cystatin, but not Avr2, reduced the DCG-04 labeling of C14^{tub}. We therefore conclude that Gr-VAP1 disturbs the active site of at least two different apoplastic papain-like cysteine proteases in two host plant species of *G. rostochiensis.*

To understand the biological significance of Gr-VAP1-induced perturbations of Rcr3^{pim}, we investigated susceptibility of roots of the tomato genotypes *Cf-2/Rcr3^{pim}, Cf-0/Rcr3^{pim}, Cf-0/Rcr3^{lyc}*, *Cf-2/rcr3-3^{pim},* and *Cf-0/rcr3-3^{pim}* to *G. rostochiensis. Rcr3^{pim}* and *Rcr3^^{yc}* are allelic variants, while *rcr3-3* is a null mutant of *Rcr3^{pim}.* The *Cf-2/Rcr3^^{yc}* genotype produces an autonecrosis phenotype and could not be tested for nematode susceptibility. The tomato genotype *Cf-O/Rcr^ ^{c}* is considered a susceptible host plant of *G. rostochiensis.* Importantly, as compared to the susceptible reference genotype *Cf-0/Rcr3^{lyc}* the infection rate of *Cf-2/Rcr3^{pim}* plants was significantly lower (Fig. 3A). The infection rates in the genotypes *Cf-0/rcr3-3* and *Cf-2/rcr3-3* were similar to that in *Cf-O/Rcr^ ^{c}.* We conclude that the resistance gene *Cf-2* confers resistance to *G. rostochiensis* in tomato, and that both Cf-2 and Rcr3^{pim} are required for the increased immunity to nematodes. Furthermore, the enhanced susceptibility of *Cf-0/Rcr3^{pim}* plants as compared to *Cf-O/Rcr^ ^{c}* plants (Fig. 3A) suggests that Rcr3^{pim}, but not Rcr3^{lyc}, is a virulence target of Gr-VAP1 .

Cf-2-mediated resistance to *C. fulvum* in tomato involves a local cell death, called hypersensitive response, at the infection site of the fungus. Our analysis of nematode-infected roots of *Cf-2/Rcr3^{pim}* tomato plants revealed a delayed hypersensitive response in most, but not all, of the feeding sites of *G. rostochiensis* (Fig 3B and C). This delayed hypersensitive response in *Cf-2/Rcr3^{pim}* plants is similar as previously described for the nematode resistance genes *H1* and *Hero.*

To test whether Gr-VAP1 is able to trigger a Cf-2-dependent hypersensitive response, we subcloned *Gr-VAP1* with its native signal peptide for secretion into the *Agrobacterium tumefaciens* strain 1D1249, which does not activate a hypersensitive response in tomato plants by itself. The *Gr-VAP1* construct as well as an empty vector control were agroinfiltrated in the leaves of the tomato genotypes *Cf-2/Rcr3^{pim}, Cf-2/rcr3-3, Cf-0/Rcr3^{pim},* and *Cf-0/Rcr3^{lyc}* (Fig. 4A). Transient expression of *Gr-VAP1* triggered a hypersensitive response in leaves of *Cf-2/Rcr3^{pim}* plants, but not in leaves of *Cf-2/rcr3-3, Cf-0/Rcr3^{pim}, and Cf-O/Rcr^ ^{c}* plants. Transient expression of *Rcr3^{pim}-His* restored the ability of Gr-VAP1 to induce a hypersensitive response in *Cf-2/rcr3-3* plants (Fig. 4B). We therefore conclude that both Cf-2 and Rcr3^{pim} are required for a Gr-VAP1-triggered hypersensitive response in tomato.

Altogether, our data demonstrate that the plant immune receptor Cf-2 displays dual resistance specificity that provides tomato plants protection against a leaf mold fungus and a

root-parasitic nematode. The dual resistance specificity of Cf-2 involves the apoplastic papain-like cysteine protease Rcr3^{pim}. Recently, it has been shown that the oomycete plant-pathogen *Phytophthora infestans* secretes cystatin-like effectors that also inhibit Rcr3^{pim}. While the perturbations of Rcr3^{pim} (or Rcr3^{pim} activity) induced by Gr-VAP1 of *G. rostochiensis* and by Avr2 of *C. fulvum* may be different, both trigger a Cf-2-mediated hypersensitive response and disease resistance. By contrast, the modifications of Rcr3^{pim} brought about by the effectors EPIC1 and EPIC2B of *P. infestans* do not trigger Cf-2-mediated defense responses, suggesting that they fall outside of the detection window of Cf-2. The next step is to investigate how Cf-2 perceives effector actions through its guardee Rcr3^{pim}, and whether this involves a specific product of the protease activity of Rcr3^{pim}. The dual resistance specificity of Cf-2 by guarding a common virulence target of divergent pathogens illustrates the power of indirect recognition of pathogen-derived molecules for plants. By monitoring the health status of critical host molecules targeted by multiple and unrelated pathogens, plants can efficiently expand the coverage of their innate immune system with a relatively small set of immune receptors. Recent findings with the *Mi-1.2* gene in tomato that confers resistance to at least four different pathogens suggests that dual and multiple resistance specificities of immune receptors may contribute significantly to the recognition repertoire of plants.

CLAIMS

1. Method for producing a plant having enhanced disease resistance to one or more nematodes compared to a control plant, said method comprising the steps of:
(a) introducing a Cf-2 gene into a plant or plant cell;
(b) introducing an Rcr3 gene encoding a RCR3 protein that binds to and/or is inhibited by Gr-VAP1 into a plant or plant cell; and
(c) regenerating a plant,
wherein at least one of steps (a) and (b) is carried out by means of transformation and/or transfection.

2. The method according to claim 1, wherein said Rcr3 gene is the *Solanum pimpinellifolium* allele of the Rcr3 gene.

3. The method according to claim 1 or 2, wherein both steps (a) and (b) are carried out by means of transformation and/or transfection.

4. Method for producing a plant having enhanced disease resistance to one or more nematodes compared to a control plant, said method comprising the steps of:
(a) introducing a Cf-2 gene into a plant or plant cell by means of transformation and/or transfection;
(b) modifying the Rcr3 gene of the plant or plant cell such that it encodes a modified RCR3 protein comprising one or more of the following amino acids at the position of an endogenous RCR3 protein as compared to the amino acid position of RCR3 protein from *Solanum lycopersicum* (SEQ ID NO:1): 197L, 222E, 229R, 241K, 284Q, 319F, and/or deletion of the amino acid corresponding to K122, and
(c) regenerating the plant.

5. Method for producing a plant having enhanced disease resistance to one or more nematodes compared to a control plant, said method comprising the steps of:
(a) providing a plant or plant cell comprising a Cf-2 gene;
(b) modifying the Rcr3 gene of the plant or plant cell such that it encodes a modified RCR3 protein comprising one or more of the following amino acids at the position of an endogenous RCR3 protein as compared to the amino acid position of RCR3 protein from *Solanum lycopersicum* (SEQ ID NO:1): 197L, 222E, 229R, 241K, 284Q, 319F, and/or deletion of the amino acid corresponding to K122, and

(c) regenerating the plant.

6. Method for producing a plant having enhanced disease resistance to one or more nematodes compared to a control plant, said method comprising the steps of:

a) introgressing a Cf-2 gene into a plant or plant cell;

b) modifying the Rcr3 gene of the plant or plant cell such that it encodes a modified RCR3 protein comprising one or more of the following amino acids at the position of an endogenous RCR3 protein as compared to the amino acid position of RCR3 protein from *Solanum lycopersicum* (SEQ ID NO:1): 197L, 222E, 229R, 241K, 284Q, 319F, and/or deletion of the amino acid corresponding to K122, and

c) regenerating the plant.

7. The method according to any one of claims 4-6, in which the endogenous Rcr3 gene of said plant or plant cell is modified using targeted mutagenesis.

8. The method according to any one of the preceding claims, in which said Cf-2 gene is derived from *Solanum pimpinellifolium.*

9. The method according to any one of the preceding claims, further comprising step:

(d) screening the regenerated plant, or a plant derived therefrom by selfing or crossing, for resistance to one or more plant pathogens and identifying a plant comprising enhanced resistance to one or more of said plant pathogens.

10. The method according to any one of the preceding claims, wherein said nematode is selected from a species selected from the group consisting of *Meloidogyne incognita, M. javanica, M. arenaria, M. hapla, M. mayaguensis, Globodera rostochiensis, G. pallida, G. Mexicana, M. graminicola, M. chitwoodi, M. fallax, Heterodera schachtii, H. zeae,* and *H. glycines.*

11. The method according to any one of the preceding claims, in which said plant or plant cell is a dicotyledonous plant or a plant cell derived from a dicotyledonous plant, in which said dicotyledonous plant preferably belongs to the family of Solanaceae, preferably to the genus of *Solanum,* preferably to the species *Solanum lycopersicum.*

12. Plant, plant cell, seed or fruit, obtainable or obtained by the method according to any one of claims 1-11.

13. Use of a modified Rcr3 gene of a *Solanum lycopersicum* plant or plant cell, said modified Rcr3 gene encoding a modified RCR3 protein comprising one or more of the following mutations with respect to an endogenous *Solanum lycopersicum* RCR3 protein (SEQ ID NO:1): 197L, 222E, 229R, 241 K, 284Q, 319F, and/or deletion of K122, and a Cf-2 gene, for the generation of a plant having enhanced disease resistance to one or more nematodes compared to a control plant.

14. The use according to claim 13, wherein the plant or plant cell is a *Solanum lycopersicum* plant or plant cell.

15. The use according to any one of claims 13-14, in which said Cf-2 gene is derived from *Solanum pimpinellifolium.*

16. Method for producing a plant having enhanced disease resistance to one or more nematodes compared to a control plant, said method comprising the steps of:

(a) introducing a chimeric gene comprising a nematode-inducible promoter, operably linked to a nucleotide sequence encoding a Cf-2 protein into a plant or plant cell;

(b) introducing a chimeric gene comprising a nematode-inducible promoter, operably linked to a nucleotide sequence encoding a RCR3 protein that binds to and/or is inhibited by Gr-VAP1, such as a *Solanum pimpinellifolium* RCR3 protein or a variant thereof, into a plant or plant cell; and

(c) regenerating a plant.

17. The method according to claim 16, in which said Cf-2 gene is derived from *Solanum pimpinellifolium.*

18. Plant, plant cell, seed or fruit, obtainable or obtained by the method according to any one of claims 16-17.

19. Plant, preferably a *Solanum lycopersicum* plant, or hybrid plant, plant cell, seed or fruit, obtainable or obtained by a method comprising the steps of:

a) preparing a first hybrid plant comprising a Cf-2 gene, preferably a *Solanum pimpinellifolium* Cf-2 gene, by crossing a plant comprising said Cf-2 gene, preferably a S. *pimpinellifolium* plant comprising said Cf-2 gene, with a sexually compatible plant, preferably a *Solanum lycopersicum* plant and selecting first hybrid plants comprising said Cf-2 gene, and

b) modifying an Rcr3 gene of the plant, preferably using targeted mutagenesis, such that it encodes a modified RCR3 protein comprising one or more of the following amino acids at the position of an endogenous RCR3 protein as compared to the amino acid position of RCR3 protein from *Solanum lycopersicum* (SEQ ID NO:1): 197L, 222E, 229R, 241K, 284Q, 319F, and/or deletion of the amino acid corresponding to K122.

20. Plant, plant cell, seed or fruit, obtainable or obtained by a method comprising the steps of:

a) introgressing a Cf-2 gene, preferably a *Solanum pimpinellifolium* Cf-2 gene into a plant, preferably a *Solanum lycopersicum* plant, and

b) modifying an Rcr3 gene of the plant, preferably using targeted mutagenesis, such that it encodes a modified RCR3 protein comprising one or more of the following amino acids at the position of an endogenous RCR3 protein as compared to the amino acid position of RCR3 protein from *Solanum lycopersicum* (SEQ ID NO:1): 197L, 222E, 229R, 241K, 284Q, 319F, and/or deletion of the amino acid corresponding to K122.

21. The plant, plant cell, seed or fruit according to claim 19 or 20, the method further comprising the step of regenerating the plant.

22. The plant, plant cell, seed or fruit according to any one of claims 19 - 21, the method further comprising the step of screening the regenerated plant, or a plant derived therefrom by selfing or crossing, for resistance to one or more nematode species and identifying a plant comprising enhanced resistance to one or more of said nematode species.

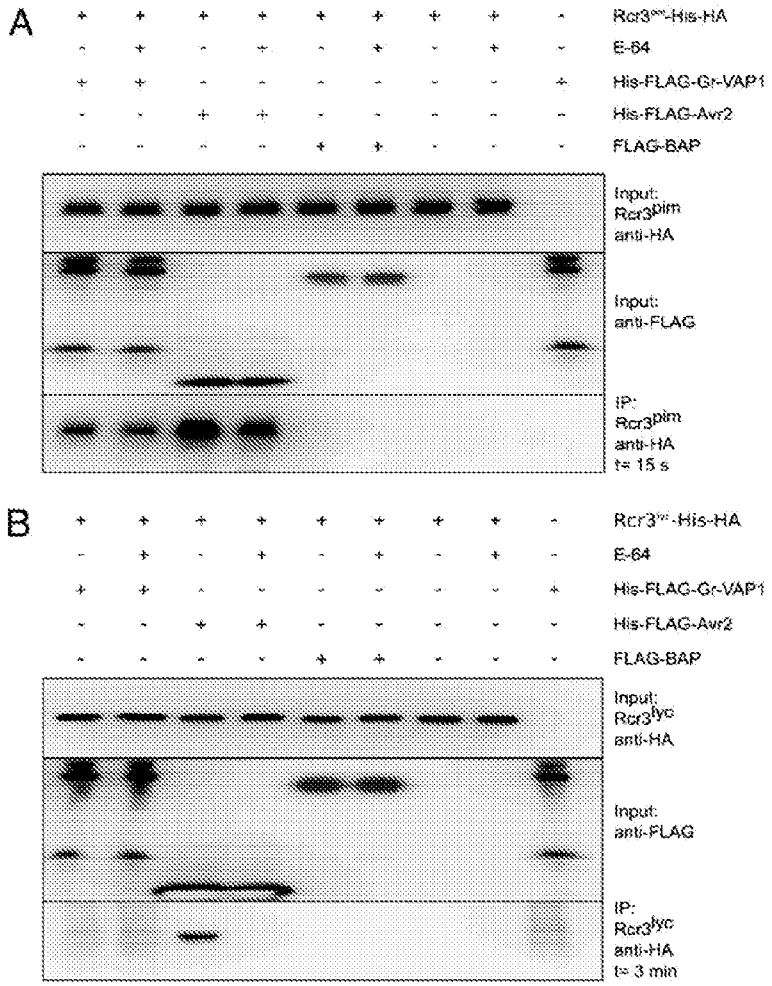
23. A nematode-resistant plant, preferably a *Solanum lycopersicum* plant, comprising an introgression comprising a Cf-2 gene, preferably an introgression from a *Solanum pimpinellifolium* plant comprising said Cf-2 gene, said plant further comprising a modified endogenous Rcr3 gene, wherein said endogenous Rcr3 gene is modified such that it encodes a modified RCR3 protein comprising one or more of the following amino acids at the position of an endogenous RCR3 protein as compared to the amino acid position of RCR3 protein from *Solanum lycopersicum* (SEQ ID NO:1): 197L, 222E, 229R, 241K, 284Q, 319F, and/or deletion of the amino acid corresponding to K122.

24. A nematode-resistant plant, preferably a *Solanum lycopersicum* plant, comprising an introgression comprising a Cf-2 gene, preferably an introgression from a *Solanum pimpinellifolium* plant comprising said Cf-2 gene, said plant further comprising an Rcr3 gene, wherein said Rcr3 gene encodes a RCR3 protein comprising one or more of the following amino acids at the position of the RCR3 protein from *Solanum lycopersicum* (SEQ ID NO:1): 197L, 222E, 229R, 241K, 284Q, 319F, and/or deletion of the amino acid corresponding to K122.

25. A nematode-resistant plant, preferably a *Solanum lycopersicum,* plant, comprising an introgression comprising a Cf-2 gene, preferably an introgression from a *Solanum pimpinellifolium* plant comprising said Cf-2 gene, said plant further comprising a modified Rcr3 gene, wherein said Rcr3 gene is modified such that it encodes a modified RCR3 protein that binds to and/or is inhibited by Gr-VAP1.

26. A plant according to claim 25, which is selected from a *Solanum lycopersicum, Solanum peruvianum, Solanum cornelliomuelleri, Solanum chilense, Solanum pennellii, Solanum habrochaites* and *Solanum chmielewski* plant.

Figure 1


A
Rcr3pim-His-HA
E-64
His-FLAG-Gr-VAP1
His-FLAG-Avr2
FLAG-BAP
Input: Rcr3pim anti-HA
Input: anti-FLAG
IP: Rcr3pim anti-HA t= 15 s
B
Rcr3lyc-His-HA
E-64
His-FLAG-Gr-VAP1
His-FLAG-Avr2
FLAG-BAP
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Input: anti-FLAG
IP: Rcr3lyc anti-HA t= 3 min

Figure 1 – Cont'd

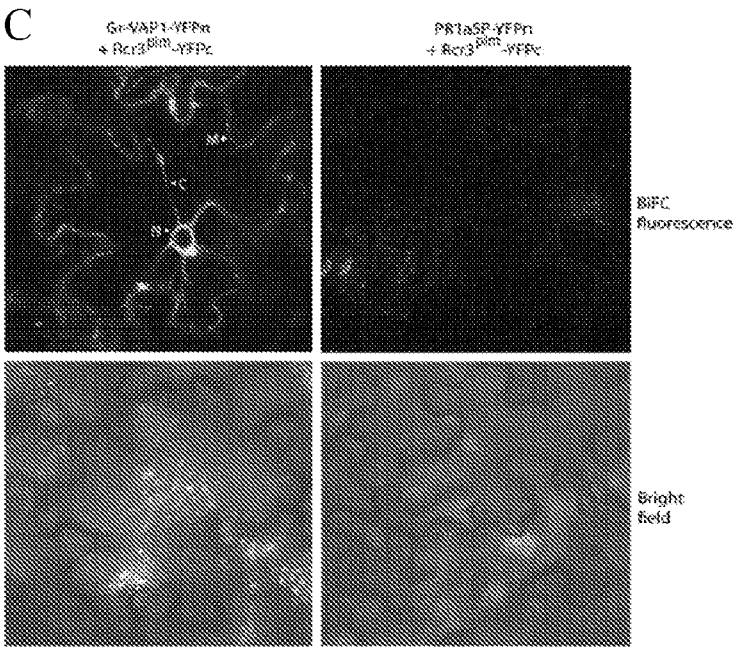
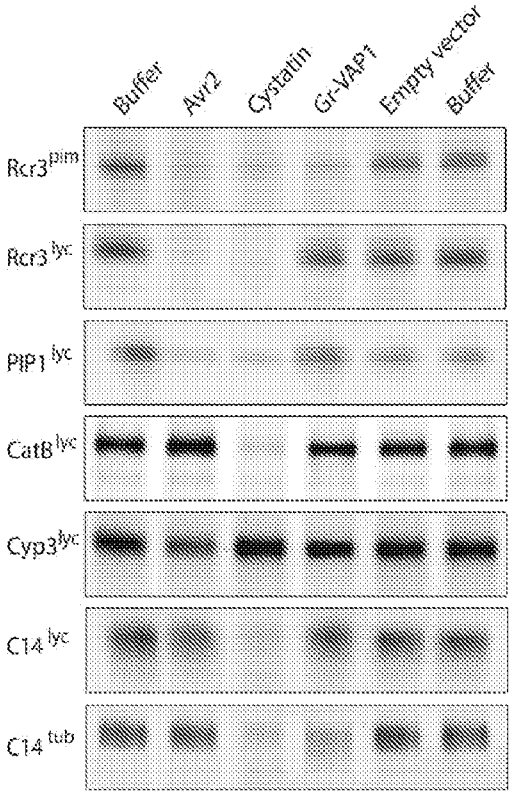


Figure 2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PIP1lyc
Cat8lyc
Cyp3lyc
C14lyc
C14tub

Figure 3

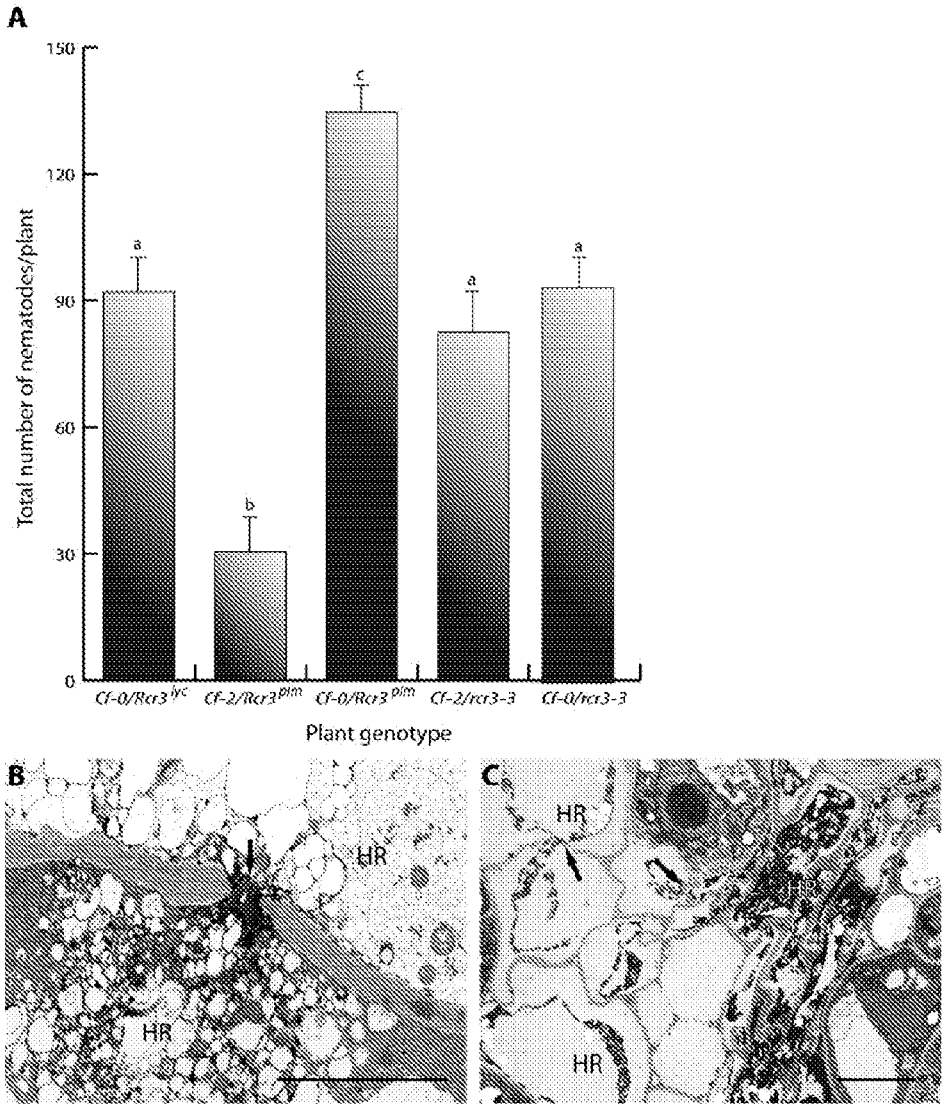
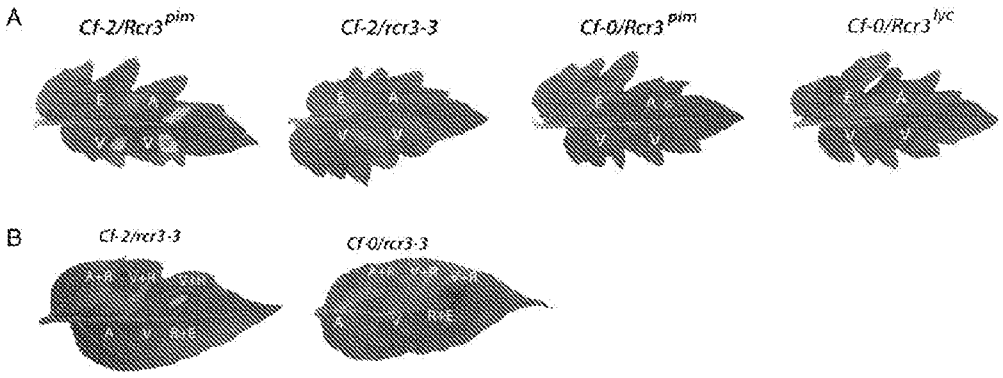

A
Total number of nematodes/plant
150
120
90
60
30
0
a
b
c
a
a
Cf-0/Rcr3 iyc
Cf-2/Rcr3 pim
Cf-0/Rcr3 pim
Cf-2/rcr3-3
Cf-0/rcr3-3
Plant genotype
B
HR
HR
C
HR
HR

Figure 4


A
Cf-2/Rcr3pim
Cf-2/rcr3-3
Cf-0/Rcr3pim
Cf-0/Rcr3lyc
B
Cf-2/rcr3-3
Cf-0/rcr3-3

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2012/050894

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/82 C12N15/29 C12N15/57 A01H1/06 A01H1/04
A01H5/00
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N A01H

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	LOZANO-TORRES, J.L., ET AL.: "Dual disease resistance mediated by the immune receptor Cf-2 in tomato requires a common virulence target of a fungus and a nematode", PNAS, vol. 109, no. 25, 6 June 2012 (2012-06-06), page 10119, XP002679103, the whole document ----	1-26

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 February 2013	27/02/2013

Name and mailing address of the ISA/	Authorized officer
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