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(54) Title: BACTERIAL RESISTANT TRANSGENIC PLANTS

(57) Abstract: A nucleic acid expression vector comprising a nucleic acid sequence encoding a dominant negative T3SS protein is disclosed. The nucleic acid expression vector further comprising a cis acting regulatory element capable of driving transcription of the nucleic acid sequence in a plant cell. Moreover, the dominant negative T3SS protein mediates assembly of a dysfunctional needle complex.

BACTERIAL RESISTANT TRANSGENIC PLANTS

FIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to bacterial resistant plants and methods of generating same.

Ralstonia solanacearum (Rs), a widely distributed, Gram-negative, soil-borne pathogen belonging to the β-subdivision of Proteobacteria, causes a lethal wilting disease of more than 200 plant species including economically important crops such as tomato, potato and banana. The bacterium enters plant roots through wounds, invades the xylem vessels and spreads rapidly to aerial parts of the plant through the vascular system. Rapidly, populations of more than 10^{10} cells per cm of stem are found. The main virulence factor of Rs is exopolysaccharide (EPS), a long (more than 106 Da) sugar polymer that clogs the xylem and causes wilting symptoms and eventually plant death.

Rs displays a remarkable ability for protein secretion of more than 100 proteins. For example, the Type II secretion system (T2SS) secretes factors including the plant cell wall-degrading pectinases, endo-glucanases, and later, the virulence EPS (Figures 1A-C). Type III secretion system (T3SS) secretes infection-promoting effector proteins (T3Es) into host cells to optimize the host environment and suppress plant defense responses following invasion (Figures 1A-C).

The Type III secretion system (T3SS) is a sophisticated molecular machinery of Gram-negative bacteria used to 'inject' (translocate) bacterial proteins (effectors) into eukaryotic cells. For this, the T3SS has to assemble into a multi-protein complex, which is comprised of distinct parts; a basal body spanning the two bacterial membranes connected with a cytoplasmic bulb, an attached needle structure resembling a molecular syringe (injectisome/pilus), and a distal needle tip structure that reorganizes into a 'translocon', which is a protein complex that inserts into the host cellular membrane. The pilus is built from only one protein subunit. Multiple subunits oligomerize into the pilus structure. This needle structure allows bacterial proteins to be transported through the inner channel, the conduit, of the needle on their way to the host cell (Figures 1A-C).

Thus, the major extracellular component of the T3SS is the needle that extends from the outer-membrane portion of the apparatus and through which runs a 25-Å channel forming the secretion conduit (the helical parameters of the needle are 5.5 subunits per turn; 4.6-Å axial rise per subunit). The needle is formed by a helical assembly of multiple copies (on the order of 100–150) of a single, small (9 kDa) protein. Though there is little homology between the primary sequence of the pilus building blocks of most of the Gram negative bacteria, it is believed that most share some structural homology. In plant pathogenic bacteria, T3SSs are encoded by hrp (hypersensitive response and pathogenicity) genes, which are so named because they are required for bacteria to cause disease in susceptible plants and to elicit the hypersensitive response in resistant plants. Hrp genes were found in almost all major gram-negative bacterial plant pathogens (e.g. *Pseudomonas syringae*, *Xanthomonas spp.*, *Ralstonia solanacearum*, and *Erwinia spp.*), illustrating a central role of the T3SS in mediating diverse plant-bacteria interactions. Thus, typically, the T3SS extracellular pilus is assembled through the stepwise polymerization of a major component (e.g. HrpY in *R. solanacearum*, HrpA in *P. syringae* and *E. amylovora,* HrpE in *Xanthomonas campestris*, MxiH in *Shigella*, PrgI in *Salmonella* and YscF in *Yersinia*).

As mentioned, although the primary function of type III effectors is to promote plant susceptibility, some effectors are recognized by plant resistance proteins which trigger defense responses, including the hypersensitive response. One method proposed to overcome plant lethal infection by gram-negative bacteria comprises enhancing plant immunity against such pathogens.

U.S. Patent Application No. 20090258825 (He et al.) discloses compositions and methods for enhancing plant defenses against pathogens (e.g. bacterial pathogens). According to their teachings, enhancing plant immunity against the *Pseudomonas syringae* virulence protein HopM1 is effected by increasing the activity of an ATMIN associated plant protection protein, such as ATMIN7.

U.S. Patent Application No. 20090044296 (Beer et al.) discloses methods of increasing plant growth or imparting disease resistance in plants by the use of nucleic acid molecules configured to increase or decrease expression of a nucleic acid molecule that encodes a HrpN-interacting protein (e.g. HIPM). Deletion analysis disclosed therein showed that the 198-aa N-terminal region of HrpN (harpin) of *Erwinia amylovora*, the

first cell-free elicitor of the hypersensitive response which plays a critical role in the virulence of this pathogen, is required for interaction with HIPM.

Moreover, bacterial wilt is difficult to control because of the soil borne nature of its causal organism. In plants infected by Rs, disease development depends on the action of the Type II and Type III protein secretion systems and mutations in one of these systems leads to non-pathogenic bacteria [Poueymiro et al., Curr. Opin. Microbiol. (2009) 12:44–52].

Roine et al. [Roine et al., FEBS Letters (1997) 417(2): 168-172] showed that once purified, HrpA, the structural protein of *Pseudomonas syringae* pv. tomato DC3000 pili, alone is sufficient for formation of filament structures undergoing self-assembly.

Taira et al. [Taira et al., Mol Microbiol. (1999) 34(4):737-44] generated insertion mutations in the hrpA gene (e.g. pentapeptide insertions) and created mutated bacteria expressing same. According to their teachings, the carboxy-terminus region of hrpA is crucial for pilus assembly and for bacterial interaction with the affected plant. Moreover, Wei et al. [Wei et al., PNAS (2000) 97(5):2247–2252] identified three single amino acid mutations at the HrpA carboxyl terminus which affect the secretion or regulatory function of the HrpA protein. These results demonstrated an essential role of the Hrp pilus structural gene in protein secretion and coordinate regulation of the type III secretion system in *Pseudomonas syringae*. Furthermore, Lee et al. [Lee et al., J. Bio. Chem. (2005) 280: 21409-17] disclosed that several pentapeptide-induced nonfunctional HrpA proteins, when expressed in bacteria, exert a strong dominant-negative effect on the function of the wild-type HrpA protein blocking the ability of *Pseudomonas syringae* to elicit host responses and cause a disease in-vivo. The dominant-negative HrpA mutants were also able to interfere with the self-assembly of wild-type HrpA into pilus in vitro.

Weber at al. [Weber and Koebnik, J. Bacteriology (2005) 187(17): 6175–6186] described hydrophobicity plot analyses of several Hrp pilin proteins, such as HrpE and HrpA from *Xanthomonas campestris* pv. vesicatoria and HrpY from *R. solanacearum*, and revealed a common domain organization. These findings suggest that plant-pathogenic bacteria, challenged with the task of overcoming the barrier of a plant cell wall, independently evolved structurally similar proteins. Weber et al. further disclose

that pentapeptide insertion mutants in the C-terminal region of HrpE inhibit Hrp pilus assembly in *X. campestris* pv. Vesicatoria. Morphology studies revealed insertion mutants with shortened Hrp pili. This dominant-negative effect suggests that the mutant variant may interfere with the assembly of the Hrp pilus. U.S. Patent Application No. 20100249234 (Yang et al.) discloses methods of reducing virulence in a bacterium, such as a HrpX/HrpY-type system or a T3SS-type system. The method comprises contacting the bacterium with an effective amount of a phenylpropanoid-type inhibitory compound.

U.S. Patent Application No. 20100099674 (Elofsson et al.) discloses methods for decreasing bacterial virulence in a plant by inhibition of the Type III secretion system using an N-substituted 7-quinolylmethyl amine, in particular one that is further substituted in 5- and 8-position on the quinoline ring.

Additional background art includes U.S. Patent Application No. 20050076406.

SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided a nucleic acid expression vector comprising a nucleic acid sequence encoding a dominant negative T3SS protein and a cis acting regulatory element capable of driving transcription of the nucleic acid sequence in a plant cell, the dominant negative T3SS protein mediates assembly of a dysfunctional needle complex.

According to an aspect of some embodiments of the present invention there is provided an isolated polynucleotide comprising a nucleic acid sequence encoding SEQ ID NO: 2, 4, 6, 8, 10 or 12.

According to an aspect of some embodiments of the present invention there is provided a nucleic acid expression vector comprising an isolated polynucleotide comprising a nucleic acid sequence encoding SEQ ID NO: 2, 4, 6, 8, 10 or 12.

According to an aspect of some embodiments of the present invention there is provided a host cell comprising the nucleic acid expression vector.

According to an aspect of some embodiments of the present invention there is provided a genetically modified plant comprising the nucleic acid expression vector.

According to an aspect of some embodiments of the present invention there is provided a genetically modified plant expressing an exogenous polynucleotide encoding a dominant negative T3SS protein as set forth in SEQ ID NO: 2, 4, 6, 8, 10 or 12.

According to an aspect of some embodiments of the present invention there is provided a method of generating a plant comprising enhanced resistance to a bacterial pathogen compared to a non modified plant, the method comprising introducing into a plant or plant cell the nucleic acid expression vector, thereby generating the plant comprising enhanced resistance to the bacterial pathogen compared to the non modified plant.

A method of evaluating resistance of a plant to a bacterial pathogen, the method comprising: (a) expressing within the plant an exogenous nucleic acid sequence encoding a dominant negative T3SS protein and a cis acting regulatory element capable of driving transcription of the nucleic acid sequence in a plant cell; (b) subjecting the plant to a bacterial pathogen; and (c) comparing the disease in the plant to a wild-type plant grown and infected with the bacterial pathogen under the same conditions, thereby evaluating resistance of the plant to the bacterial pathogen.

According to some embodiments of the invention, the nucleic acid sequence comprises SEQ ID NO: 1, 3, 5, 7, 9 or 11.

According to some embodiments of the invention, the nucleic acid sequence comprises SEQ ID NOs: 20-65.

According to some embodiments of the invention, the nucleic acid sequence encodes for the polypeptide set forth in SEQ ID NO: 2, 4, 6, 8, 10 or 12.

According to some embodiments of the invention, the nucleic acid expression vector further comprises an additional nucleic acid sequence encoding an endoplasmic reticulum signal peptide upstream of the nucleic acid sequence .

According to some embodiments of the invention, the cis acting regulatory element comprises a promoter sequence.

According to some embodiments of the invention, the promoter sequence is a constitutive promoter.

According to some embodiments of the invention, the constitutive promoter is CaMV 35S promoter.

According to some embodiments of the invention, the dominant negative T3SS protein is generated by introduction of a mutation selected from the group consisting of an insertion mutation, a deletion mutation and a substitution mutation.

According to some embodiments of the invention, the insertion mutation comprises an intercalating blocking element.

According to some embodiments of the invention, the T3SS protein is a T3SS structural protein.

According to some embodiments of the invention, the T3SS structural protein is a HRP protein .

According to some embodiments of the invention, the T3SS protein is selected from the group consisting of a Ralstonia solanacearum HrpY protein, a Pseudomonas syringae HrpA protein, a Erwinia amylovora HrpA protein, a Xanthomonas campestris HrpE protein, a *Erwinia pyrifoliae* HrpA protein and a Xanthomonas oryzae HrpE protein .

According to some embodiments of the invention, the T3SS protein is a Ralstonia solanacearum translocon protein.

According to some embodiments of the invention, the Ralstonia solanacearum translocon protein is selected from the group consisting of PopF1 and PopF2 .

According to some embodiments of the invention, the host cell being a plant cell.

According to some embodiments of the invention, the plant comprises enhanced resistance to a bacterial pathogen compared to a non modified plant.

According to some embodiments of the invention, the bacterial pathogen is a gram-negative bacteria.

According to some embodiments of the invention, the gram-negative bacteria is selected from the group consisting of a Ralstonia solanacearum, a Pseudomonas syringae, a Erwinia amylovora, a Xanthomonas campestris and a Xanthomonas oryzae.

According to some embodiments of the invention, the gram-negative bacteria is a Proteobacteria species.

According to some embodiments of the invention, the Proteobacteria is Ralstonia solanacearum.

According to some embodiments of the invention, the plant is selected from the group consisting of a crop plant, a decorative plant, and a tree .

According to some embodiments of the invention, the plant is a Solanaceae plant.

According to some embodiments of the invention, the plant is selected from the group consisting of a tomato plant, a potato plant, an eggplant plant, a banana plant, a sweet pepper plant, an olive plant, an apple plant, a pear plant, a firethorn plant, a flowering crabapple plant, a hawthorn plant, a cotoneaster plant, a quince plant, a mountain ash plant, an arabidopsis plant, a geranium, a ginger plant, a tobacco plant and a eucalyptus plant.

According to some embodiments of the invention, the plant is a tomato plant.

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

BRIEF DESCRIPTION OF THE DRAWINGS

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

In the drawings:

FIG. 1A is a picture illustrating Type II secretion system (T2SS), Type III secretion system (T3SS) and Type IV pili in gram-negative bacteria. The picture was adapted from Donnenberg M.S., Nature (2000) 406: 768-774.

FIGs. 1B-C are pictures adapted from Buttner and He, Plant Physiology (2009) 150: 1656–64 illustrating the T3SS complex in plant (Figure 1B) and animal (Figure 1C) pathogenic bacteria. The secretion apparatus spans both bacterial membranes and is associated with a cytoplasmic ATPase. Plant pathogenic bacteria share a pilus that presumably spans the plant cell wall. Animal pathogenic bacteria has a short needle

which is linked via the so-called tip complex (missing in plant pathogens) to the translocon. The translocon forms a channel in the host plasma membrane and allows transport of effector proteins into the host cell cytosol.

FIGs. 2A-F are pictures illustrating *Ralstonia solanacearum* (Rs) HrpY protein (SEQ ID NO: 14) aligned with *Shigella* T3SS needle monomer MxiH (SEQ ID NO: 15, Figure 2A), structural models of *Shigella* MxiH (Figures 2B-E) and overall needle structure (Figure 2F). Pictures of MxiH and needle structures were adapted from Deane et al., PNAS 2006 103: 12529-33.

FIGs. 3A-E are pictures illustrating the dominant negative proteins T3SS Intercalating Blocking Elements 1 (Figure 3A, SEQ ID NO: 2) and 2 (Figure 3C, SEQ ID NO: 4), the predicted model of the structure of T3SS IBEs 1 and 2 (Figures 3B and 3D, respectively), a model of interaction with the needle and needle conduit (Figure 3E) and plant secretion signals from sp|Q56YT0|LAC3_At Laccase or the tr|Q6TDS6|Q6TDS6_GOSAR Secretory laccase Gossypium arboreum (SEQ ID NOs: 16 and 17, respectively). Pictures 3B, 3D and 3E were adapted from Deane et al., PNAS 2006 103: 12529-33.

FIGs. 4A-C are pictures illustrating the dominant negative protein T3SS Intercalating Blocking Element 3 (Figure 4A, SEQ ID NO: 6), the predicted model of the structure of T3SS IBE 3 (Figures 4B) and model of interaction with the needle (Figures 4C). Pictures 4B and 4C were adapted from Deane et al., PNAS 2006 103: 12529-33.

FIGs. 5A-C are pictures illustrating the dominant negative protein T3SS Intercalating Blocking Element 4 (Figure 5A, SEQ ID NO: 8), the predicted model of the blocking element (Figure 5C) and the needle (Figure 5B). Pictures 5B and 5C were adapted from Deane et al., PNAS 2006 103: 12529-33. Of note, duplicated tail may interact with different monomers disrupting and blocking the needle channel.

FIGs. 6A-D are pictures illustrating the dominant negative proteins T3SS Intercalating Blocking Elements 5 (Figure 6A, SEQ ID NO: 10) and 6 (Figure 6C, SEQ ID NO: 12) and the predicted model of the structure of the T3SS Intercalating Blocking Elements 5 & 6 (Figures 6B and 6D respectively). Of note, HrpY head and tail alpha-helices are disrupted by prolines and such deformations may block the needle channel

and disrupt functionality of the needle. Pictures 6B and 6D were adapted from Deane et al., PNAS 2006 103: 12529-33.

FIGs. 7A-C are pictures illustrating the different *Ralstonia solanacearum* (Rs) HrpY dominant negative proteins. Figure 7A shows the T-DNA cloning map. Each Intercalating Blocking Element (IBE) was cloned downstream to CaMV 35S promoter and upstream to NOS terminator using *XbaI* and *SacI* sites. Figure 7B shows the model of interaction with the needle; and Figure 7C shows the different HrpY mutants (SEQ ID NOs: 2, 4, 6, 8, 10 and 12). Picture 7B was adapted from Deane et al., PNAS 2006 103: 12529-33.

FIG. 8 is a picture adapted from Taira et al., Mol Microbiol. (1999) 34(4):737-44 illustrating insertion mutations and their location in the hrpA gene (SEQ ID NO: 18). In short, location of the insertions in the 496 bp BamHI±EcoRI fragment encoding the HrpA pilus are marked as lollipops with mutant numbers above. Each insertion consists of 10 transposon-derived basepairs and 5 bp upstream of the insertion label duplicated distal to the 10 bp. The amino acid sequence (SEQ ID NO: 19) is written below the nucleotide sequence. The Hrp boxes in the promoter are boxed; the putative ribosome binding site is underlined. Amino-terminal protein processing sites are marked with arrows below the amino acid sequence. The boxed mutation numbers with arrowheads indicate the start and end points of four deletion mutations.

FIG. 9 is an alignment of the *Ralstonia solanacearum* HrpY polypeptide variants (i.e. slight sequence changes between strains, SEQ ID NOs: 14 and 70-86).

FIGs. 10A-C are pictures illustrating PCR and sqRT-PCR analysis of tomato plants expressing wilt resistant (WiltR) HrpY mutant 6. Tomato plants were transformed with constructs carrying HrpY mutant 6 and plants were further analyzed by genomic PCR and semi-quantitative RT-PCR. Events expressing these HrpY mutants were detected.

FIGs. 11A-C are pictures illustrating PCR and sqRT-PCR analysis of tomato plants expressing wilt resistant (WiltR) HrpY mutant 1. Tomato plants were transformed with constructs carrying HrpY mutant 1 and plants were further analyzed by genomic PCR and semi-quantitative RT-PCR. Events expressing these HrpY mutants were detected.

FIGs. 12A-C are pictures illustrating PCR and sqRT-PCR analysis of tomato plants expressing wilt resistant (WiltR) HrpY mutant 2. Tomato plants were transformed with constructs carrying HrpY mutant 2 and plants were further analyzed by genomic PCR and semi-quantitative RT-PCR. Events expressing these HrpY mutants were detected.

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to bacterial resistant plants and methods of generating same.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

While reducing some embodiments of the present invention to practice, the present inventors have generated dominant negative bacterial type III protein secretion system (T3SS) proteins, which are expressed in plant cells and secreted therefrom. The novel dominant negative T3SS proteins of the present invention intercalate within the T3SS needle structure during its assembly and block the bacterial needle channel, thereby protecting plants from bacterial infection.

The design of the dominant negative T3SS proteins of the present invention is based on preserving and utilizing the native T3SS protein (e.g. HrpY) subunit-subunit interaction sites while incorporating translationally fused channel-blocking peptides or deforming structures of the T3SS protein (e.g. HrpY alpha-helices) which prevent bacterial effectors from being secreted from the bacteria into the plant cells. Plant secretion signals added thereto enable the expression of the dominant negative proteins in the plant cells, secretion from the plant cells and accessibility of the dominant negative T3SS proteins during bacterial pilus assembly in close proximity to the plant cell wall. Thus, for example and as shown in the Examples section which follows, the

present inventors have generated intercalating blocking elements of T3SS needle channel (T3SS-IBEs) of gram-negative bacteria. T3SS-IBEs of *Ralstonia solanacearum* (SEQ ID NOs: 2, 4, 6, 8, 10 and 12) were generated using structural modifications of HrpY protein (SEQ ID NO: 14), the building block monomer of the *Rs* needle. The present inventors have further generated expression vectors comprising these T3SS-IBEs for transformation of plant cells. Moreover, the present inventors have illustrated transformation of tomato plants with *Ralstonia solanacearum* HrpY mutants 1, 2 or 6 and expression of same (see Figures 11A-C, 12A-C and 10A-C, respectively). Additionally, the present inventors have contemplated over-expression of modified Rs translocon proteins (PopF1) in transgenic plants. Over-expression of these proteins leads to an arrest in T3SS assembly due to interactions with a premature needle and, thus, deactivation thereof. Thus, modified PopF1 proteins are incorporated into the translocon gate and block it. Taken together, the present teachings may serve as powerful tools in the field of agriculture transgenic technologies for generation of bacterial resistant plants.

Thus, according to one aspect of the present invention there is provided a method of generating a plant comprising enhanced resistance to a bacterial pathogen compared to a non modified plant, the method comprising introducing into a plant or plant cell the nucleic acid expression vector, thereby generating the plant comprising enhanced resistance to the bacterial pathogen compared to the non modified plant.

The term "'plant" as used herein encompasses whole plants, ancestors and progeny of the plants and plant parts, including seeds, shoots, stems, roots (including tubers), and plant cells, tissues and organs. The plant may be in any form including suspension cultures, embryos, meristematic regions, callus tissue, leaves, gametophytes, sporophytes, pollen, and microspores. Plants that are particularly useful in the methods of the invention include all plants which belong to the superfamily Viridiplantee, in particular monocotyledonous and dicotyledonous plants including a fodder or forage legume, ornamental plant, food crop, tree, or shrub selected from the list comprising Acacia spp., Acer spp., Actinidia spp., Aesculus spp., Agathis australis, Albizia amara, Alsophila tricolor, Andropogon spp., Arachis spp, Areca catechu, Astelia fragrans, Astragalus cicer, Baikiaea plurijuga, Betula spp., Brassica spp., Bruguiera gymnorrhiza, Burkea africana, Butea frondosa, Cadaba farinosa, Calliandra spp, Camellia sinensis,

Canna indica, Capsicum spp., Cassia spp., Centroema pubescens, Chacoomeles spp., Cinnamomum cassia, Coffea arabica, Colophospermum mopane, Coronillia varia, Cotoneaster serotina, Crataegus spp., Cucumis spp., Cupressus spp., Cyathea dealbata, Cydonia oblonga, Cryptomeria japonica, Cymbopogon spp., Cynthea dealbata, Cydonia oblonga, Dalbergia monetaria, Davallia divaricata, Desmodium spp., Dicksonia squarosa, Dibeteropogon amplectens, Dioclea spp, Dolichos spp., Dorycnium rectum, Echinochloa pyramidalis, Ehraffia spp., Eleusine coracana, Eragrestis spp., Erythrina spp., Eucalyptus spp., Euclea schimperi, Eulalia vi/losa, Pagopyrum spp., Feijoa sellowlana, Fragaria spp., Flemingia spp, Freycinetia banksli, Geranium thunbergii, GinAgo biloba, Glycine javanica, Gliricidia spp, Gossypium hirsutum, Grevillea spp., Guibourtia coleosperma, Hedysarum spp., Hemaffhia altissima, Heteropogon contoffus, Hordeum vulgare, Hyparrhenia rufa, Hypericum erectum, Hypeffhelia dissolute, Indigo incamata, Iris spp., Leptarrhena pyrolifolia, Lespediza spp., Lettuca spp., Leucaena leucocephala, Loudetia simplex, Lotonus bainesli, Lotus spp., Macrotyloma axillare, Malus spp., Manihot esculenta, Medicago saliva, Metasequoia glyptostroboides, Musa sapientum, Nicotianum spp., Onobrychis spp., Ornithopus spp., Oryza spp., Peltophorum africanum, Pennisetum spp., Persea gratissima, Petunia spp., Phaseolus spp., Phoenix canariensis, Phormium cookianum, Photinia spp., Picea glauca, Pinus spp., Pisum sativam, Podocarpus totara, Pogonarthria fleckii, Pogonaffhria squarrosa, Populus spp., Prosopis cineraria, Pseudotsuga menziesii, Pterolobium stellatum, Pyrus communis, Quercus spp., Rhaphiolepsis umbellata, Rhopalostylis sapida, Rhus natalensis, Ribes grossularia, Ribes spp., Robinia pseudoacacia, Rosa spp., Rubus spp., Salix spp., Schyzachyrium sanguineum, Sciadopitys vefficillata, Sequoia sempervirens, Sequoiadendron giganteum, Sorghum bicolor, Spinacia spp., Sporobolus fimbriatus, Stiburus alopecuroides, Stylosanthos humilis, Tadehagi spp, Taxodium distichum, Themeda triandra, Trifolium spp., Triticum spp., Tsuga heterophylla, Vaccinium spp., Vicia spp., Vitis vinifera, Watsonia pyramidata, Zantedeschia aethiopica, Zea mays, amaranth, artichoke, asparagus, broccoli, Brussels sprouts, cabbage, canola, carrot, cauliflower, celery, collard greens, flax, kale, lentil, oilseed rape, okra, onion, potato, rice, soybean, straw, sugar beet, sugar cane, sunflower, tomato, squash tea, trees. Alternatively algae and other non-Viridiplantae can be used for the methods of the present invention.

According to a specific embodiment the plant is a Solanaceae plant.

According to a specific embodiment the plant is a Solanum plant.

According to another specific embodiment, the Solanum plant is a tomato (Lycopersicum esculentum).

According to another specific embodiment the plant comprises a potato (Solanum tuberosum); a tomato (Lycopersicum esculentum); an aubergine (egg plant) (Solanum melongena); a banana, (Musa spp); a geranium (common name) (Pelargonium); a ginger (Zingiber officinale); a tobacco (Nicotiana tabacum); a sweet pepper (Capsicum spp); an olive (Olea europea) an arabidopsis plant, a eucalyptus, an apple, a flowering crabapple, a pear, a firethorn, a hawthorn, a cotoneaster, a quince or a mountain ash plant.

As used herein the term "bacterial pathogen" refers to any type of virulent bacterial species or strains which infects plants and include, without being limited to, Pseudomonas spp., Erwinia-related strains, *Ralstonia solanacearum* and *Xanthomonas campestris*. The bacterium may be a Pseudomonas spp including P. aureofaciens, P. chlororaphis, P. fluorescens, P. marginalis, Pseudomonas syringae, P. tolaasii, *P.* agarici and P. viridiflava. The bacterium may be an Erwinia-related strain including Dickeya dadantii (Erwinia chrysanthemi), Erwinia carotovora, Erwinia atroseptica and Erwinia amylovora. The bacterium may be a *Xanthomonas campestris*-related strain including Xanthomonas campestris pv. campestris (Xcc) and Xanthomonas oryzae.

According to an embodiment of the present invention, the bacteria is a gram-negative bacteria.

According to a specific embodiment, the gram-negative bacteria is a Proteobacteria species.

According to another specific embodiment, the Proteobacteria is Ralstonia solanacearum.

According to another specific embodiment, the gram-negative bacteria is selected from the group consisting of Ralstonia solanacearum, Pseudomonas syringae, Erwinia amylovora, Erwinia Psidii, Erwinia pyrifoliae, Xanthomonas campestris and Xanthomonas oryzae.

As used herein the phrase "enhanced resistance" refers to reducing the virulence of the bacteria and hence reducing susceptibility of the host plant as compared to a non

modified plant infected with the same bacterial pathogen. Reducing bacterial virulence according to the present teachings is effected by expression of dominant negative proteins associated with bacterial virulence (e.g. needle complex, as described in further detail hereinbelow) and may affect any step of the bacterial life cycle when it is associated with a host, including without limitation, the adherence, invasion, replication, evasion of host defenses and transmittal to a new host.

Enhanced resistance to bacterial pathogens may be manifested in the form of reduced symptoms in a host, and thus may be detected by monitoring the host for a reduced reaction to the bacteria associated therewith. Enhanced resistance may be at least about a 1 % reduction, at least about a 5 % reduction, at least about a 10 % reduction, at least about a 20 % reduction, at least about a 30 % reduction, at least about a 40 % reduction, at least about a 50 % reduction, at least about a 60 % reduction, at least about a 70 % reduction, at least about a 80 % reduction, at least about a 90 % reduction, or at least about a 100 % reduction of symptoms associated with a bacterial pathogen, as measured by any assay known to those of skill in the art, when measured against a suitable control (e.g. a non modified plant grown under the same conditions).

The methods of the present invention are effected by introducing into the plant a nucleic acid expression vector comprising a nucleic acid sequence encoding a dominant negative T3SS protein and a cis acting regulatory element capable of driving transcription of the nucleic acid sequence in a plant cell, the dominant negative T3SS protein mediates assembly of a dysfunctional needle complex.

The term "T3SS" as used herein refers to the type III secretion system (also named TTSS) of bacteria (e.g. gram negative bacteria) which typically functions as a needle-like structure to secrete proteins directly from the bacterial cell. The T3SS needle complex generally starts at the cytoplasm of the bacterium, crosses the two membranes and protrudes out of the cell (see e.g. Figure 1A). The part anchored in the membrane is the base (or basal body) of the T3SS. The extracellular part is the needle (also named pilus). The final structure serving as the gate to the host cell cytoplasm is the translocon. (see Figures 1B-C). A so-called inner rod connects the needle to the base.

As used herein the term "T3SS protein" refers to a protein which makes up the T3SS secretion complex. These include the structural proteins, i.e. those which build the bases, the inner rod, the needle, the tip or the translocon. The needle itself is typically

made out of many units of a single T3SS protein. Thus, the majority of the different T3SS proteins are those that build the base and those that are secreted into the host.

According to an embodiment of the present invention, the T3SS protein is a protein which makes up the T3SS needle structure such as HRP (hypersensitive response and pathogenicity) protein. Exemplary HRP proteins includes, without being limited to, *Ralstonia solanacearum* HrpY protein, *Pseudomonas syringae* HrpA protein, *Erwinia amylovora* HrpA protein, *Erwinia pyrifoliae* HrpA protein and *Xanthomonas campestris* HrpE protein (for exemplary proteins, see Table 1, below, incorporated herein from Buttner and He, Plant Physiology (2009) 150:1656-1664].

Table 1: Exemplary T3SS proteins

Protein	**Predicted protein function**	**Bacterial species**
HrpA	Pilus protein	*Erwinia amylovora*
HrpK	Translocon protein	
HrpA	Pilus protein	*Pseudomonas syringae* pv tomato
HrpK1	Translocon protein	
HrpY	Pilus protein	*Ralstonia solanacearum*
PopF1	Translocon protein	
PopF2		
HrpExcv	Pilus protein	*Xanthomonas* spp.
HrpFxcv	Translocon protein	
HrpFxoo		
HrpA	Pilus protein	*Erwinia pyrifoliae*

Of note: xcv - X. campestris pv vesicatoria, xoo – X. oryzae pv oryzae

According to a specific embodiment, the wild-type *Ralstonia solanacearum* HrpY polypeptide is as set in SEQ ID NO: 14.

According to another embodiment, the *Ralstonia solanacearum* HrpY polypeptide comprises variants as set forth in SEQ ID NO: 70-86.

According to a specific embodiment, the wild-type *Pseudomonas syringae* HrpA polypeptide is as set in SEQ ID NO: 19.

According to a specific embodiment, the wild-type *Erwinia amylovora* HrpA polypeptide is as set in SEQ ID NO: 88.

According to a specific embodiment, the wild-type *Xanthomonas campestris* HrpE polypeptide is as set in SEQ ID NO: 90.

According to a specific embodiment, the wild-type Xanthomonas oryzae HrpE polypeptide is as set in SEQ ID NO: 92.

According to another embodiment, the T3SS protein is a translocon protein such as the *Ralstonia solanacearum* translocon proteins PopF1 or PopF2 (SEQ ID NOs: 67 and 69, respectively).

According to a specific embodiment, the wild-type *Erwinia pyrifoliae* HrpA polypeptide is as set in SEQ ID NO: 100.

The phrase "dominant negative T3SS protein" as used herein refers to a T3SS protein which has a structurally altered gene product that interacts with the wild type T3SS protein secreted from the bacteria but mediates the formation of a dysfunctional needle complex (e.g. one which is not able to or comprises a reduced ability as compared to wild-type protein to penetrate a host cell or transport effector proteins into the host cell). The bacterial dysfunctional needle complex may be structurally deformed (e.g. partially or fully blocked or distorted in such a way which renders it less capable of transferring effector proteins to a host cell) or may partially assemble or not assemble at all. Typically the dominant negative T3SS protein of the present invention reduces infectivity and pathogenicity of the bacteria. Methods of measuring infectivity are well known in the art.

Thus, the dominant negative T3SS protein reduces the assembly and/or functionality of the needle complex and consequently the infectivity of the pathogenic bacteria by about 5 %, by about 10 %, by about 20 %, by about 30 %, by about 40 %, by about 50 %, by about 60 %, by about 70 %, by about 80 %, by about 90 % or by about 100 %, as compared to bacteria having a needle structure composed of wild type T3SS proteins.

Of note, according to a specific embodiment, the dominant negative protein is expressed exogenously to the bacteria by the plant cell.

Typically, the dominant negative T3SS protein is encoded by a gene comprising one or more mutations in the wild type protein coding sequence such as an insertion

mutation, a deletion mutation or a substitution mutation. These mutations may comprise a single nucleic acid alteration in the wild type T3SS protein (e.g., inclusion of a beta breaker amino acid such as a proline or a synthetic mimetic thereof) or alternatively may comprise 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more nucleic acid alterations. Alternatively, the mutation may comprise insertion of a single peptide (3, 4, 5, 10 amino acids in length) or of several peptides (e.g. pentapeptide insertion) into the T3SS protein.

Exemplary single amino acid mutations which may be implemented in the peptides of the present invention include replacement of glycine at location 23 of hrpA gene with alanine, replacement of alanine at location 54 of hrpA gene with glutamic acid, replacement of lysine at location 93 of hrpA gene with isoleucine, replacement of aspartic acid at location 95 of hrpA gene with serine, replacement of isoleucine at location 101 of hrpA gene with threonine, replacement of isoleucine at location 111 of hrpA gene with proline.

Exemplary pentapeptide insertions which may be inserted into the peptides of the present invention are set forth in SEQ ID NOs: 20-65 (see Table 2, below).

It will be appreciated that the mutations may be effected at any location in the T3SS gene which results in a dominant negative protein. Exemplary locations of nucleic acid insertions and deletions are depicted for HrpA gene, see Figure 8 and in Table 2 below [incorporated herein from Taira et al., Mol Microbiol. (1999) 34(4):737-44].

As mentioned and according to a specific embodiment, the dominant negative T3SS protein of the present invention is one which maintains protein-protein interaction sites which allows it to bind with high affinity to the cognate wild-type bacterial protein and form a needle structure, however, due to the mutations in the dominant negative proteins, the resultant needle structure is dysfunctional.

Thus, the present invention contemplates any mutation in or to a T3SS gene which renders the needle complex dysfunctional.

According to an embodiment of the present invention, the insertion mutation comprises an intercalating blocking element (IBE). Dominant negative T3SS proteins comprising IBEs typically form subunit-subunit interactions with the cognate proteins while incorporating translationally fused channel-blocking elements (e.g. peptides) or

deforming structures of the T3SS protein (e.g. HrpY alpha-helices) which prevent bacterial effectors from being secreted from the bacteria into the plant cells (see Example 1 of the Examples section which follows).

According to an embodiment of this aspect of the present invention, the nucleic acid sequence encodes for a peptide as set forth in SEQ ID NOs: 2, 4, 6, 8, 10 or 12.

According to another embodiment, the dominant-negative T3SS proteins are capable of arresting T3SS assembly due to interactions with a premature needle (e.g. the dominant negative translocon proteins interact with the needle ahead of time, thus, interfering with the T3SS assembly and deactivate it (see Example 3 of the Examples section which follows).

Table 2: Exemplary pentapeptide insertions and locations for insertions and deletions of nucleic acids in the HrpA gene

	Pentapeptide	**Location of insertion**
		8
		9
		19
		39
		49
		52
		58
		59
		61
		70
		72
		86
		91
SEQ ID NO: 20	MRPHS	122
SEQ ID NO: 21	GAAAI	138
SEQ ID NO: 22	CGRIG	139
SEQ ID NO: 23	CGRSA	148
SEQ ID NO: 24	GAAAV	153
SEQ ID NO: 25	CGRIG	163
SEQ ID NO: 26	CGRSG	166
SEQ ID NO: 27	VRPQQ	176
SEQ ID NO: 28	GAAAQ	177
SEQ ID NO: 29	NAAAV	183
SEQ ID NO: 30	TAAAN	186
SEQ ID NO: 31	MRPHS	197
SEQ ID NO: 32	TAAAA	204
SEQ ID NO: 33	LRPHT	206
SEQ ID NO: 34	CGRTF	226

SEQ ID NO: 35	VRPHL	227
SEQ ID NO: 36	MRPQG	230
SEQ ID NO: 37	CGRTG	235
SEQ ID NO: 38	CGRSD	238
SEQ ID NO: 39	VRPQS	248
SEQ ID NO: 40	VAAAS	249
SEQ ID NO: 41	DAAAV	252
SEQ ID NO: 42	NAAAA	264
SEQ ID NO: 43	CGRTS	271
SEQ ID NO: 44	MRPHA	308
SEQ ID NO: 45	VRPQQ	314
SEQ ID NO: 46	CGRTQ	319
SEQ ID NO: 47	CGRKE	322
SEQ ID NO: 48	NAAAM	330
SEQ ID NO: 49	DAAAM	345
SEQ ID NO: 50	AAAAN	357
SEQ ID NO: 51	VRPHQ	365
SEQ ID NO: 52	AAAAG	369
SEQ ID NO: 53	MRPHS	383
SEQ ID NO: 54	TAAAS	384
SEQ ID NO: 55	CGRTN	388
SEQ ID NO: 56	AAAAT	405
SEQ ID NO: 57	AAAAT	408
SEQ ID NO: 58	CGRTA	409
SEQ ID NO: 59	AAAAT	411
SEQ ID NO: 60	MRPQT	413
SEQ ID NO: 61	AAAAN	417
SEQ ID NO: 62	CGRNA	418
SEQ ID NO: 63	CGRIS	430
SEQ ID NO: 64	YAAAS	432
SEQ ID NO: 65	CGRSY	433
		451
		453
		455
		461
		464
		479
		493
		Location of deletion
		138-177
		138-204
		137-249
		138-264

Nucleic acid sequences according to this aspect of the present invention can be a complementary polynucleotide sequence (cDNA), a genomic polynucleotide sequence and/or a composite polynucleotide sequences (e.g., a combination of the above).

As used herein the phrase "complementary polynucleotide sequence" refers to a sequence, which results from reverse transcription of messenger RNA using a reverse transcriptase or any other RNA dependent DNA polymerase. Such a sequence can be subsequently amplified *in vivo* or *in vitro* using a DNA dependent DNA polymerase.

As used herein the phrase "genomic polynucleotide sequence" refers to a sequence derived (isolated) from a chromosome and thus it represents a contiguous portion of a chromosome.

As used herein the phrase "composite polynucleotide sequence" refers to a sequence, which is at least partially complementary and at least partially genomic. A composite sequence can include some exonal sequences required to encode the polypeptide of the present invention, as well as some intronic sequences interposing therebetween. The intronic sequences can be of any source, including of other genes, and typically will include conserved splicing signal sequences. Such intronic sequences may further include cis acting expression regulatory elements, as described in further detail below.

According to a specific embodiment the nucleic acid sequence comprises an insertion such as set forth in SEQ ID NO: 1, 3, 5, 7, 9 and 11.

Constructs useful in the methods according to the present invention may be constructed using recombinant DNA technology well known to persons skilled in the art. The gene constructs may be inserted into vectors, which may be commercially available, suitable for transforming into plants and suitable for expression of the gene of interest in the transformed cells. The genetic construct can be an expression vector wherein the heterologous nucleic acid sequence is operably linked to a cis-acting regulatory element allowing expression in the plant cells.

As used herein, the phrase "cis acting regulatory element" refers to a polynucleotide sequence, preferably a promoter, which binds a trans acting regulator and regulates the transcription of a coding sequence located downstream thereto.

As used herein, the phrase "operably linked" refers to a functional positioning of the cis-regulatory element (e.g., promoter) so as to allow regulating expression of the selected nucleic acid sequence. For example, a promoter sequence may be located upstream of the selected nucleic acid sequence in terms of the direction of transcription and translation.

Preferably, the promoter in the nucleic acid construct of the present invention is a plant promoter which serves for directing expression of the heterologous nucleic acid molecule within plant cells.

It will be appreciated that novel nucleic acid sequences encoding intercalating elements such as set forth in SEQ ID NOs: 2, 4, 6 8 10 or 12 are contemplated per se or as part of a nucleic acid expression vector for expression in bacteria or plant cells.

As used herein the phrase "plant promoter" refers to a promoter sequence, including any additional regulatory elements added thereto or contained therein, is at least capable of inducing, conferring, activating or enhancing expression in a plant cell, tissue or organ, preferably a monocotyledonous or dicotyledonous plant cell, tissue, or organ. Such a promoter can be derived from a plant, bacterial, viral, fungal or animal origin. Such a promoter can be constitutive, i.e., capable of directing high level of gene expression in a plurality of plant tissues, tissue specific, i.e., capable of directing gene expression in a particular plant tissue or tissues, inducible, i.e., capable of directing gene expression under a stimulus, or chimeric, i.e., formed of portions of at least two different promoters.

Examples of constitutive plant promoters include, without being limited to, CaMV35S and CaMV19S promoters, FMV34S promoter, sugarcane bacilliform badnavirus promoter, CsVMV promoter, *Arabidopsis* ACT2/ACT8 actin promoter, *Arabidopsis* ubiquitin UBQ1 promoter, barley leaf thionin BTH6 promoter, and rice actin promoter.

According to a specific embodiment, the promoter is a constitutive promoter, such as a CaMV 35S promoter.

Other exemplary promoters useful for the methods of some embodiments of the invention are presented in Tables 3, 4, 5 and 6.

Table 3
Exemplary constitutive promoters for use in the performance of some embodiments of the invention

Reference	*Expression Pattern*	*Gene Source*
McElroy etal, Plant Cell, 2: 163-171, 1990	constitutive	Actin
Odell et al, Nature, 313: 810-812, 1985	constitutive	CAMV 35S

Nilsson et al., Physiol. Plant 100:456-462, 1997	constitutive	CaMV 19S
de Pater et al, Plant J Nov;2(6):837-44, 1992	constitutive	GOS2
Christensen et al, Plant Mol. Biol. 18: 675-689, 1992	constitutive	Ubiquitin
Bucholz et al, Plant Mol Biol. 25(5):837-43, 1994	constitutive	Rice cyclophilin
Lepetit et al, Mol. Gen. Genet. 231: 276-285, 1992	constitutive	Maize H3 histone
An et al, Plant J. 10(1);107-121, 1996	constitutive	Actin 2

Table 4

Exemplary seed-preferred promoters for use in the performance of some embodiments of the invention

Reference	***Expression Pattern***	***Gene Source***
Simon, et al., Plant Mol. Biol. 5. 191, 1985; Scofield, etal., J. Biol. Chem. 262: 12202, 1987.; Baszczynski, et al., Plant Mol. Biol. 14: 633, 1990.	Seed	Seed specific genes
Pearson' et al., Plant Mol. Biol. 18: 235- 245, 1992.	Seed	Brazil Nut albumin
Ellis, et al.Plant Mol. Biol. 10: 203-214, 1988	Seed	Legumin
Takaiwa, et al., Mol. Gen. Genet. 208: 15-22, 1986; Takaiwa, et al., FEBS Letts. 221: 43-47, 1987	Seed	Glutelin (rice)
Matzke et al Plant Mol Biol, 143).323-32 1990	Seed	Zein
Stalberg, et al, Planta 199: 515-519, 1996	Seed	napA
Mol Gen Genet 216:81-90, 1989; NAR 17:461-2,	Endosperm	wheat LMW and HMW, glutenin-1
Albanietal, Plant Cell, 9: 171- 184, 1997	Seed	Wheat SPA
EMBO3:1409-15, 1984	Endosperm	wheat a, b and g gliadins
	Endosperm	Barley ltrl promoter
Theor Appl Gen 98:1253-62, 1999; Plant J 4:343-55, 1993; Mol Gen Genet 250:750- 60, 1996	Endosperm	barley B1, C, D hordein
Mena et al, The Plant Journal, 116(1): 53- 62, 1998	Endosperm	Barley DOF
EP99106056.7	Endosperm	Biz2
Vicente-Carbajosa et al.,	Endosperm	Synthetic promoter

Plant J. 13: 629-640, 1998		
Wu et al, Plant Cell Physiology 39(8) 885- 889, 1998	Endosperm	rice prolamin NRP33
Wu et al, Plant Cell Physiology 398) 885-889, 1998	Endosperm	rice -globulin Glb-1
Sato et al, Proc. Nati. Acad. Sci. USA, 93: 8117-8122	Emryo	rice OSH1
Nakase et al. Plant Mol. Biol. 33: 513-S22, 1997	Endosperm	rice alpha-globulin REB/OHP-1
Trans Res 6:157-68, 1997	Endosperm	rice ADP-glucose PP
Plant J 12:235-46, 1997	Endosperm	maize ESR gene family
PMB 32:1029-35, 1996	Endosperm	sorgum gamma- kafirin
Postma-Haarsma ef al, Plant Mol. Biol. 39:257-71, 1999	Emryo	KNOX
Wu et at, J. Biochem., 123:386, 1998	Embryo and aleuton	rice oleosin
Cummins, etal., Plant Mol. Biol. 19: 873- 876, 1992	Seed (embryo and dry seed)	sunflower oleosin

Table 5

Exemplary flower-specific promoters for use in the performance of the invention

Reference	***Expression Pattern***	***Gene Source***
www.salus.medium.edu/mmg/tierney/html	Flowers	AtPRP4
Van der Meer, et al., Plant Mol. Biol. 15, 95-109, 1990.	Flowers	chalene synthase (chsA)
Twell et al Mol. Gen Genet. 217:240-245 (1989)	Anther	LAT52
	Flowers	apetala- 3

Table 6

Alternative rice promoters for use in the performance of the invention

expression	***Gene***	***PRO #***
transfer layer of embryo + calli	Metallothionein Mte	PR00001
transfer layer of embryo	putative beta-amylase	PR00005
Weak in roots	Putative cellulose synthase	PR00009
	lipase (putative)	PR00012
	Transferase (putative)	PR00014
	peptidyl prolyl cis-trans isomerase (putative)	PR00016
	Unknown	PR00019
	prp protein (putative)	PR00020
	noduline (putative)	PR00029

seed	Proteinase inhibitor Rgpi9	PR00058
Weak in young flowers	beta expansine EXPB9	PR00061
young tissues+calli+embryo	Structural protein	PR00063
	xylosidase (putative)	PR00069
strong in endosperm	Prolamine 10Kda	PR00075
strong in endosperm	allergen RA2	PR00076
strong in endosperm	prolamine RP7	PR00077
	CBP80	PR00078
	starch branching enzyme I	PR00079
transfer layer of embryo + calli	Metallothioneine-like ML2	PR00080
shoot	putative caffeoyl- CoA 3-0 methyltransferase	PR00081
strong in endosperm	prolamine RM9	PR00087
strong in endosperm	prolamine RP6	PR00090
strong in endosperm	prolamine RP5	PR00091
	allergen RA5	PR00092
embryo	putative methionine aminopeptidase	PR00095
	ras-related GTP binding protein	PR00098
	beta expansine EXPB1	PR00104
	Glycine rich protein	PR00105
	metallothionein like protein (putative)	PR00108
	RCc3 strong root	PR00110
weak discrimination center / shoot meristem	uclacyanin 3-like protein	PR00111
very weak meristem specific	26S proteasome regulatory particle non-ATPase subunit 11	PR00116
weak in endosperm	putative 40S ribosomal protein	PR00117
very weak in shoot	chlorophyll a/lo-binding protein precursor (Cab27)	PR00122
Strong leaves	putative protochlorophyllide reductase	PR00123
strong discrimination center shoot meristem	metallothionein RiCMT	PR00126
Strong constitutive	GOS2	PR00129
	GOS9	PR00131
very weak meristem specific	chitinase Cht-3	PR00133
Strong in endosperm	alpha- globulin	PR00135
Weak in endosperm	alanine aminotransferase	PR00136
	Cyclin A2	PR00138
	Cyclin D2	PR00139
	Cyclin D3	PR00140
Shoot and seed	Cyclophyllin 2	PR00141
medium constitutive	sucrose synthase SS1 (barley)	PR00146
weak in endosperm	trypsin inhibitor ITR1 (barley)	PR00147
strong constitutive	ubiquitine 2 with intron	PR00149
Embryo and stress	WSI18	PR00151

	HVA22 homologue (putative)	PR00156
	EL2	PR00157
medium constitutive in young plants	Aquaporine	PR00169
Strong constitutive	High mobility group protein	PR00170
weak constitutive	reversibly glycosylated protein RGP1	PR00171
shoot	cytosolic MDH	PR00173
Embryo and stress	RAB21	PR00175
	CDPK7	PR00176
very weak in meristem	Cdc2-1	PR00177
	sucrose synthase 3	PR00197
	OsVP1	PRO0198
very weak in young plant meristem	OSH1	PRO0200
	putative chlorophyllase	PRO0208
	OsNRT1	PRO0210
	EXP3	PRO0211
	phosphate transporter OjPT1	PRO0216
aleurone + embryo	oleosin 18kd	PRO0218
	ubiquitine 2 without intron	PRO0219
	RFL	PRO0220
not detected	maize UBI delta intron	PRO0221
	glutelin-1	PRO0223
	fragment of prolamin RP6 promoter	PRO0224
	4xABRE	PRO0225
	glutelin OSGLUA3	PRO0226
	BLZ-2_short (barley)	PRO0227
	BLZ-2_long (barley)	PR00228

The nucleic acid construct of the present invention may also comprise an additional nucleic acid sequence encoding an endoplasmic reticulum signal peptide that allows transport of the dominant negative T3SS propeptide to the endoplasmic reticulum and through the secretory pathway. Such a signal peptide is typically linked in frame to the amino terminus of a polypeptide (i.e. upstream thereto) and directs the encoded polypeptide into a cell's secretory pathway and its final secretion therefrom (e.g. to the apoplast).

Exemplary secretion signal sequences which direct polypeptides via the ER to the extracellular space include the plant secretion leader peptide from sp|Q56YT0|LAC3_At Laccase (SEQ ID NO: 16) and the plant secretion leader peptide from tr|Q6TDS6|Q6TDS6_GOSAR (SEQ ID NO: 17).

Additional exemplary signal peptides that may be used herein include the tobacco pathogenesis related protein (PR-S) signal sequence (Sijmons *et al.*, 1990, Bio/technology, 8:217-221), lectin signal sequence (Boehn *et al.*, 2000, Transgenic Res, 9(6):477-86), signal sequence from the hydroxyproline-rich glycoprotein from Phaseolus vulgaris (Yan *et al.*, 1997, Plant Phyiol. 115(3):915-24 and Corbin *et al.*, 1987, Mol Cell Biol 7(12):4337-44), potato patatin signal sequence (Iturriaga, G *et al.*, 1989, Plant Cell 1:381-390 and Bevan *et al.*, 1986, Nuc. Acids Res. 41:4625-4638.) and the barley alpha amylase signal sequence (Rasmussen and Johansson, 1992, Plant Mol. Biol. 18(2):423-7).

According to an embodiment of the present invention, the nucleic acid construct of the present invention may further comprise a translation enhancer such as an omega translation enhancer.

Nucleic acid sequences of the polypeptides of some embodiments of the invention may be optimized for plant expression. Examples of such sequence modifications include, but are not limited to, an altered G/C content to more closely approach that typically found in the plant species of interest, and the removal of codons atypically found in the plant species commonly referred to as codon optimization.

The phrase "codon optimization" refers to the selection of appropriate DNA nucleotides for use within a structural gene or fragment thereof that approaches codon usage within the plant of interest. Therefore, an optimized gene or nucleic acid sequence refers to a gene in which the nucleotide sequence of a native or naturally occurring gene has been modified in order to utilize statistically-preferred or statistically-favored codons within the plant. The nucleotide sequence typically is examined at the DNA level and the coding region optimized for expression in the plant species determined using any suitable procedure, for example as described in Sardana *et al.* (1996, Plant Cell Reports 15:677-681). In this method, the standard deviation of codon usage, a measure of codon usage bias, may be calculated by first finding the squared proportional deviation of usage of each codon of the native gene relative to that of highly expressed plant genes, followed by a calculation of the average squared deviation. The formula used is: 1 SDCU = n = 1 N [(Xn - Yn) / Yn] 2 / N, where Xn refers to the frequency of usage of codon n in highly expressed plant genes, where Yn to the frequency of usage of codon n in the gene of interest and N refers to the total

number of codons in the gene of interest. A table of codon usage from highly expressed genes of dicotyledonous plants is compiled using the data of Murray *et al.* (1989, Nuc Acids Res. 17:477-498).

One method of optimizing the nucleic acid sequence in accordance with the preferred codon usage for a particular plant cell type is based on the direct use, without performing any extra statistical calculations, of codon optimization tables such as those provided on-line at the Codon Usage Database through the NIAS (National Institute of Agrobiological Sciences) DNA bank in Japan (www.kazusa.or.jp/codon/). The Codon Usage Database contains codon usage tables for a number of different species, with each codon usage table having been statistically determined based on the data present in Genbank.

By using the above tables to determine the most preferred or most favored codons for each amino acid in a particular species (for example, rice), a naturally-occurring nucleotide sequence encoding a protein of interest can be codon optimized for that particular plant species. This is effected by replacing codons that may have a low statistical incidence in the particular species genome with corresponding codons, in regard to an amino acid, that are statistically more favored. However, one or more less-favored codons may be selected to delete existing restriction sites, to create new ones at potentially useful junctions (5' and 3' ends to add signal peptide or termination cassettes, internal sites that might be used to cut and splice segments together to produce a correct full-length sequence), or to eliminate nucleotide sequences that may negatively effect mRNA stability or expression.

The naturally-occurring encoding nucleotide sequence may already, in advance of any modification, contain a number of codons that correspond to a statistically-favored codon in a particular plant species. Therefore, codon optimization of the native nucleotide sequence may comprise determining which codons, within the native nucleotide sequence, are not statistically-favored with regards to a particular plant, and modifying these codons in accordance with a codon usage table of the particular plant to produce a codon optimized derivative. A modified nucleotide sequence may be fully or partially optimized for plant codon usage provided that the protein encoded by the modified nucleotide sequence is produced at a level higher than the protein encoded by the corresponding naturally occurring or native gene. Construction of synthetic genes

by altering the codon usage is described in for example PCT Patent Application 93/07278.

Thus, some embodiments of the invention encompasses nucleic acid sequences described hereinabove; fragments thereof, sequences hybridizable therewith, sequences homologous thereto, sequences orthologous thereto, sequences encoding similar polypeptides with different codon usage, altered sequences characterized by mutations, such as deletion, insertion or substitution of one or more nucleotides, either naturally occurring or man induced, either randomly or in a targeted fashion.

Plant cells may be transformed stably or transiently with the nucleic acid constructs of some embodiments of the invention. In stable transformation, the nucleic acid molecule of some embodiments of the invention is integrated into the plant genome and as such it represents a stable and inherited trait. In transient transformation, the nucleic acid molecule is expressed by the cell transformed but it is not integrated into the genome and as such it represents a transient trait.

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

The principle methods of causing stable integration of exogenous DNA into plant genomic DNA include two main approaches:

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

(ii) direct DNA uptake: Paszkowski et al., in Cell Culture and Somatic Cell Genetics of Plants, Vol. 6, Molecular Biology of Plant Nuclear Genes eds. Schell, J., and Vasil, L. K., Academic Publishers, San Diego, Calif. (1989) p. 52-68; including methods for direct uptake of DNA into protoplasts, Toriyama, K. et al. (1988) Bio/Technology 6:1072-1074. DNA uptake induced by brief electric shock of plant cells: Zhang et al. Plant Cell Rep. (1988) 7:379-384. Fromm et al. Nature (1986)

319:791-793. DNA injection into plant cells or tissues by particle bombardment, Klein et al. Bio/Technology (1988) 6:559-563; McCabe et al. Bio/Technology (1988) 6:923-926; Sanford, Physiol. Plant. (1990) 79:206-209; by the use of micropipette systems: Neuhaus et al., Theor. Appl. Genet. (1987) 75:30-36; Neuhaus and Spangenberg, Physiol. Plant. (1990) 79:213-217; glass fibers or silicon carbide whisker transformation of cell cultures, embryos or callus tissue, U.S. Pat. No. 5,464,765 or by the direct incubation of DNA with germinating pollen, DeWet et al. in Experimental Manipulation of Ovule Tissue, eds. Chapman, G. P. and Mantell, S. H. and Daniels, W. Longman, London, (1985) p. 197-209; and Ohta, Proc. Natl. Acad. Sci. USA (1986) 83:715-719.

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

There are various methods of direct DNA transfer into plant cells. In electroporation, the protoplasts are briefly exposed to a strong electric field. In microinjection, the DNA is mechanically injected directly into the cells using very small micropipettes. In microparticle bombardment, the DNA is adsorbed on microprojectiles such as magnesium sulfate crystals or tungsten particles, and the microprojectiles are physically accelerated into cells or plant tissues.

Following stable transformation plant propagation is exercised. The most common method of plant propagation is by seed. Regeneration by seed propagation, however, has the deficiency that due to heterozygosity there is a lack of uniformity in the crop, since seeds are produced by plants according to the genetic variances governed by Mendelian rules. Basically, each seed is genetically different and each will grow with its own specific traits. Therefore, it is preferred that the transformed plant be

produced such that the regenerated plant has the identical traits and characteristics of the parent transgenic plant. Therefore, it is preferred that the transformed plant be regenerated by micropropagation which provides a rapid, consistent reproduction of the transformed plants.

Micropropagation is a process of growing new generation plants from a single piece of tissue that has been excised from a selected parent plant or cultivar. This process permits the mass reproduction of plants having the preferred tissue expressing the fusion protein. The new generation plants which are produced are genetically identical to, and have all of the characteristics of, the original plant. Micropropagation allows mass production of quality plant material in a short period of time and offers a rapid multiplication of selected cultivars in the preservation of the characteristics of the original transgenic or transformed plant. The advantages of cloning plants are the speed of plant multiplication and the quality and uniformity of plants produced.

Micropropagation is a multi-stage procedure that requires alteration of culture medium or growth conditions between stages. Thus, the micropropagation process involves four basic stages: Stage one, initial tissue culturing; stage two, tissue culture multiplication; stage three, differentiation and plant formation; and stage four, greenhouse culturing and hardening. During stage one, initial tissue culturing, the tissue culture is established and certified contaminant-free. During stage two, the initial tissue culture is multiplied until a sufficient number of tissue samples are produced to meet production goals. During stage three, the tissue samples grown in stage two are divided and grown into individual plantlets. At stage four, the transformed plantlets are transferred to a greenhouse for hardening where the plants' tolerance to light is gradually increased so that it can be grown in the natural environment.

Although stable transformation is presently preferred, transient transformation of leaf cells, meristematic cells or the whole plant is also envisaged by some embodiments of the invention.

Transient transformation can be effected by any of the direct DNA transfer methods described above or by viral infection using modified plant viruses.

Viruses that have been shown to be useful for the transformation of plant hosts include CaMV, TMV and BV. Transformation of plants using plant viruses is described in U.S. Pat. No. 4,855,237 (BGV), EP-A 67,553 (TMV), Japanese Published

Application No. 63-14693 (TMV), EPA 194,809 (BV), EPA 278,667 (BV); and Gluzman, Y. et al., Communications in Molecular Biology: Viral Vectors, Cold Spring Harbor Laboratory, New York, pp. 172-189 (1988). Pseudovirus particles for use in expressing foreign DNA in many hosts, including plants, is described in WO 87/06261.

Construction of plant RNA viruses for the introduction and expression of non-viral exogenous nucleic acid sequences in plants is demonstrated by the above references as well as by Dawson, W. O. et al., Virology (1989) 172:285-292; Takamatsu et al. EMBO J. (1987) 6:307-311; French et al. Science (1986) 231:1294-1297; and Takamatsu et al. FEBS Letters (1990) 269:73-76.

When the virus is a DNA virus, suitable modifications can be made to the virus itself. Alternatively, the virus can first be cloned into a bacterial plasmid for ease of constructing the desired viral vector with the foreign DNA. The virus can then be excised from the plasmid. If the virus is a DNA virus, a bacterial origin of replication can be attached to the viral DNA, which is then replicated by the bacteria. Transcription and translation of this DNA will produce the coat protein which will encapsidate the viral DNA. If the virus is an RNA virus, the virus is generally cloned as a cDNA and inserted into a plasmid. The plasmid is then used to make all of the constructions. The RNA virus is then produced by transcribing the viral sequence of the plasmid and translation of the viral genes to produce the coat protein(s) which encapsidate the viral RNA.

Construction of plant RNA viruses for the introduction and expression in plants of non-viral exogenous nucleic acid sequences such as those included in the construct of some embodiments of the invention is demonstrated by the above references as well as in U.S. Pat. No. 5,316,931.

In one embodiment, a plant viral nucleic acid is provided in which the native coat protein coding sequence has been deleted from a viral nucleic acid, a non-native plant viral coat protein coding sequence and a non-native promoter, preferably the subgenomic promoter of the non-native coat protein coding sequence, capable of expression in the plant host, packaging of the recombinant plant viral nucleic acid, and ensuring a systemic infection of the host by the recombinant plant viral nucleic acid, has been inserted. Alternatively, the coat protein gene may be inactivated by insertion of the non-native nucleic acid sequence within it, such that a protein is produced. The

recombinant plant viral nucleic acid may contain one or more additional non-native subgenomic promoters. Each non-native subgenomic promoter is capable of transcribing or expressing adjacent genes or nucleic acid sequences in the plant host and incapable of recombination with each other and with native subgenomic promoters. Non-native (foreign) nucleic acid sequences may be inserted adjacent the native plant viral subgenomic promoter or the native and a non-native plant viral subgenomic promoters if more than one nucleic acid sequence is included. The non-native nucleic acid sequences are transcribed or expressed in the host plant under control of the subgenomic promoter to produce the desired products.

In a second embodiment, a recombinant plant viral nucleic acid is provided as in the first embodiment except that the native coat protein coding sequence is placed adjacent one of the non-native coat protein subgenomic promoters instead of a non-native coat protein coding sequence.

In a third embodiment, a recombinant plant viral nucleic acid is provided in which the native coat protein gene is adjacent its subgenomic promoter and one or more non-native subgenomic promoters have been inserted into the viral nucleic acid. The inserted non-native subgenomic promoters are capable of transcribing or expressing adjacent genes in a plant host and are incapable of recombination with each other and with native subgenomic promoters. Non-native nucleic acid sequences may be inserted adjacent the non-native subgenomic plant viral promoters such that said sequences are transcribed or expressed in the host plant under control of the subgenomic promoters to produce the desired product.

In a fourth embodiment, a recombinant plant viral nucleic acid is provided as in the third embodiment except that the native coat protein coding sequence is replaced by a non-native coat protein coding sequence.

The viral vectors are encapsidated by the coat proteins encoded by the recombinant plant viral nucleic acid to produce a recombinant plant virus. The recombinant plant viral nucleic acid or recombinant plant virus is used to infect appropriate host plants. The recombinant plant viral nucleic acid is capable of replication in the host, systemic spread in the host, and transcription or expression of foreign gene(s) (isolated nucleic acid) in the host to produce the desired protein.

In addition to the above, the nucleic acid molecule of some embodiments of the invention can also be introduced into a chloroplast genome thereby enabling chloroplast expression.

A technique for introducing exogenous nucleic acid sequences to the genome of the chloroplasts is known. This technique involves the following procedures. First, plant cells are chemically treated so as to reduce the number of chloroplasts per cell to about one. Then, the exogenous nucleic acid is introduced via particle bombardment into the cells with the aim of introducing at least one exogenous nucleic acid molecule into the chloroplasts. The exogenous nucleic acid is selected such that it is integratable into the chloroplast's genome via homologous recombination which is readily effected by enzymes inherent to the chloroplast. To this end, the exogenous nucleic acid includes, in addition to a gene of interest, at least one nucleic acid stretch which is derived from the chloroplast's genome. In addition, the exogenous nucleic acid includes a selectable marker, which serves by sequential selection procedures to ascertain that all or substantially all of the copies of the chloroplast genomes following such selection will include the exogenous nucleic acid. Further details relating to this technique are found in U.S. Pat. Nos. 4,945,050; and 5,693,507 which are incorporated herein by reference. A polypeptide can thus be produced by the protein expression system of the chloroplast and become integrated into the chloroplast's inner membrane.

According to an additional aspect of the invention there is provided a host cell comprising the nucleic acid expression vector of the present invention.

A "host cell" of the present invention refers to a new individual cell arising as a result of the introduction into the cell of the nucleic acid expression vector comprising the nucleic acid sequence encoding a dominant negative T3SS protein. According to a specific embodiment, the host cell is a plant cell. The host cell may contain the nucleic acid construct as an extra-chromosomally (episomal) replicating molecule, or alternatively, may comprises the chimeric gene integrated in the nuclear or plastid genome of the host cell.

According to a further aspect of the invention there is provided a genetically modified plant comprising the nucleic acid expression vector of the present invention.

According to a further aspect of the invention there is provided a genetically modified plant expressing an exogenous polynucleotide encoding a dominant negative T3SS protein as set forth in SEQ ID NO: 2, 4, 6, 8, 10 or 12.

According to a specific embodiment, the genetically modified plant expressing the dominant negative T3SS protein comprises enhanced resistance to a bacterial pathogen compared to a non modified plant (as described in detail hereinabove).

It will be appreciated that when referring to a genetically modified plant or plant cell, the present inventors also refer to progeny arising therefrom.

Progeny resulting from breeding or from transforming plants can be selected, by verifying presence of exogenous mRNA and/or polypeptides by using nucleic acid or protein probes (e.g. antibodies). Alternatively, expression of the dominant negative T3SS proteins of the present invention may be verified by measuring enhanced resistance to bacterial pathogens by infecting the genetically modified plant and a wild-type (i.e. non-modified plant of the same type) and comparing the disease in the plant (e.g. observing the wilting of the plant).

The present invention further provides methods of evaluating resistance of a plant to a bacterial pathogen, the method comprising: (a) expressing within the plant an exogenous nucleic acid sequence encoding a dominant negative T3SS protein and a cis acting regulatory element capable of driving transcription of the nucleic acid sequence in a plant cell; (b) subjecting the plant to a bacterial pathogen; and (c) comparing the disease in the plant to a wild-type plant grown and infected with the bacterial pathogen under the same conditions.

The bacterial pathogens as described herein may cause a variety of diseases in plants. Thus, for example, *R. solanacearum* may cause wilting disease, *P. agarici* may cause drippy gill disease (e.g. in cultivated mushrooms), *P. tolaasii* may cause bacterial blotch (e.g. in cultivated mushrooms), X. campestris may causes black rot (e.g. in crucifers such as Brassica and Arabidopsis), *X. oryzae* may cause bacterial blight (e.g. in rice), *E. amylovora* may cause fireblight disease (e.g. in apples and pears), *E. carotovora* may cause bacterial soft rot disease.

Thus, for example, for wilting disease, symptoms are typically scored on a daily basis for 2 to 4 weeks by a rater (blind to treatment identity) on a 0 to 4 disease index, where 0 indicates no disease, 1 indicates 1 to 25 % of leaves wilted, 2 indicates 25 to 50

% of leaves wilted, 3 indicates 51 to 75 % of leaves wilted, and 4 indicates 76 to 100 % of leaves wilted.

As used herein the term “about” refers to ± 10 %.

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

The term “consisting of means “including and limited to”.

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

As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

Throughout this application, various embodiments of this invention may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases “ranging/ranges between” a first indicate number and a second indicate number and “ranging/ranges from” a first indicate number “to” a second indicate number are used herein interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals therebetween.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners,

means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

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

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

EXAMPLES

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

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange,

Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., Eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

EXAMPLE 1

Generation and Expression of Intercalating Blocking Elements of Ralstonia solanacearum type III secretion system (T3SS) needle channel in plants

MATERIALS AND EXPERIMENTAL PROCEDURES

Gene synthesis, codon usage expression:

T3SS-IBE variations were designed based on the 3D template of a T3SS needle from *Shigela flexneri* (MxiH) previously described [Deane et al., PNAS 2006 103: 12529-33] representing the hypothetical natural structure model of HrpY (Figures 2A-F).

Ralstonia solanacearum (Rs) HrpY intercalating blocking element (hY-IBE) genes were synthetically synthesized and optimized for target plant codon usage. Plant specific secretion leader peptides were fused to the 5' of each hY-IBE to transport and localize the mature proteins in the apoplast or cell wall.

Cloning in Binary Vector and transformation:

Synthetic fragments consisting of IBE's 1-6 coding regions with 5' untranslated enhancer were cloned downstream to a CaMV 35S constitutive promoter and upstream to a NOS terminator in a plant transformation vector based on pBI121 plasmid (NCBI genebank ID# AF485783) using XbaI and SacI restriction sites (Figure 7A).

An agro-transformation protocol was used for Tomato plants, Arabidopsis plants and Eucalyptus plants as previously described for tobacco plants [see e.g. Svab, Z., P. Hajdukiewicz and P. Maliga. (1975) Transgenic tobacco plants by co-cultivation of leaf disks with pPZP Agrobacterium binary vectors. In "Methods in Plant Molecular Biology-A Laboratory Manual", P. Maliga, D. Klessig, A. Cashmore, W. Gruissem and J. Varner, eds. Cold Spring Harbor Press: 55- 77] and for eucalyptus plants [Spokevicius AV., Van Beveren K., Leitch MA and Bossinger G. (2005) Agrobacterium-mediated in vitro transformation of wood-producing stem segments in eucalypts. Plant Cell Reports, Volume 23(9), 617-624].

Transformation of tomato plants

Tomato plants were transformed as previously described [Qiu et al., Scientia Horticulturae 112 (2007) 172–175]. In short:

Plant material

Seeds of tomato, L. esculentum cv M82 were surface sterilized for 30 s in a 70 % alcohol and washed with sterilized water for 10 s and then sterilized for 10 min in a 1 % hypochlorite solution and washed two times with sterilized water for 30 min before sowing on Medium A (as described in Table 7 below). Seeds were sown in a Magenta box and germinated at 24 °C during a 16 h light period and 8 h dark period. Cotyledons of half upright seedling were used after 4–5 days of germination.

Media, antibiotics and hormones

The media MSB5 (M0404) were obtained as powders from Sigma Chemical Co., and stored at 2–8 °C. Sucrose and glucose were stored at room temperature. Kanamycin, carbenicillin, cefotaxime, rifamicin, ZR, IAA, IBA were further used in the plant mediums (as described in Table 7 below).

Table 7: Plant media used in the tomato transformation protocol (incorporated herein from Qiu et al., Scientia Horticulturae 112 (2007) 172–175)

Composition of the various media

MSB5 salts	Medium					
	A, 0.5×	B, 1×	B1, 1×	C, 1×	D, 1×	E, 1×
Sucrose (%)	1	3	3	3	N	1
Glucose	N	N	N	N	1%	N
Agar (Daichin) (%)	0.60	0.60	N	0.60	0.60	0.60
pH	5.8	5.8	5.8	5.8	5.8	5.8
IAA 0.1 mg/L	—	+	—	+	+	—
ZR 2 mg/L	—	+	—	+	+	—
IBA 0.1 mg/L	—	—	—	—	—	+
Cefotaxime 500 mg/L	—	—	—	+	+	+
Carbenicillin 500 mg/L	—	—	—	—	+	—
Kanamycin (mg/L)	—	—	—	—	100	30

Bacterial strains and plasmids

For transformation experiments, Agrobacterium containing the gene of interest was used. The binary vector used in this study was pBI121 which contained the nptII gene as selection maker; The Agrobacterium strains used in this study harbored a rifampicin selection maker. Bacteria were grown overnight in LB medium with antibiotic (rifamicin 30 mg/L, kanamycin 100 mg/L), diluted to OD600 = 0.2 and grown to expected OD 600 in LB without antibiotics. Bacterial suspensions were centrifuged at 4000 rpm for 15 min in a 50 mL Falcon tube. Bacteria were resuspended in B1 medium, and used for cocultivation experiments.

Transformation protocol

The cotyledons were prepared as follows: The excision of the cotyledons from the seedling was done extremely carefully to prevent the issue from bruising. Isolated cotyledons were cut on the basal and the lateral side only and placed upside up onto Medium B (as described in Table 7 above). Approximately 50 explants were placed on a single Petri dish and incubated overnight. The next day explants were carefully submerged in the Agrobacterium inoculum in a Petri dish for 20 min. They were blotted dry on sterile paper and transferred to the new Medium B. After 72 h, explants were

transferred to plates containing Medium C (as described in Table 7 above). After incubation for another 72 h, the explants were transferred to selection Medium D (as described in Table 7 above). Every 3 weeks the explants were subcultured to the same medium. After approximately 6–8 weeks, shoots were excised and transferred to Medium E (as described in Table 7 above). Transformation frequency was expressed as the percentage of the number of cotyledons from which shoots were recovered, with regard to the total number of explants incubated.

Expression and cloning confirmation:

Plant genome integration and expression of T3SS-IBE's was analyzed using conventional molecular methods such as PCR, RT-PCR [as previously described, see e.g. Sambrook J. and Russell DW., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press; 3rd edition (January 15, 2001)] with IBE's 1-6 specific primers and Western analysis with anti-IBE's polyclonal antibody. Specifically, the PCR primers used for HrpY mutant 1 were Forward primer: TCTCTTTGCTCTCCTTTATAGCCCTAC and Reverse primer: TCGCAGCGTCTAACATATCTTGTTGTC (SEQ ID NOs: 95-96, respectively); the primers used for HrpY mutant 2 were Forward primer: GTCACATGGTTCGTTGGTGTACTCTTC and Reverse primer: CATCTGGGTTCTATTCAGCGCATTTTG (SEQ ID NOs: 97-98, respectively); and the primers used for or HrpY mutant 6 were Forward primer: GTCTTGTTCCTGTCTACCTTGCTCC and Reverse primer: GAGATTAGGTCTTTCGCAGCTTTGG (SEQ ID NOs: 93-94, respectively).

BioAssays:

Transgenic plants are subjected to a bioassay for testing the resistance level of each transgenic line compared to wild type (i.e. not expressing the IBE gene). Three bioassay methods are applied:

1. Soaked soil - Unwounded 19 to 21 day old plants are inoculated by pouring a bacterial suspension onto the soil to a final density of approximately 1×10^8 CFU/g soil, followed by incubation at 28 °C. Control plants are mock-inoculated with sterile water. Symptoms are scored daily by a rater blind to treatment identity on a 0-to-4 disease index, where 0 indicates no disease, 1 indicates 1 to 25 % of leaves wilted, 2 indicates 25 to 50 % of leaves wilted, 3 indicates 51 to 75 % of leaves wilted, and 4 indicates 76

to 100 % of leaves wilted. Each experiment encompasses 16 plants per treatment, and experiments are repeated at least three times.

2. Petiole inoculation – Lower leaf of unwounded 19 to 21 day old plants are cut and 2 µl of bacteria suspension with a final density of approximately 1×10^8 CFU/ml is dropped on the open petiole. Control plants are mock-inoculated with sterile water. Symptoms are scored daily by a rater blind to treatment identity on a 0-to-4 disease index, where 0 indicates no disease, 1 indicates 1 to 25 % of leaves wilted, 2 indicates 25 to 50 % of leaves wilted, 3 indicates 51 to 75 % of leaves wilted, and 4 indicates 76 to 100 % of leaves wilted. Each experiment encompasses 16 plants per treatment, and experiments are repeated at least three times.

3. Stem inoculation - Unwounded 19 to 21 day old plants are inoculated by cutting the stem with a sterile knife vertically. The wound, 1 cm long and 0.5 cm deep is injected with 100 µl of bacteria suspension with a final density of approximately 1×10^8 CFU/ml. Control plants are mock-inoculated with sterile water. Symptoms are scored daily by a rater blind to treatment identity on a 0-to-4 disease index, where 0 indicates no disease, 1 indicates 1 to 25 % of leaves wilted, 2 indicates 25 to 50 % of leaves wilted, 3 indicates 51 to 75 % of leaves wilted, and 4 indicates 76 to 100 % of leaves wilted. Each experiment contained 16 plants per treatment, and experiments are repeated at least three times.

RESULTS

The present inventors generated plant-expressed blocking elements of *Ralstonia solanacearum* (*Rs*) type III secretion system (T3SS) needle channel (Intercalating Blocking Elements of T3SS or T3SS-IBE), for protection of plants from wilt disease, using structural modifications of HrpY protein, the building monomer of the needle. The structurally modified HrpY (SEQ ID NOs: 2, 4, 6, 8, 10 and 12 and depicted in detail in Figures 2A-F, 3A-D, 4A-C, 5A-C and 6A-D), expressed in transgenic plants (crops and woody), is incorporated into the native pilus of *Rs*, functionally deactivating it and preventing or decreasing Rs bacterial infection in the transgenic plant compared to wild type plants. The transgenic plant that expresses the T3SS-IBEs is designed to secrete the T3SS-IBE outside of the cell where the T3SS-IBE is assembled into the pilus of the attacking *Rs* rendering the pilus non-functional or dys-functional. Bacteria with structurally modified plant-derived protein intercalated in its pilus will render the

T3SS nonfunctional and thus are not able to overcome the plant's natural defense. Such transgenic resistant plants are able to resist infection by *Rs*.

T3SS-IBE variations were designed based on the 3D template of a T3SS needle from *Shigela flexneri* (MxiH) previously described [Deane et al., PNAS 2006 103: 12529-33]. Based on this model, structural modifications of *Rs* HrpY were planned generating modified T3SS needle monomers that intercalate within the needle structure and block the needle channel, the conduit, in which plant cell wall-degrading pectinases, endo-glucanases, and virulence EPS and effector proteins are translocated into or through the host cell wall (Figures 1A-C). Thus, the structurally modified pilus comprises protein domains which are located in the conduit of the pilus and thus are understood to functionally and physically block the conduit. Such structurally unstable pilus also terminate their assembly early resulting in relatively short pili further damaging their functionality and ability to transfer proteins to the plant. This rational design is based on preserving and utilizing the native HrpY subunit-subunit interaction sites while incorporating translationally fused channel-blocking peptide and/or deforming structures of alpha-helices. Plant secretion signals were included in these T3SS-IBEs to enable secretion from the plant cell to the extracellular space.

As described in the 'Materials and Experimental Procedures' section above, wilt resistant (WiltR) tomato plants were generated by transforming the tomato plants with constructs carrying *Ralstonia solanacearum* HrpY mutants 1, 2 or 6 (SEQ ID NOs: 1, 3 and 11, respectively). These plants were further analyzed by genomic PCR and semi-quantitative RT-PCR using specific primers for HrpY mutant 1 (SEQ ID NOs: 95-96), HrpY mutant 2 (SEQ ID NOs: 97-98) or HrpY mutant 6 (SEQ ID NOs: 93-94). Expression of the HrpY mutants 1, 2 or 6 was determined (see Figures 11A-C, 12A-C and 10A-C, respectively) in the transformed tomato plants.

EXAMPLE 2

Generation and Expression of T3SS-IBEs of different bacteria in plants

In addition to the IBEs described in Example 1 above, other IBEs are being developed and identified for other gram negative bacteria using the methods described above. For example, IBEs are developed by mapping binding regions of pilus building

block proteins, identifying candidate peptides that bind and integrate into the native pilus during its in vivo formation, modifying the candidate peptide to render the conduit incapable of secreting effector proteins and producing modified candidate IBEs.

Modifications may include those based on any of the following principles:

Conceptually the native proteins can be modified on the basis of one or more of several different approaches including the following:

1. Addition of a translation fusion to the N- terminal region of the native protein as in IBEs 1, 2 and 3 with (IBE 1&3) or without (IBE 2) and before (IBE 1) or after (IBE 3) the native N-terminal domain (see Figures 3A-D, 4A-C and 7C).

2. Addition of a translational fusion to the C-terminal region of the native protein as in IBE 4 with or without an amino acid bridge which allows rotational movement of the translational fusion fragment (see Figures 5A-C and 7C). Such a bridge can be one or more glycine or alanine residues for example.

3. Addition of Proline amino acid to random points along the native building block sequence as in IBE 5 or 6 (see Figures 6A-D and 7C).

4. Pentapeptide inserts.

EXAMPLE 3

Generation and Expression of Modified Ralstonia solanacearum translocon proteins (PopF1) in plants

Another approach taken by the present inventors is to over-express wild-type (wt) and modified Rs translocon proteins (e.g. PopF1 or PopF2) in transgenic plants. PopF1 and PopF2 are building blocks of the needle gate and play an important role in virulence and hypersensitive response (HR) in plants Wt and modified PopF1/F2 proteins arrest T3SS assembly due to interactions with a premature needle. The bacterial controlled needle extension and the translocon proteins are normally extracted at the final stage of the process. Transgenic translocon proteins, which interact with the needle prematurely, interfere with the controlled sequential T3SS assembly and deactivate it. Thus, modified PopF1/F2 proteins are incorporated into the translocon gate and block it or structurally deform it to cause dysfunctionality. Taken together, the

present teachings will enable exportation of the wt and modified PopF1 proteins to the apoplast/cell wall by the transgenic plants.

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

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

WHAT IS CLAIMED IS:

1. A nucleic acid expression vector comprising a nucleic acid sequence encoding a dominant negative T3SS protein and a cis acting regulatory element capable of driving transcription of said nucleic acid sequence in a plant cell, said dominant negative T3SS protein mediates assembly of a dysfunctional needle complex.

2. The nucleic acid expression vector of claim 1, wherein said nucleic acid sequence comprises SEQ ID NO: 1, 3, 5, 7, 9 or 11.

3. The nucleic acid expression vector of claim 1, wherein said nucleic acid sequence comprises SEQ ID NOs: 20-65.

4. The nucleic acid expression vector of claim 1 or 2, wherein said nucleic acid sequence encodes for the polypeptide set forth in SEQ ID NO: 2, 4, 6, 8, 10 or 12.

5. The nucleic acid expression vector of claim 1, further comprising an additional nucleic acid sequence encoding an endoplasmic reticulum signal peptide upstream of said nucleic acid sequence.

6. The nucleic acid expression vector of claim 1, wherein said cis acting regulatory element comprises a promoter sequence.

7. The nucleic acid expression vector of claim 6, wherein said promoter sequence is a constitutive promoter.

8. The nucleic acid expression vector of claim 7, wherein said constitutive promoter is CaMV 35S promoter.

9. The nucleic acid expression vector of claim 1, wherein said dominant negative T3SS protein is generated by introduction of a mutation selected from the

group consisting of an insertion mutation, a deletion mutation and a substitution mutation.

10. The nucleic acid expression vector of claim 9, wherein said insertion mutation comprises an intercalating blocking element.

11. The nucleic acid expression vector of claim 1, wherein said T3SS protein is a T3SS structural protein.

12. The nucleic acid expression vector of claim 11, wherein said T3SS structural protein is a HRP protein.

13. The nucleic acid expression vector of claim 1, wherein said T3SS protein is selected from the group consisting of a *Ralstonia solanacearum* HrpY protein, a *Pseudomonas syringae* HrpA protein, a *Erwinia amylovora* HrpA protein, a *Xanthomonas campestris* HrpE protein, a *Erwinia pyrifoliae* HrpA protein and a *Xanthomonas oryzae* HrpE protein.

14. The nucleic acid expression vector of claim 1, wherein said T3SS protein is a *Ralstonia solanacearum* translocon protein.

15. The nucleic acid expression vector of claim 14, wherein said *Ralstonia solanacearum* translocon protein is selected from the group consisting of PopF1 and PopF2.

16. An isolated polynucleotide comprising a nucleic acid sequence encoding SEQ ID NO: 2, 4, 6, 8, 10 or 12.

17. A nucleic acid expression vector comprising the polynucleotide of claim 16.

18. A host cell comprising the nucleic acid expression vector of any of claims 1-15 or 17.

19. The host cell of claim 18 being a plant cell.

20. A genetically modified plant comprising the nucleic acid expression vector of any of claims 1-15.

21. A genetically modified plant expressing an exogenous polynucleotide encoding a dominant negative T3SS protein as set forth in SEQ ID NO: 2, 4, 6, 8, 10 or 12.

22. The genetically modified plant of claim 20, wherein said plant comprises enhanced resistance to a bacterial pathogen compared to a non modified plant.

23. A method of generating a plant comprising enhanced resistance to a bacterial pathogen compared to a non modified plant, the method comprising introducing into a plant or plant cell the nucleic acid expression vector of any of claims 1-15, thereby generating the plant comprising enhanced resistance to the bacterial pathogen compared to the non modified plant.

24. A method of evaluating resistance of a plant to a bacterial pathogen, the method comprising:

(a) expressing within the plant an exogenous nucleic acid sequence encoding a dominant negative T3SS protein and a cis acting regulatory element capable of driving transcription of said nucleic acid sequence in a plant cell;

(b) subjecting the plant to a bacterial pathogen; and

(c) comparing the disease in said plant to a wild-type plant grown and infected with said bacterial pathogen under the same conditions, thereby evaluating resistance of the plant to the bacterial pathogen.

25. The genetically modified plant or method of claims 22, 23 or 24, wherein said bacterial pathogen is a gram-negative bacteria.

26. The genetically modified plant or method of claim 25, wherein said gram-negative bacteria is selected from the group consisting of *a Ralstonia solanacearum, a Pseudomonas syringae*, *a Erwinia amylovora*, *a Xanthomonas campestris* and a *Xanthomonas oryzae*.

27. The genetically modified plant or method of claim 25, wherein said gram-negative bacteria is a Proteobacteria species.

28. The genetically modified plant or method of claim 27, wherein said Proteobacteria is *Ralstonia solanacearum*.

29. The genetically modified plant or method of any of claims 20, 21, 23 or 24, wherein said plant is selected from the group consisting of a crop plant, a decorative plant, and a tree.

30. The genetically modified plant or method of any of claims 20, 21, 23 or 24, wherein said plant is a Solanaceae plant.

31. The genetically modified plant or method of any of claims 20, 21, 23 or 24, wherein said plant is selected from the group consisting of a tomato plant, a potato plant, an eggplant plant, a banana plant, a sweet pepper plant, an olive plant, an apple plant, a pear plant, a firethorn plant, a flowering crabapple plant, a hawthorn plant, a cotoneaster plant, a quince plant, a mountain ash plant, an arabidopsis plant, a geranium, a ginger plant, a tobacco plant and a eucalyptus plant.

32. The genetically modified plant or method of any of claims 20, 21, 23 or 24, wherein said plant is a tomato plant.

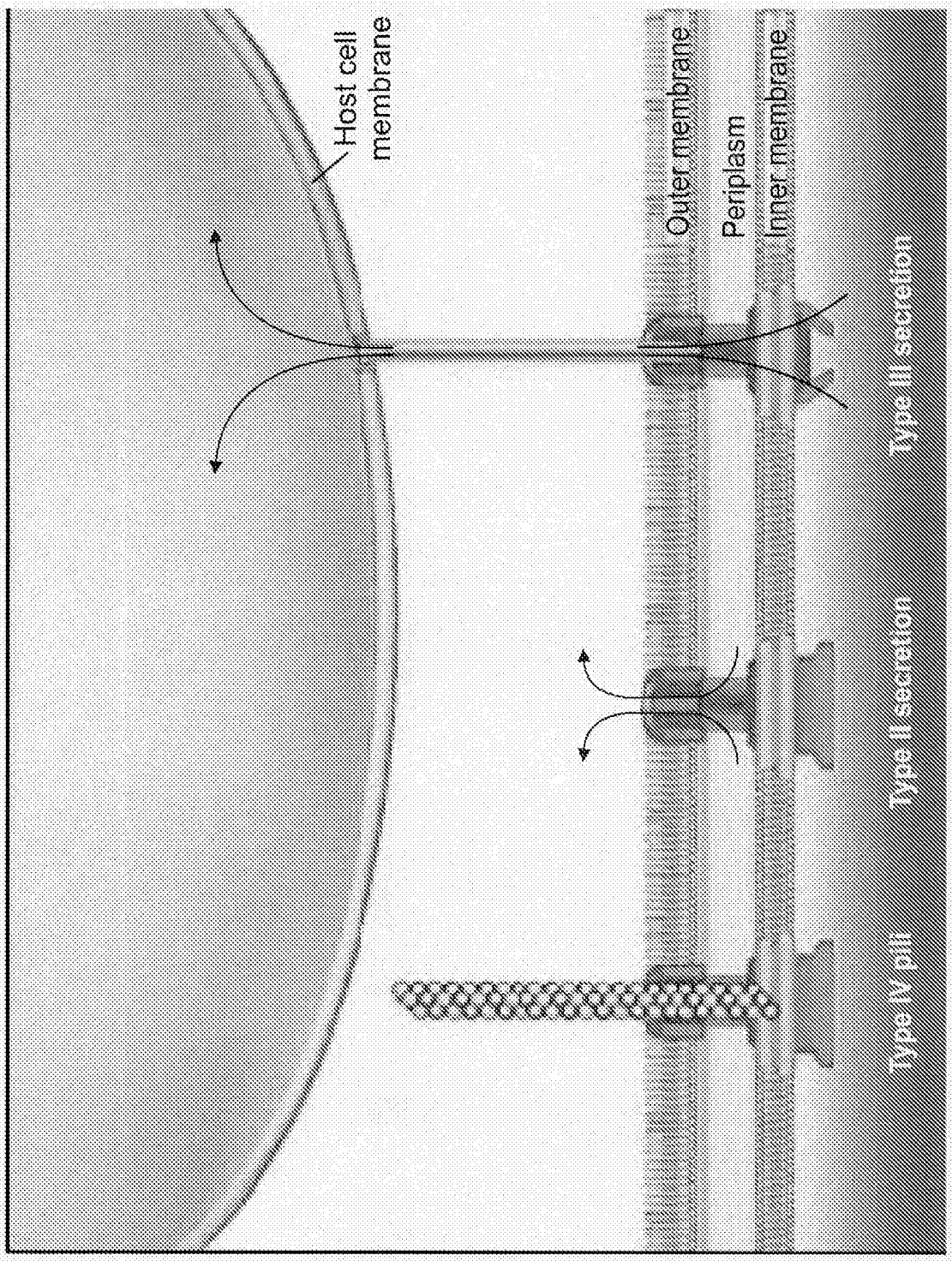
Host cell membrane
Outer membrane
Periplasm
Inner membrane
Type III secretion
Type II secretion
Type IV pili

FIG. 1A

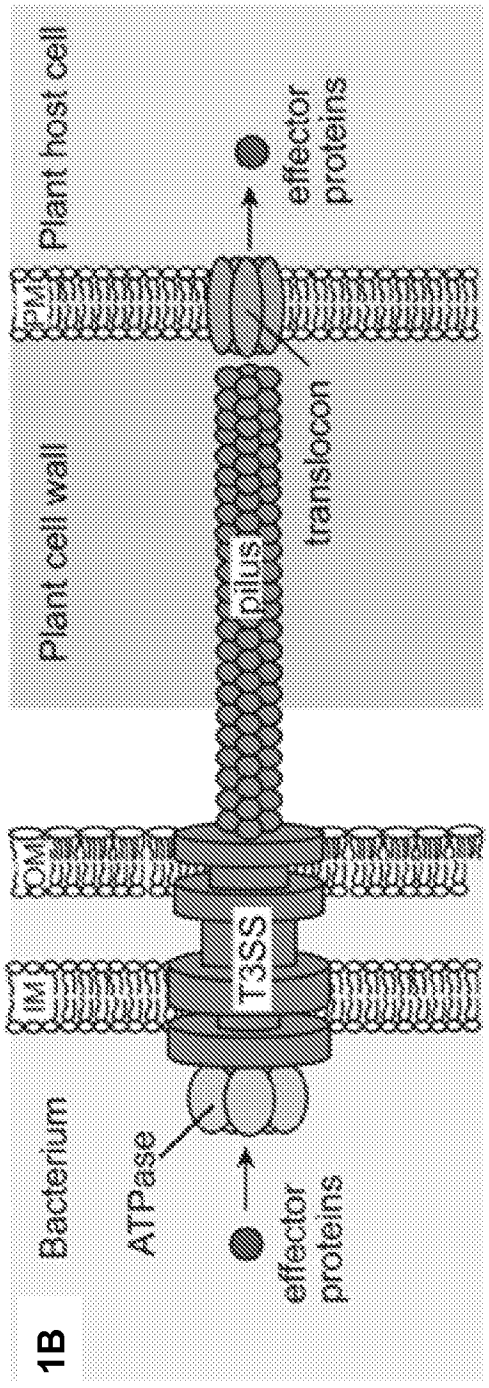
Plant host cell
effector proteins
PM
Plant cell wall
pilus
translocon
OM
T3SS
IM
Bacterium
ATPase
effector proteins

FIG. 1B

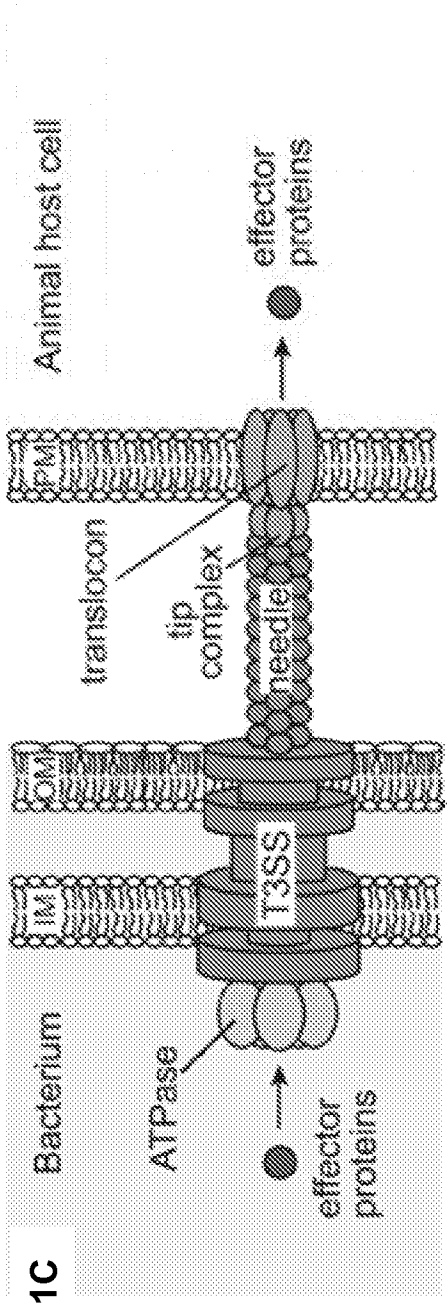
Animal host cell
effector proteins
PM
translocon
tip complex
needle
OM
T3SS
IM
Bacterium
ATPase
effector proteins

FIG. 1C

>Q52483|Q52483_RALSO HrpY protein - Ralstonia solanacearum

FIG. 2A

```
1         10        20        30        40        50        60        70        80
MAGVPKPNTTNTTSTTSTFQSFANGVDDAASRTGFQAQYQAITAQGQQDMLDAAKMQNALNRTQMLAKLMEAGPKAARDLIS*  SEQ ID NO: 14
|..|.|.|.1.....||2.....3.....4.....5.....6.....7.....8...
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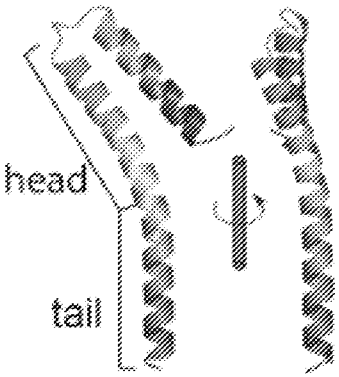

FIG. 2B

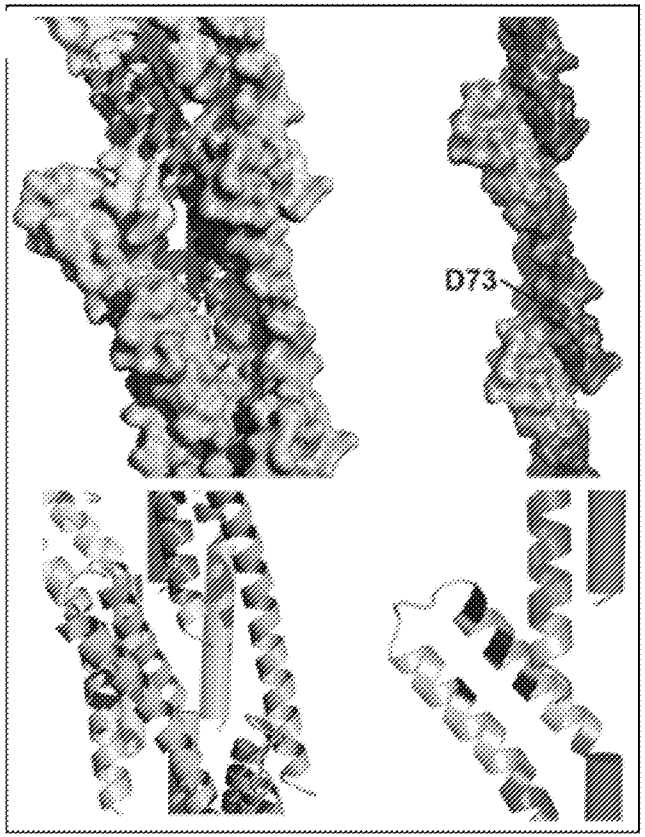

FIG. 2C

FIG. 2D

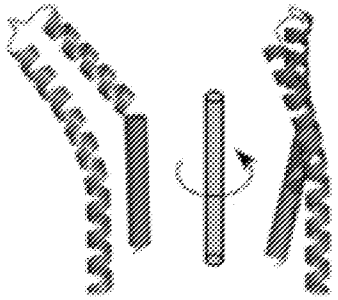

FIG. 2E

FIG. 2F

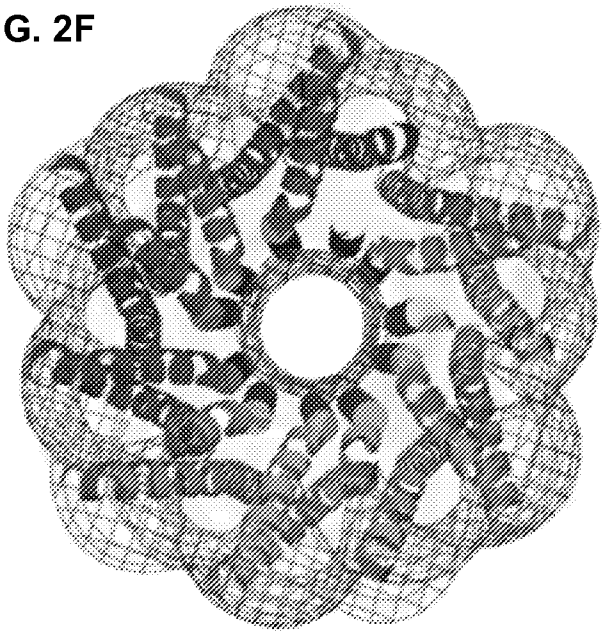

FIG. 3A SEQ ID NO: 2

>T3SS Blocking element 1
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QGQQDMLDAAKMQNALNRTQMLAKLMEAGPKAAKDLIS*

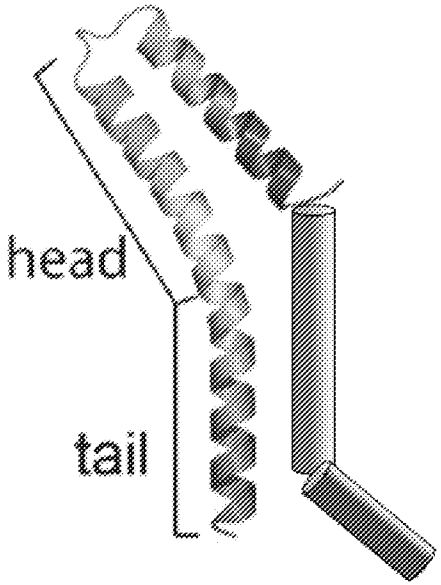


FIG. 3B

FIG. 3C SEQ ID NO: 4

>T3SS Blocking element 2
MGLQQGLVTWFVGVLFLSTLLLSNADVHHYEFFVRPNGV
DDAASRTGFQAQYQAITAQGQQDMLDAAKMQNALNRTQMLA
KLMEAGPKAAKDLIS*

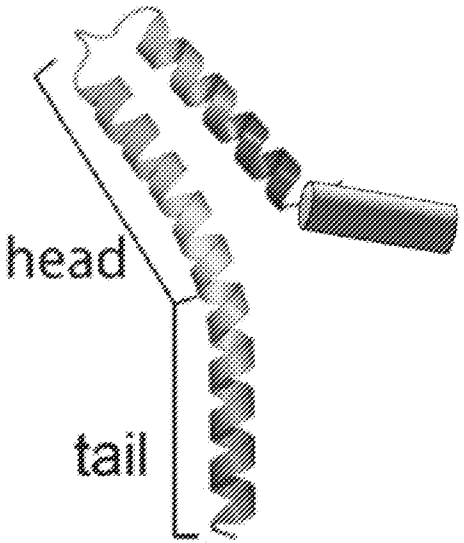
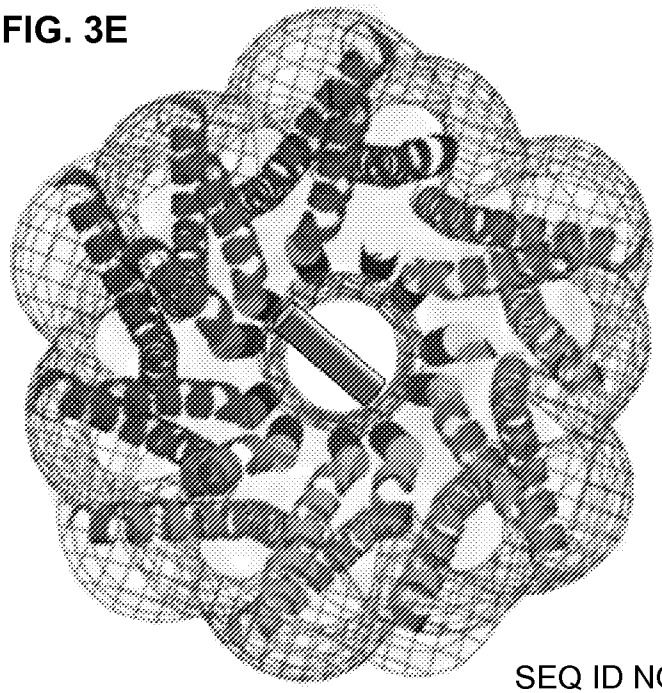


FIG. 3D

FIG. 3E



SEQ ID NO: 16

>sp|Q56YT0|LAC3_ARATH Laccase
MESFRRFSLLSFIALLAYFAFLASA EHHVHQ

SEQ ID NO: 17

>tr|Q6TDS6|Q6TDS6_GOSAR Secretory laccase Gossypium arboreum
MGLQQGLVTWFVGVLFLSTLLLSNA DVHHYE

FIG. 4A SEQ ID NO: 6

>T3ss Blocking element 3

MESFRRFSLLSFIALLAYFAFLASAMAGVPKPNTNTTSTTS
TFQSVTVGNDDWTLSSLSETFDSFANGVDDAASRTGFQA
QYQAITAQGQDMLDAAKMQNALNRTQMLAKLMEAGPKAAKD
LIS*

FIG. 4B

FIG. 4C

FIG. 5A SEQ ID NO: 8

>T3SS Blocking element 4

MGLQQGLVTWFVGVLFLSTLLLSNAMAGVPKPNTTNTT
STTSTPQSFANGVDDAASRTGFQAQYQAITAQGQQDML
DAAKMQNALNRTQMLAKLMEAGPKAAKDLISQGQMLA
KLMEAGPKAAKDLIS*

FIG. 5B

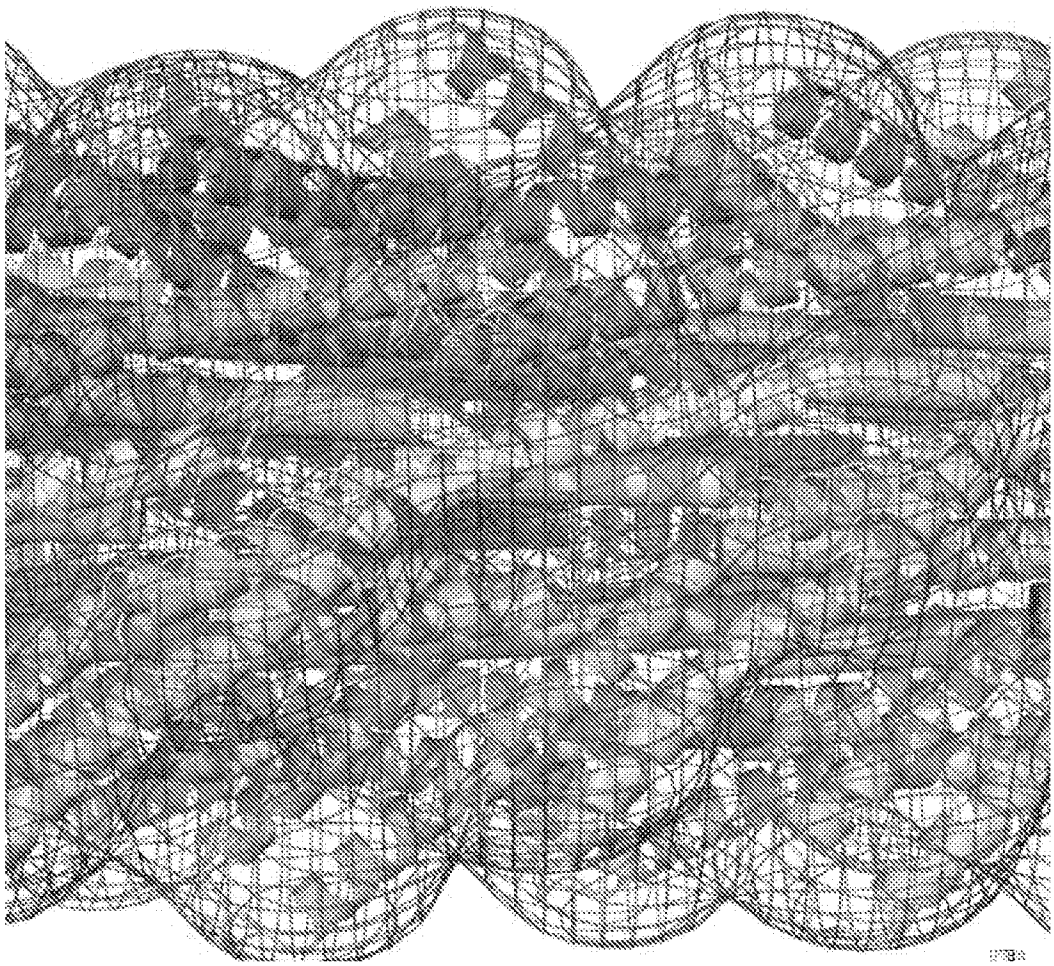


FIG. 5C

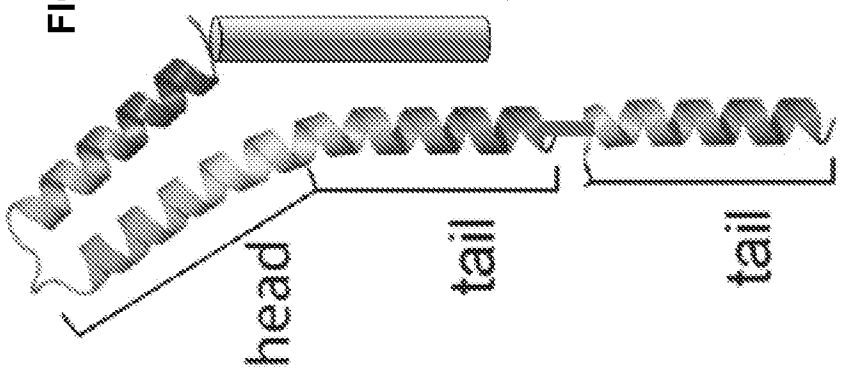


FIG. 6A SEQ ID NO: 10

>T3SS Blocking element 5
MESFRRFSLLSFIALLAYFAFLASAMAGVPKPNTTNTTSTTS
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LNRTQMLAKLMEAGPKAAKDLIS*

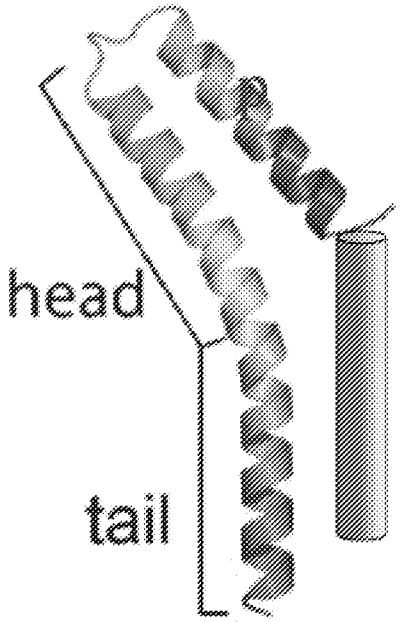

FIG. 6B

FIG. 6C SEQ ID NO: 12

>T3SS Blocking element 6
MGLQQGLVTWFVGVLFLSTLLLLSNAMAGVPKPNTTNTT
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DAAKMQNALNRTQMLAKLMEAPPKAAKDLIS*

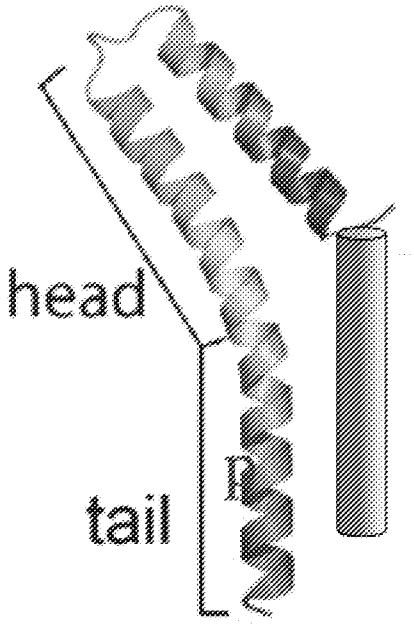


FIG. 6D

FIG. 7A

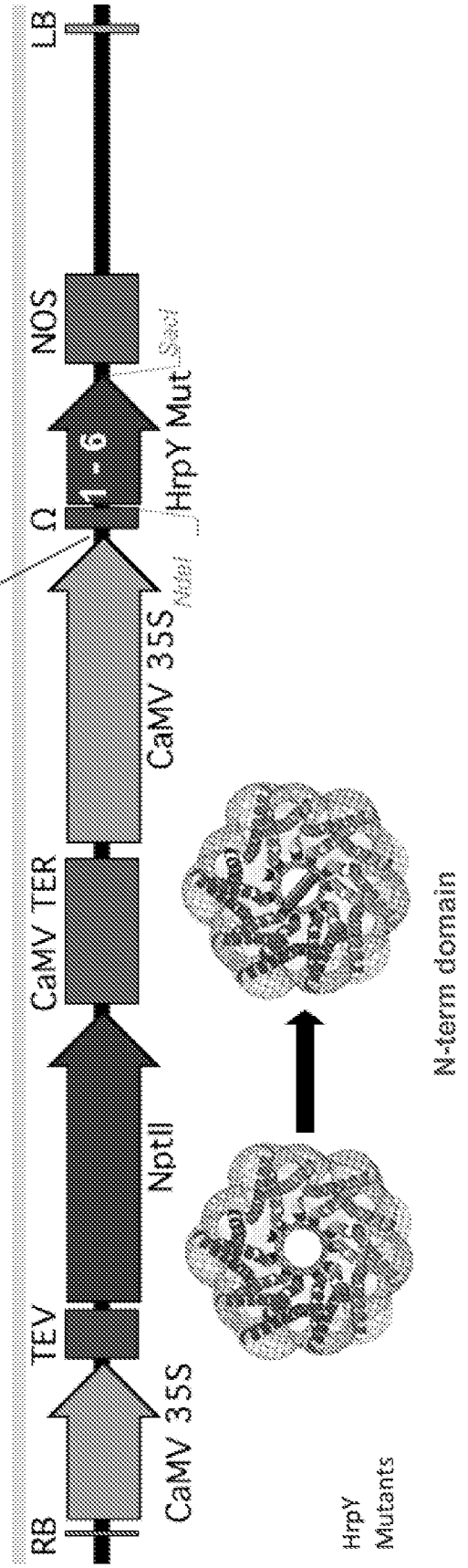
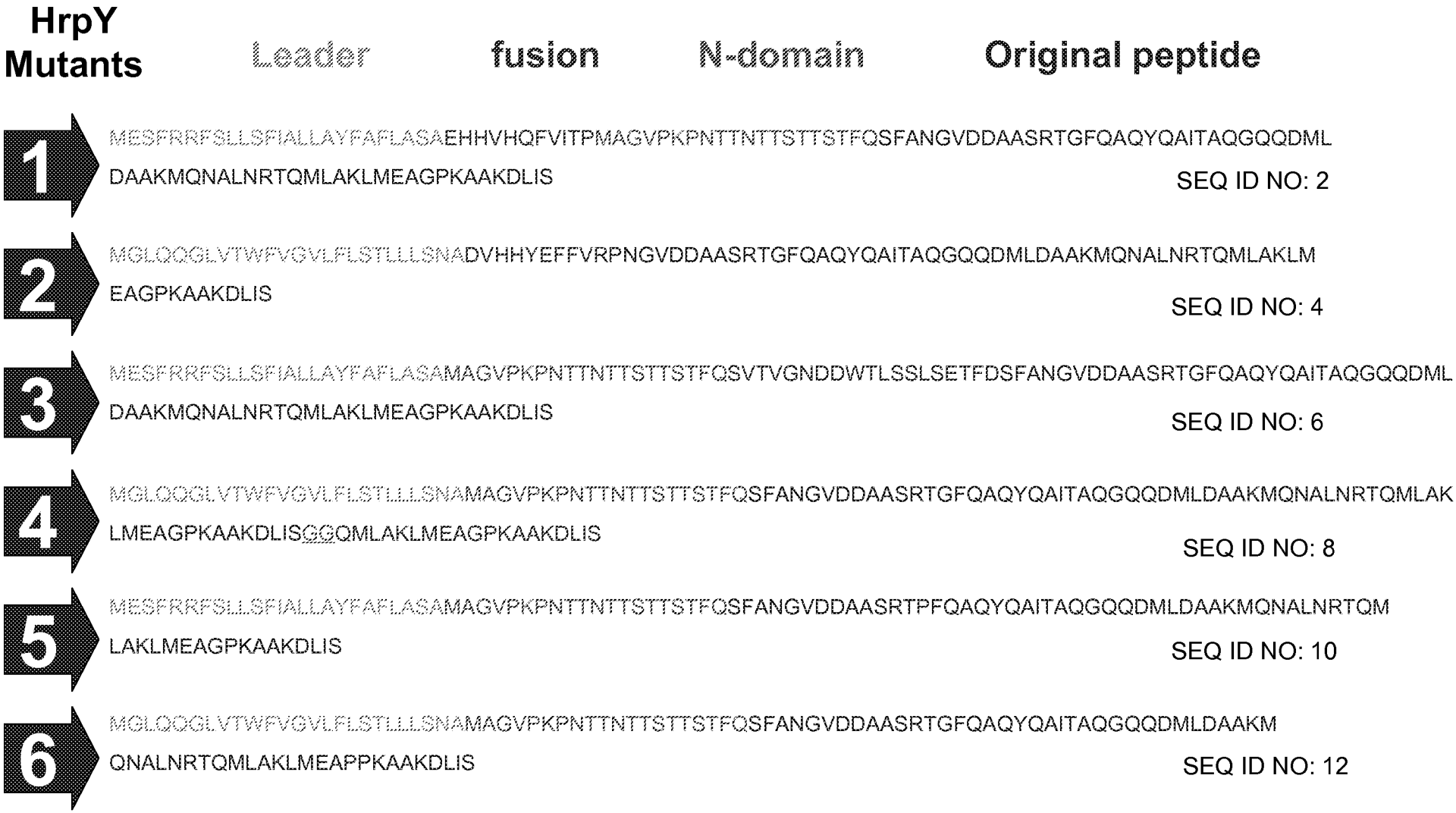

Mutated HrpY - Pilus interference
RB
CaMV 35S
TEV
NptII
CaMV TER
XbaI
CaMV 35S
NdeI
Ω
1 - 6
HrpY Mut
SacI
NOS
LB
HrpY Mutants
N-term domain

FIG. 7B

FIG. 7C


HrpY Mutants
Leader
fusion
N-domain
Original peptide
1
MESFRRFSLLSFIALLAYFAFLASAEHHVHQFVITPMAGVPKPNTTNTTSTTSTFQSFANGVDDAASRTGFQAQYQAITAQGQQDML
DAAKMQNALNRTQMLAKLMEAGPKAAKDLIS
SEQ ID NO: 2
2
MGLQQGLVTWFVGVLFLSTLLLSNADVHHYEFFVRPNGVDDAASRTGFQAQYQAITAQGQQDMLDAAKMQNALNRTQMLAKLM
EAGPKAAKDLIS
SEQ ID NO: 4
3
MESFRRFSLLSFIALLAYFAFLASAMAGVPKPNTTNTTSTTSTFQSVTVGNDDWTLSSLSETFDSFANGVDDAASRTGFQAQYQAITAQGQQDML
DAAKMQNALNRTQMLAKLMEAGPKAAKDLIS
SEQ ID NO: 6
4
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LMEAGPKAAKDLISGGQMLAKLMEAGPKAAKDLIS
SEQ ID NO: 8
5
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LAKLMEAGPKAAKDLIS
SEQ ID NO: 10
6
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SEQ ID NO: 12

SEQ ID NOs: 18 and 19

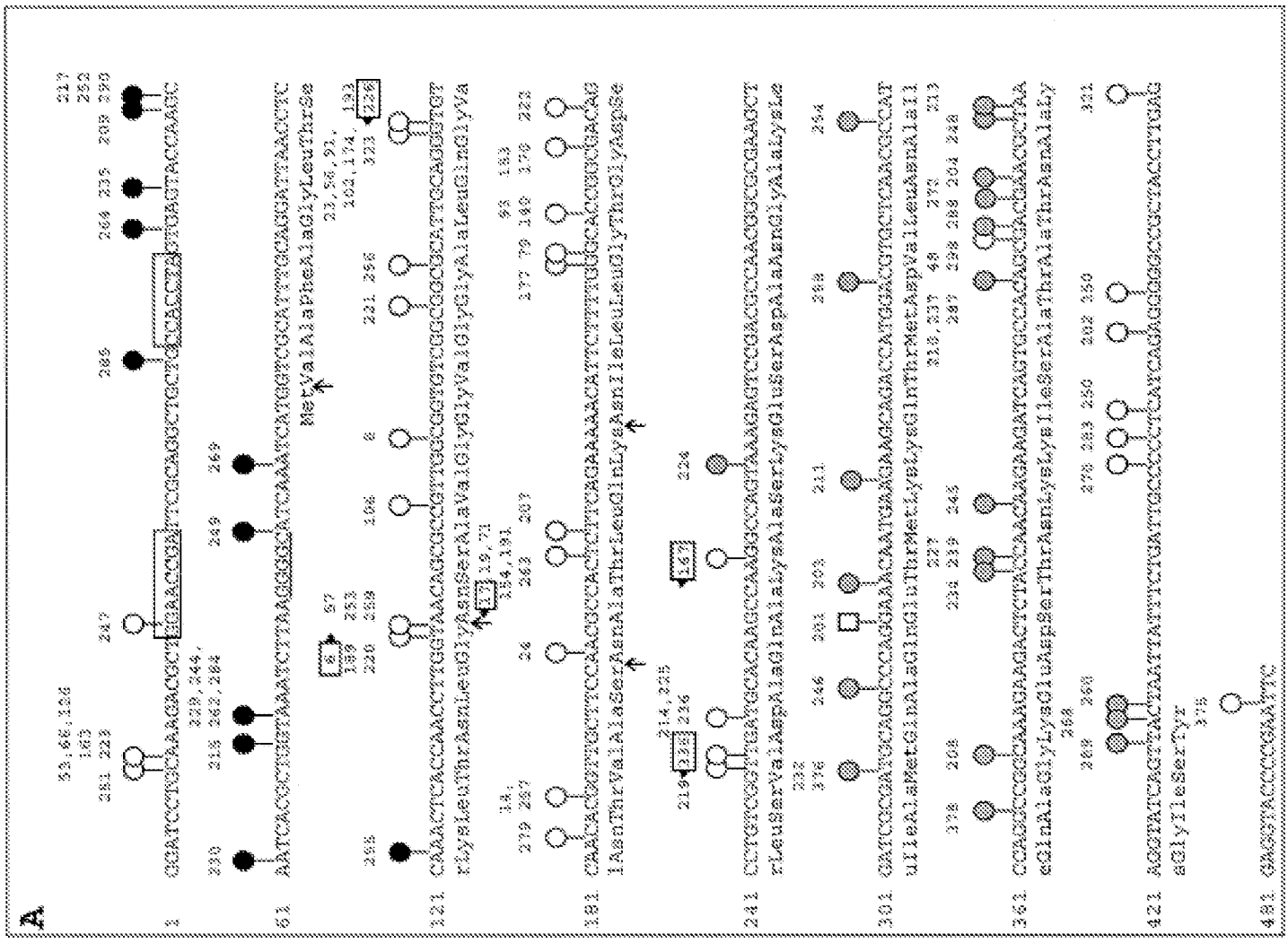
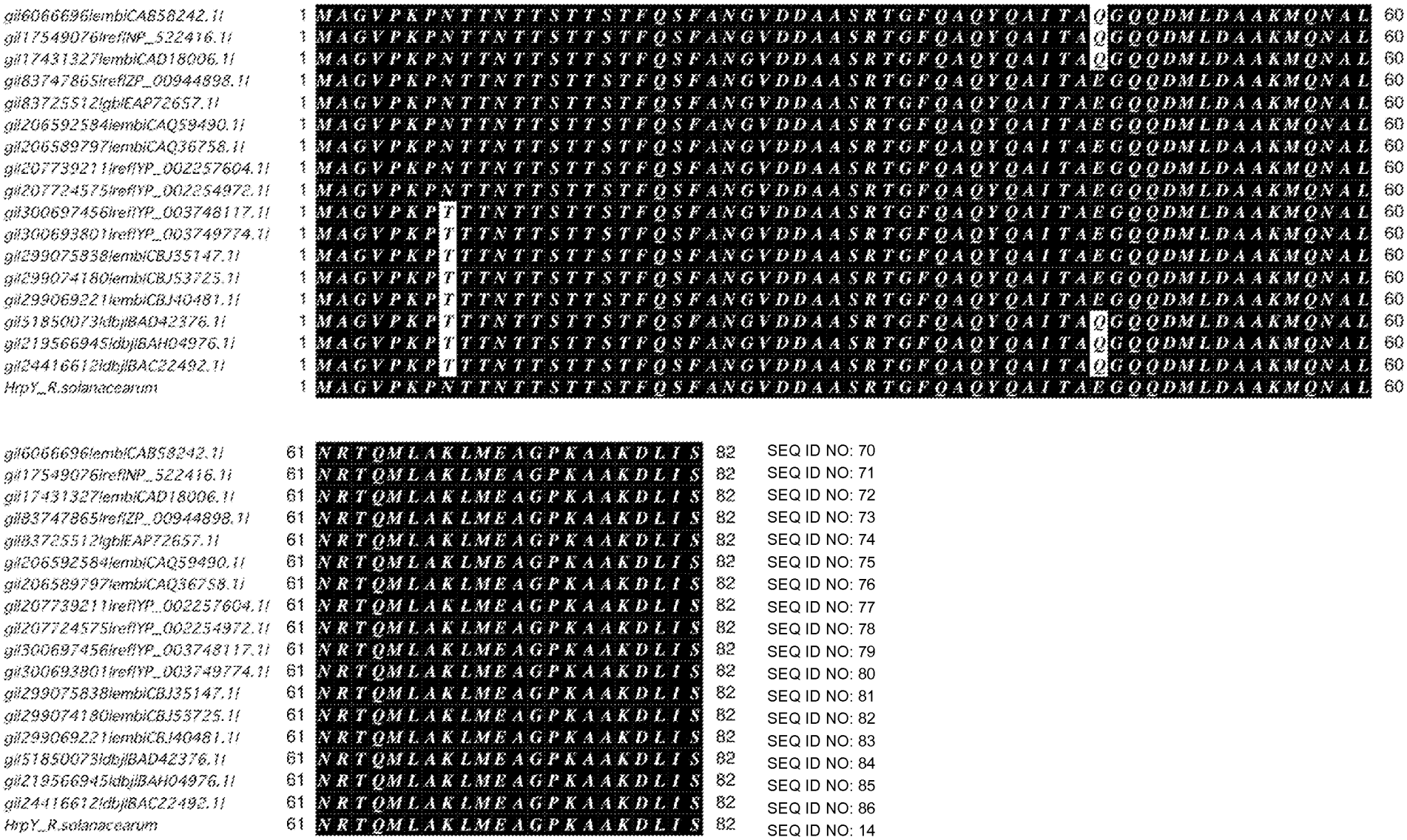


FIG. 8

FIG. 9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gi|24416612|dbj|BAC22492.1| 1 MAGVPKPTTTNTTSTTSTFQSFANGVDDAASRTGFQAQYQAITAQGQQDMLDAAKMQNAL 60
HrpY_R.solanacearum 1 MAGVPKPNTTNTTSTTSTFQSFANGVDDAASRTGFQAQYQAITAEGQQDMLDAAKMQNAL 60
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gi|300697456|ref|YP_003748117.1| 61 NRTQMLAKLMEAGPKAAKDLIS 82 SEQ ID NO: 79
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gi|299074180|emb|CBJ53725.1| 61 NRTQMLAKLMEAGPKAAKDLIS 82 SEQ ID NO: 82
gi|299069221|emb|CBJ40481.1| 61 NRTQMLAKLMEAGPKAAKDLIS 82 SEQ ID NO: 83
gi|51850073|dbj|BAD42376.1| 61 NRTQMLAKLMEAGPKAAKDLIS 82 SEQ ID NO: 84
gi|219566945|dbj|BAH04976.1| 61 NRTQMLAKLMEAGPKAAKDLIS 82 SEQ ID NO: 85
gi|24416612|dbj|BAC22492.1| 61 NRTQMLAKLMEAGPKAAKDLIS 82 SEQ ID NO: 86
HrpY_R.solanacearum 61 NRTQMLAKLMEAGPKAAKDLIS 82 SEQ ID NO: 14

FIG. 10A

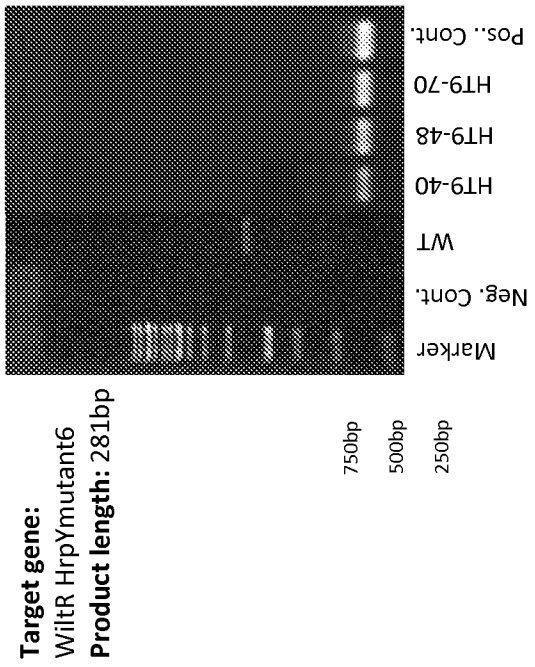

FIG. 10B

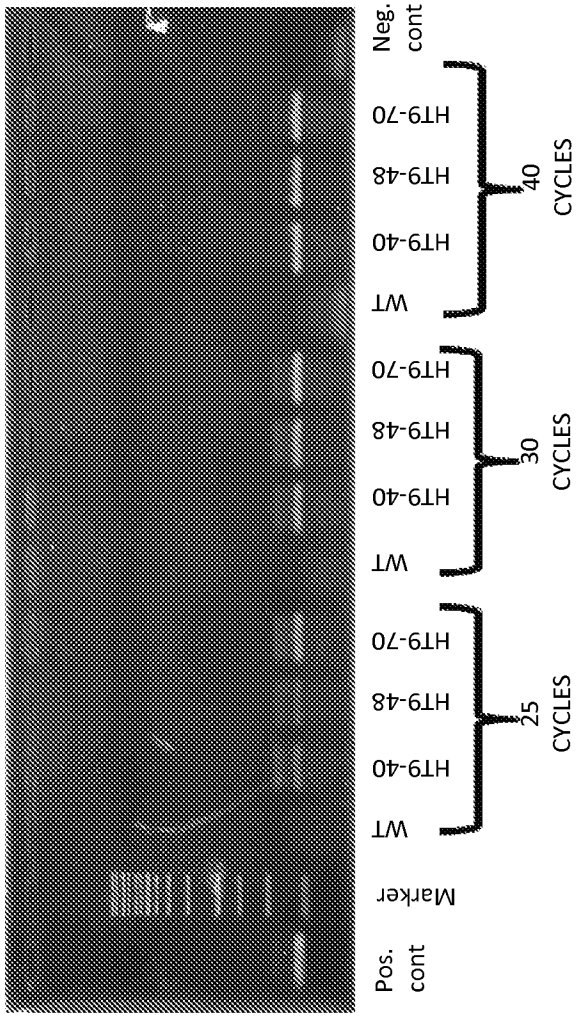

FIG. 10C

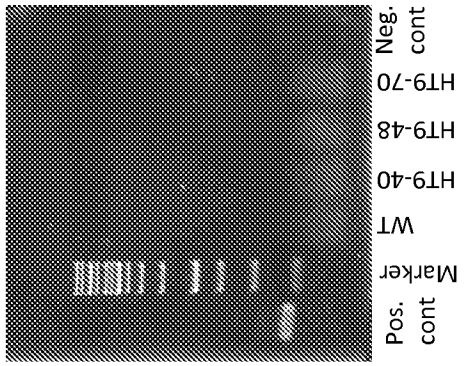

FIG. 11A

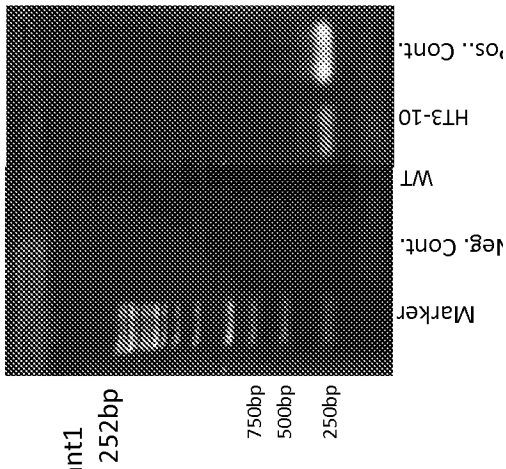

FIG. 11B

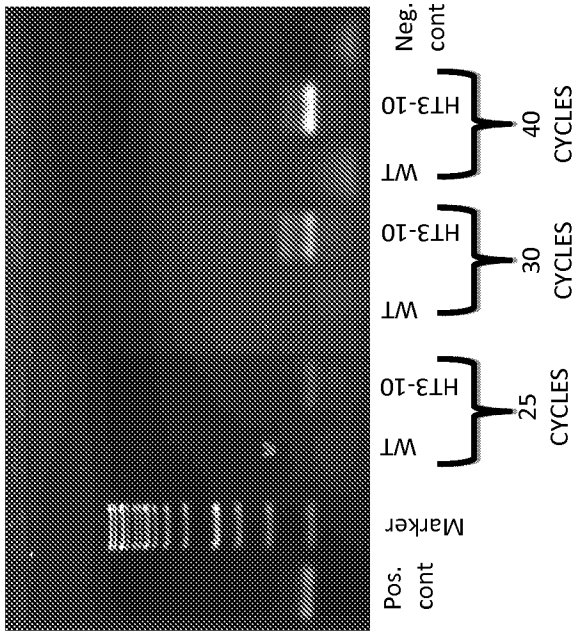

FIG. 11C

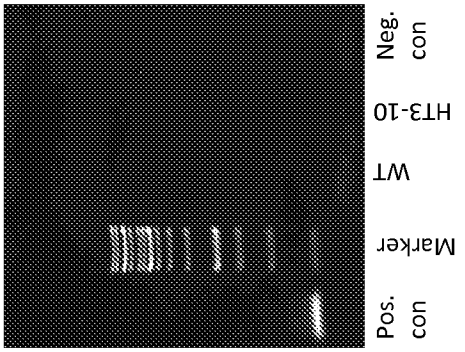

FIG. 12A

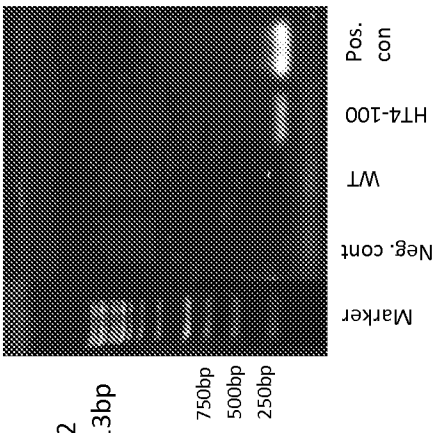

FIG. 12B

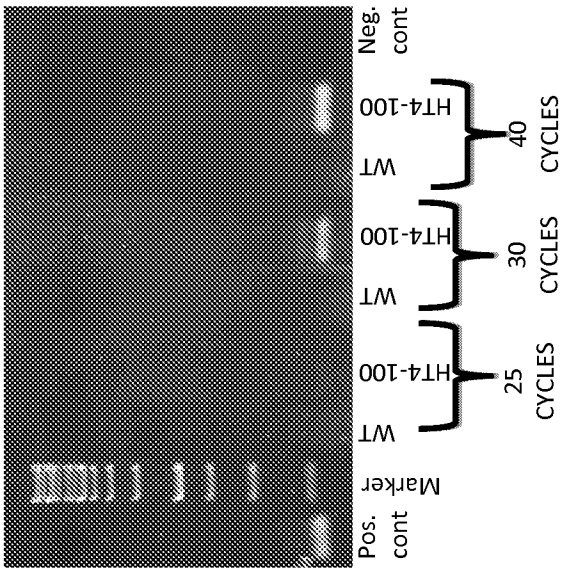

FIG. 12C