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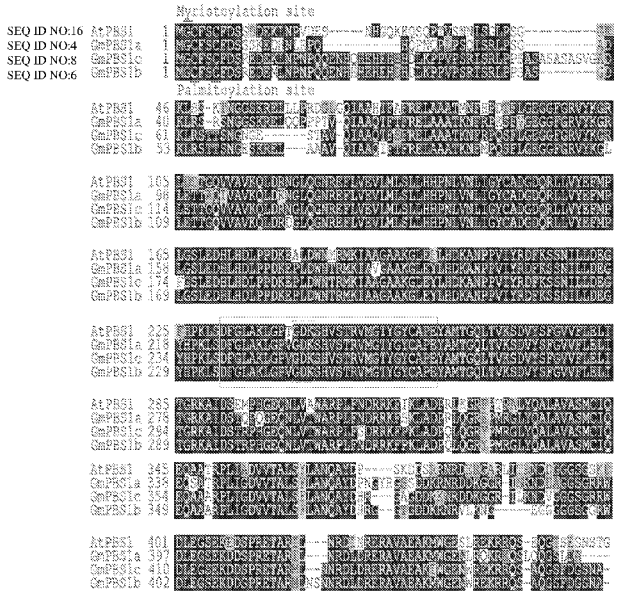
(54) Title: COMPOSITIONS AND SYSTEMS FOR CONFERRING DISEASE RESISTANCE IN SOYBEAN PLANTS AND METHODS OF USE THEREOF

FIG. 1B



(57) Abstract: Compositions, systems and methods are provided for conferring disease resistance to plant pathogens that use proteases to target plant substrate proteins inside plant cells. Briefly, the compositions, systems and methods are based upon plant substrate proteins that are targeted by pathogen-specific proteases and that activate nucleotide binding site-leucine rich repeat (NB-LRR) disease resistance proteins when cleaved by the protease. These substrate proteins are modified such that the endogenous protease recognition sequence is replaced by a protease recognition sequence specific to a different pathogen protease (i.e., a heterologous protease recognition sequence). The modified plant substrate protein therefore can be used in connection with its corresponding NB-LRR protein to activate resistance in response to cleavage by the heterologous pathogen-specific protease. When activated by the plant pathogen-specific protease, the pair initiates host defense responses thereto, including programmed cell death.

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[Continued on next page]

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COMPOSITIONS AND SYSTEMS FOR CONFERRING DISEASE RESISTANCE IN SOYBEAN PLANTS AND METHODS OF USE THEREOF

CROSS-REFERENCE OT RELATED APPLICATION

[0001] This application claims the benefit to U.S. Provisional Patent Application No. 62/482,074, filed on April 5, 2017, which is hereby incorporated by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made with government support under IOS1551452 awarded by the National Science Foundation. The Government has certain rights in the invention.

INCORPORATION OF SEQUENCE LISTING

[0003] A computer readable form of the Sequence Listing containing the file named "2017-128-01_ST25.txt", which is 38,907 bytes in size (as measured in Microsoft WINDOWS® Explorer), is provided herein and is herein incorporated by reference. This Sequence Listing consists of SEQ ID NOs:1-16.

BACKGROUND

[0004] The present disclosure relates generally to plant genetics and plant molecular biology, and more particularly relates to compositions, systems and methods of conferring disease resistance to soybean plant pathogens that express pathogen-specific proteases based on recognition of the pathogen-specific proteases in the soybean plant cell.

[0005] Plant diseases are a serious limitation on agricultural productivity and influence the development and history of agricultural practices. A variety of plant pathogens are responsible for plant diseases including bacteria, fungi, insects, nematodes and viruses.

[0006] Incidence of plant diseases can be controlled by agronomic practices that include conventional breeding techniques, crop rotation and use of synthetic agrochemicals. Conventional breeding methods, however, are time-consuming and require continuous effort to maintain disease resistance as plant pathogens evolve. *See*, Grover & Gowthaman (2003) *Curr. Sci.* 84:330-340. Likewise, agrochemicals increase costs to farmers and cause harmful effects on

the ecosystem. Because of such concerns, regulators have banned or limited the use of some of the most harmful agrochemicals.

[0007] Agricultural scientists now can enhance plant pathogen resistance by genetically engineering plants to express anti-pathogen polypeptides. For example, potatoes and tobacco plants have been developed that exhibit an increased resistance to foliar and soil-borne fungal pathogens. *See*, Lorito *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 95:7860-7865. In addition, transgenic barley has been developed that exhibit an increased resistance to fungal pathogens. *See*, Horvath *et al.* (2003) *Proc. Natl. Acad. Sci. USA* 100:364-369. Moreover, transgenic corn and cotton plants have been developed to produce *Cry* endotoxins. *See*, *e.g.*, Aronson (2002) *Cell Mol. Life Sci.* 59:417-425; and Schnepf *et al.* (1998) *Microbiol. Mol. Biol. Rev.* 62:775-806. Other crops, including potatoes, have been genetically engineered to contain similar endotoxins. *See*, Hussein *et al.* (2006) *J. Chem. Ecol.* 32:1-8; Kalushkov & Nedved (2005) *J. Appl. Entomol.* 129:401-406 and Dangl *et al.* (2013) *Science* 341: 746-751.

[0008] In light of the significant impact of plant pathogens on the yield and quality of plants, additional compositions, systems and methods are needed for protecting plants, and in particular, soybean plants, from plant pathogens.

BRIEF SUMMARY

[0009] Compositions, systems and methods are provided for conferring disease resistance to plant pathogens that express pathogen-specific proteases by modifying at least one member of a protein pair used by plants to detect the pathogen-specific proteases. These protein pairs enable plants to activate endogenous defense systems in response to the pathogen-specific proteases. Briefly, the compositions, systems and methods are based upon a protein pair in which one member of the pair is a nucleotide binding-leucine rich repeat (NB-LRR) disease resistance protein and the other member of the pair is a substrate protein of a pathogen-specific protease that physically associates with its native/corresponding NB-LRR protein and that activates the NB-LRR protein when cleaved by the pathogen-specific protease. The specificity of such pairs for a given pathogen-specific protease can be engineered by replacing an endogenous protease recognition sequence in the substrate protein with a recognition sequence for a pathogen-specific protease of interest (*i.e.*, a heterologous protease recognition sequence).

[0010] The compositions include recombinant nucleic acid molecules having a nucleotide sequence that encodes a modified substrate protein (also referred to herein as a "fusion protein")

of a pathogen-specific protease, where the modified substrate protein has a heterologous protease recognition sequence. The heterologous protease recognition sequence can be within, for example, an exposed loop of the modified substrate protein. Optionally, the recombinant nucleic acid molecule can have a nucleotide sequence that encodes a NB-LRR protein so that the nucleic acid molecule encodes the protein pair. For example, in one embodiment, a recombinant nucleic acid molecule having a nucleotide sequence that encodes the NB-LRR protein can be co-transformed with the recombinant nucleic acid molecules having a nucleotide sequence that encodes a modified substrate protein of a pathogen-specific protease so that the modified substrate protein and the NB-LRR protein are co-expressed. The NB-LRR protein can associate with, and can be activated by, the modified substrate protein of the pathogen-specific protease.

[0011] The compositions also include isolated, modified substrate proteins of pathogen-specific proteases as described herein, as well as active fragments and variants thereof.

[0012] The compositions also include nucleic acid constructs, such as expression cassettes and vectors, having a nucleotide sequence that encodes a modified substrate protein of a pathogen-specific protease as described herein operably linked to a promoter that drives expression in a plant cell, plant part or plant. Such a nucleic acid construct can be used to provide a modified substrate protein to a plant cell, plant part or plant that natively expresses the corresponding NB-LRR protein. The modified substrate protein can associate with, and can activate, the NB-LRR protein.

[0013] Optionally, the constructs, including expression cassettes and vectors, can include a nucleotide sequence that encodes a NB-LRR protein operably linked to a promoter that drives expression in a plant cell, plant part or plant. The nucleic acid constructs having a nucleotide sequence that encodes a modified substrate protein of a pathogen-specific protease and the nucleic acid constructs having a nucleotide sequence that encodes a NB-LRR protein can be co-expressed in a plant cell, plant part or plant. The NB-LRR protein can associate with, and can be activated by, the modified substrate protein of the pathogen-specific protease. Such a nucleic acid construct can be used to provide the protein pair to a plant cell, plant part or plant that does not natively express both members of the protein pair.

[0014] The compositions also include transformed plant cells, plant parts and plants having a nucleotide sequence that encodes at least one modified substrate protein of a pathogen-specific protease as described herein operably linked to a promoter that drives expression in a plant cell,

plant part or plant. Optionally, the plant cells, plant parts and plants are transformed to include a nucleotide sequence that encodes a NB-LRR protein operably linked to a promoter that drives expression in the plant cell, plant part or plant. The NB-LRR protein can associate with, and can be activated by, the modified substrate protein of the pathogen-specific protease.

[0015] The systems include a nucleic acid construct having a nucleotide sequence for a first promoter that drives expression in a plant cell, plant part or plant operably linked to a nucleotide sequence that encodes a modified substrate protein of a pathogen-specific protease as described herein and a nucleotide sequence for a second promoter that drives expression in a plant cell, plant part or plant operably linked to a nucleotide sequence that encodes a NB-LRR protein. The NB-LRR protein can associate with, and can be activated by, the modified substrate protein. Such systems can be used to provide the protein pair to a plant cell, plant part or plant that does not natively express both members of the protein pair.

[0016] The systems also include a first nucleic acid construct having nucleotide sequence for a promoter that drives expression in a plant cell, plant part or plant operably linked to a nucleotide sequence that encodes a modified substrate protein of a pathogen-specific protease as described herein, and a second nucleic acid construct having a nucleotide sequence for a promoter that drives expression in a plant cell, plant part or plant operably linked to a nucleotide sequence that encodes a NB-LRR protein. Additional nucleic acid constructs also can be included in the system, where each construct has a nucleotide sequence that encodes a distinct modified substrate protein, each having a heterologous recognition sequence for a separate pathogen-specific protease. Although each modified substrate protein has a heterologous recognition sequence distinct from one another, each can associate with, and can activate, the NB-LRR protein. Alternatively, the first nucleic acid construct can encode more than one modified substrate protein, where each modified substrate protein has a heterologous recognition sequence distinct from one another and where each can associate with, and can activate, the NB-LRR protein. Alternatively, the second nucleic acid construct can encode one or more modified substrate proteins, where each modified substrate protein has a heterologous recognition sequence distinct from one another and where each can associate with, and can activate, the NB-LRR protein. Such systems can be used to provide the protein pair to a plant cell, plant part or plant that does not natively express the protein pair or can be used to provide more than one modified substrate protein to a plant cell, plant part or plant.

[0017] By way of example, the substrate protein of the pathogen-specific protease can be a PBS1 homolog from *Glycine max* (soybean) (e.g., PBS1 homolog GmPBS1a (SEQ ID NO:4). GmPBS1b (SEQ ID NO:6), and GmPBS1c (SEQ ID NO:8)). The PBS1 homolog is modified to include a heterologous protease recognition sequence. As understood by those skilled in the art, "PBS1" refers to avrPphB susceptible 1. As understood by those skilled in the art, "avrPphB" refers to the bacterial avirulence from *Pseudomonas syringae* that encodes the "AvrPphB" polypeptide having a role in plant-*P. syringae* interactions.

[0018] In some embodiments, the present disclosure is directed to the fusion protein encoded by the nucleotide sequence. By way of example, the fusion protein can include a *G. max* AvrPphB susceptible 1 (GmPBS1) substrate protein and a heterologous pathogen-specific protease recognition sequence. Exemplary GmPBS1 substrate proteins including a heterologous pathogen-specific protease recognition sequence includes proteins having an amino acid sequence of SEQ ID NOs:10, 12 or 14.

[0019] In view of the foregoing, the methods include introducing into a plant cell, plant part or plant at least one nucleic acid molecule, construct, expression cassette or vector as described herein to confer disease resistance to plant pathogens that express pathogen-specific proteases.

[0020] The compositions, systems and methods therefore find use in conferring disease resistance to plant pathogens by transferring to plant cells, plant parts or plants nucleotide sequences that encode at least one modified substrate protein of a pathogen-specific protease and optionally that encode a NB-LRR protein when such NB-LRR protein is not native to the plant cell, plant part or plant. The pair is thus engineered to be specific for a plant pathogen-specific protease by including in the modified substrate protein a heterologous protease recognition sequence for that plant pathogen-specific protease. When activated by the plant pathogen-specific protease, the pair initiates host defense responses thereto, including programmed cell death.

[0021] These and other features, objects and advantages of the present disclosure will become better understood from the description that follows. In the description, reference is made to the accompanying drawings, which form a part hereof.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022] The features, objects and advantages other than those set forth above will become more readily apparent when consideration is given to the detailed description below. Such detailed description makes reference to the following drawings, wherein:

[0023] FIGS. 1A & 1B depict three genes within the *Glycine max* genome that encode proteins with significant amino acid homology to Arabidopsis PBS1 (AtPBS1; At5g13160) (SEQ ID NO:16).

[0024] FIG. 2 depicts modified soybean PBS1 substrate proteins to function as 'decoys' for the SMV NIa protease.

[0025] FIGS. 3A & 3B depict recognition of AvrPphB in soybean.

[0026] FIGS. 4A & 4B depict the effects of activating an AvrPphB-specific R protein in soybean on resistance to Soybean Mosaic Virus (SMV).

[0027] FIGS. 5A-5C depict the effects of activating RPS5 in Arabidopsis on resistance to Turnip Mosaic Virus (TuMV).

[0028] FIGS. 6A & 6B depict that overexpression of $PBS1^{TuMV}$ confers resistance to infection by TuMV. FIG. 6A are ultraviolet light images of Arabidopsis plants expressing the $PBS1^{TuMV}$ decoy protein. FIG. 6B shows immunoblot analysis of $PBS1^{TuMV}$ and viral protein levels in transgenic lines.

[0029] FIG. 7A depicts that AvrPphB was recognized in soybean, barley and wheat.

[0030] FIG. 7B are immunoblots showing cleavage of PBS1 proteins from Arabidopsis (At), soybean (Gm) and barley (Hv) by AvrPphB.

[0031] While the present disclosure is susceptible to various modifications and alternative forms, exemplary embodiments thereof are shown by way of example in the drawings and are herein described in detail. It should be understood, however, that the description of exemplary embodiments is not intended to limit the disclosure to the particular forms disclosed, but on the contrary, the intention is to cover all modifications, equivalents and alternatives falling within the scope of the disclosure as defined by the embodiments above and the claims below.

Reference should therefore be made to the embodiments above and claims below for interpreting the scope of the present disclosure.

DETAILED DESCRIPTION

[0032] The compositions, systems and methods now will be described more fully hereinafter with reference to the accompanying drawings, in which some, but not all embodiments of the present disclosure are shown. Indeed, the present disclosure may be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are provided so that this disclosure will satisfy applicable legal requirements.

[0033] Likewise, many modifications and other embodiments of the compositions, systems and methods described herein will come to mind to one of skill in the art to which the present disclosure pertains having the benefit of the teachings presented in the foregoing descriptions and the associated drawings. Therefore, it is to be understood that the present disclosure is not to be limited to the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended claims. Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation.

[0034] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of skill in the art to which the present disclosure pertains. Moreover, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one element is present, unless the context clearly requires that there be one and only one element. The indefinite article "a" or "an" thus usually includes "at least one."

[0035] Many plant pathogens employ proteases as virulence factors, including bacteria, fungi and viruses. As used herein, "plant pathogen" or "pathogen" means an organism that interferes with or is harmful to plant development and/or growth. Examples of plant pathogens include, but are not limited to, bacteria (*e.g.*, *Xanthomonas* spp. and *Pseudomonas* spp.), fungi (*e.g.*, members in the phylum Ascomycetes or Basidiomycetes, and fungal-like organisms including Oomycetes such as *Pythium* spp. and *Phytophthora* spp.), insects, nematodes (*e.g.*, soil-transmitted nematodes including *Clonorchis* spp., *Fasciola* spp., *Heterodera* spp., *Globodera* spp., *Opisthorchis* spp. and *Paragonimus* spp.), protozoans (*e.g.*, *Phytomonas* spp.), and viruses (*e.g.*, Soybean Mosaic Virus (SMV), Turnip Mosaic Virus (TMV), *Comovirus* spp.,

Cucumovirus spp., *Cytorhabdovirus* spp., *Luteovirus* spp., *Nepovirus* spp., *Potyvirus* spp., *Tobamovirus* spp., *Tombusvirus* spp. and *Tospovirus* spp.).

[0036] Plants, however, contain innate disease resistance against a majority of plant pathogens. Natural variation for resistance to plant pathogens has been identified by plant breeders and pathologists and can be bred into many plants. These natural disease resistance genes provide high levels of resistance (or immunity) to plant pathogens and represent an economical and environmentally friendly form of plant protection.

[0037] Innate disease resistance in plants to plant pathogens typically is governed by the presence of dominant or semidominant resistance (R) genes in the plant and dominant avirulence (*avr*) genes in the pathogen. In Arabidopsis, an example of this is the dominant R gene *RPS5*, which mediates recognition of the *avrPphB* gene from *Pseudomonas syringae*. Recognition of the AvrPphB protein by the RPS5 protein activates RPS5, which then initiates a disease resistance response that culminates in programmed cell death of cells surrounding the bacteria.

[0038] The AvrPphB protein also elicits a cell death response in most varieties of soybean (*Glycine max*), indicating that these varieties of soybean possess an R gene functionally analogous to *RPS5*. Soybean contains three genes co-orthologous to PBS1(*GmPBS1a*, *GmPBS1b*, and *GmPBS1c*). AvrPphB induces cleavage of all three soybean PBS1 proteins, and AvrPphB protease activity is required to activate a cell death response in soybean. These findings indicate that recognition of AvrPphB in soybean likely occurs by the same mechanism as previously described in Arabidopsis.

[0039] The present disclosure therefore provides compositions, systems and methods for conferring additional disease resistance to plant pathogens that express specific proteases in plant cells, plant parts or plants by using a modified substrate of a pathogen-specific protease that has a heterologous protease recognition sequence in connection with its corresponding NB-LRR protein.

Compositions

Recombinant Nucleic and Amino Acid Molecules

[0040] Compositions of the present disclosure include recombinant nucleic and amino acid sequences for modified substrate proteins of pathogen-specific proteases in which an endogenous

protease recognition sequence within the substrates are replaced with a heterologous protease recognition sequence.

[0041] In one aspect, the present disclosure is directed to a recombinant nucleic acid molecule comprising a nucleotide sequence that encodes at least one substrate protein of a plant pathogen-specific protease and a heterologous pathogen-specific protease recognition sequence within the substrate protein. The substrate protein can be, for example, *Glycine max* AvrPphB susceptible 1 (GmPBS1). More particularly, the nucleotide sequence encoding at least one substrate protein of a plant pathogen-specific protease and a heterologous pathogen-specific protease recognition sequence within the substrate protein can be one or more of SEQ ID NO:9, SEQ ID NO:11 or SEQ ID NO:13. The nucleotide sequence may encode one or more substrate proteins such as GmPBS1a (SEQ ID NO: 10), GmPBS1b (SEQ ID NO: 12), or GmPBS1c (SEQ ID NO: 14).

[0042] As used herein, a "nucleic acid" sequence means a DNA or RNA sequence. The term encompasses sequences that include any of the known base analogues of DNA and RNA such as, but not limited to 4-acetylcytosine, 8-hydroxy-N6-methyladenosine, aziridinylcytosine, pseudoisocytosine, 5-(carboxyhydroxylmethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, dihydrouracil, inosine, N6-isopentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, oxybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, -uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, and 2,6-diaminopurine.

[0043] As used herein, "recombinant," when used in connection with a nucleic acid molecule, means a molecule that has been created or modified through deliberate human intervention such as by genetic engineering. For example, a recombinant nucleic acid molecule is one having a nucleotide sequence that has been modified to include an artificial nucleotide sequence or to include some other nucleotide sequence that is not present within its native (non-recombinant) form.

[0044] Further, a recombinant nucleic acid molecule has a structure that is not identical to that of any naturally occurring nucleic acid molecule or to that of any fragment of a naturally occurring genomic nucleic acid molecule spanning more than one gene. A recombinant nucleic acid molecule also includes, without limitation, (a) a nucleic acid molecule having a sequence of a naturally occurring genomic or extrachromosomal nucleic acid molecule, but which is not flanked by the coding sequences that flank the sequence in its natural position; (b) a nucleic acid molecule incorporated into a construct, expression cassette or vector, or into a host cell's genome such that the resulting polynucleotide is not identical to any naturally occurring vector or genomic DNA; (c) a separate nucleic acid molecule such as a cDNA, a genomic fragment, a fragment produced by polymerase chain reaction (PCR) or a restriction fragment; and (d) a recombinant nucleic acid molecule having a nucleotide sequence that is part of a hybrid gene (*i.e.*, a gene encoding a fusion protein). As such, a recombinant nucleic acid molecule can be modified (chemically or enzymatically) or unmodified DNA or RNA, whether fully or partially single-stranded or double-stranded or even triple-stranded.

[0045] A nucleic acid molecule (or its complement) that can hybridize to any of the uninterrupted nucleotide sequences described herein, under either highly stringent or moderately stringent hybridization conditions, also is within the scope of the present disclosure.

[0046] As used herein, "stringent conditions" means conditions under which one nucleic acid molecule will hybridize to its target to a detectably greater degree than to other sequences (*e.g.*, at least two-fold over background). Stringent conditions can be sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences that are 100% complementary to the nucleic acid molecule can be identified (*i.e.*, homologous probing). Alternatively, the stringent condition can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (*i.e.*, heterologous probing).

[0047] Typically, stringent conditions can be one in which the salt concentration is less than about 1.5 M Na^+, typically about 0.01 M to 1.0 M Na^+ (or other salts) at about pH 7.0 to 8.3, and a temperature of at least 30°C for short molecules (*e.g.*, 10 to 50 nucleotides) and of at least 60°C for long molecules (*e.g.*, greater than 50 nucleotides). Stringent conditions also can be achieved by adding destabilizing agents such as formamide.

[0048] As used herein, "about" means within a statistically meaningful range of a value or values such as a stated concentration, length, molecular weight, pH, sequence identity, time frame, temperature or volume. Such a value or range can be within an order of magnitude, typically within 20%, more typically within 10%, and even more typically within 5% of a given value or range. The allowable variation encompassed by "about" will depend upon the particular system under study, and can be readily appreciated by one of skill in the art.

[0049] An exemplary low stringent condition includes hybridizing with a buffer solution of about 30% to about 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at about 37°C, and washing in about 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at about 50°C to about 55°C. Wash buffers optionally can comprise about 0.1% to about 1% SDS.

[0050] An exemplary moderate stringent condition includes hybridizing in about 40% to about 45% formamide, 1.0 M NaCl, 1% SDS at about 37°C, and washing in about 0.5X to 1X SSC at about 55°C to about 60°C. Wash buffers optionally can comprise about 0.1% to about 1% SDS.

[0051] An exemplary high stringent condition includes hybridizing in about 50% formamide, 1 M NaCl, 1% SDS at about 37°C, and washing in about 0.1X SSC at about 60°C to about 65°C. Wash buffers optionally can comprise about 0.1% to about 1% SDS.

[0052] The duration of hybridizing generally can be less than 24 hours, usually about 4 hours to about 12 hours. The duration of the washing can be at least a length of time sufficient to reach equilibrium. Additional guidance regarding such conditions is readily available in the art, for example, in *Molecular Cloning: A Laboratory Manual*, 3rd ed. (Sambrook & Russell eds., Cold Spring Harbor Press 2001); and *Current Protocols in Molecular Biology* (Ausubel *et al.* eds., John Wiley & Sons 1995).

[0053] An example of a recombinant nucleic acid molecule encoding a modified substrate protein of a pathogen-specific protease therefore includes a nucleotide sequence that encodes PBS1 in which its endogenous AvrPphB cleavage site (SEQ ID NO:1) is replaced with a heterologous AvrRpt2 cleavage site (SEQ ID NO:2).

[0054] Methods for synthesizing nucleic acid molecules are well known in the art, such as cloning and digestion of the appropriate sequences, as well as direct chemical synthesis (*e.g.*, ink-jet deposition and electrochemical synthesis). Methods of cloning nucleic acid molecules are

described, for example, in Ausubel *et al.* (1995), *supra*; Copeland *et al.* (2001) *Nat. Rev. Genet.* 2:769-779; *PCR Cloning Protocols*, 2nd ed. (Chen & Janes eds., Humana Press 2002); and Sambrook & Russell (2001), *supra*. Methods of direct chemical synthesis of nucleic acid molecules include, but are not limited to, the phosphotriester methods of Reese (1978) *Tetrahedron* 34:3143-3179 and Narang *et al.* (1979) *Methods Enzymol.* 68:90-98; the phosphodiester method of Brown *et al.* (1979) *Methods Enzymol.* 68:109-151; the diethylphosphoramidate method of Beaucage *et al.* (1981) *Tetrahedron Lett.* 22:1859-1862; and the solid support methods of Fodor *et al.* (1991) *Science* 251:767-773; Pease *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:5022-5026; and Singh-Gasson *et al.* (1999) *Nature Biotechnol.* 17:974-978; as well as US Patent No. 4,485,066. *See also*, Peattie (1979) *Proc. Natl. Acad. Sci. USA* 76:1760-1764; as well as EP Patent No. 1 721 908; Int'l Patent Application Publication Nos. WO 2004/022770 and WO 2005/082923; US Patent Application Publication No. 2009/0062521; and US Patent Nos. 6,521,427; 6,818,395 and 7,521,178.

[0055] In addition to the full-length nucleotide sequence of a nucleic acid molecule encoding a modified substrate/fusion protein, it is intended that the nucleic acid molecule can be a fragment or variant thereof that is capable of functioning as a substrate. For nucleotide sequences, "fragment" means a portion of a nucleotide sequence of a nucleic acid molecule, for example, a portion of the nucleotide sequence encoding a modified substrate protein. Fragments of a nucleotide sequence may retain the biological activity of the reference nucleic acid molecule. For example, less than the entire sequence disclosed in SEQ ID NO:10 can be used and will encode a modified substrate protein that interacts with a pathogen-specific protease and that retains its ability to interact with its corresponding NB-LRR protein. Likewise, a fragment of a nucleotide sequence encoding the modified substrate protein can be used if that fragment encodes a modified substrate protein that interacts with a pathogen-specific protease and that retains its ability to interact with its corresponding NB-LRR protein. Alternatively, fragments of a nucleotide sequence that can be used as hybridization probes generally do not need to retain biological activity. Thus, fragments of the nucleic acid molecules can be at least 10, 15, 20, 25, 50, 75, 100, 125, 150, 175, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850 or 900 nucleotides, or up to the number of nucleotides present in a full-length nucleic acid molecule.

[0056] A fragment of the nucleic acid molecule therefore can include a functionally/biologically active portion, or it can include a fragment that can be used as a

hybridization probe or PCR primer. A biologically active portion of the nucleic acid molecule can be prepared by isolating part of the sequence of the nucleic acid molecule, operably linking that fragment to a promoter, expressing the nucleotide sequence encoding the protein, and assessing the amount or activity of the protein. Methods of assaying protein expression are well known in the art. *See*, *e.g.*, Chan *et al.* (1994) *J. Biol. Chem.* 269:17635-17641; Freyssinet & Thomas (1998) *Pure & Appl. Chem.* 70:61-66; and Kirby *et al.* (2007) *Adv. Clin. Chem.* 44:247-292; as well as US Patent Application Publication Nos. 2009/0183286 and 2009/0217424; and US Patent Nos. 7,294,711 and 7,408,055. Likewise, kits for assaying protein expression are commercially available, for example, from Applied Biosystems, Inc. (Foster City, CA), Caliper Life Sciences (Hopkinton, MA), Promega (Madison, WI), and SABiosciences (Frederick, MD). Protein expression also can be assayed using other methods well known in the art, including, but not limited to, Western blot analysis, enzyme-linked immunosorbent assay, and the like. *See*, *e.g.*, Sambrook & Russel (2001), *supra.* Moreover, methods of assaying pathogen-specific protease substrate protein activity are well known in the art. *See*, DeYoung *et al.* (2012), *supra.*

[0057] For nucleotide sequences, "variant" means a substantially similar nucleotide sequence to a nucleotide sequence of a recombinant nucleic acid molecule as described herein, for example, a substantially similar nucleotide sequence encoding a modified substrate protein. For nucleotide sequences, a variant comprises a nucleotide sequence having deletions (*i.e.*, truncations) at the 5' and/or 3' end, deletions and/or additions of one or more nucleotides at one or more internal sites compared to the nucleotide sequence of the recombinant nucleic acid molecules as described herein; and/or substitution of one or more nucleotides at one or more sites compared to the nucleotide sequence of the recombinant nucleic acid molecules described herein. One of skill in the art understands that variants are constructed in a manner to maintain the open reading frame.

[0058] Conservative variants include those nucleotide sequences that, because of the degeneracy of the genetic code (*see*, Table 1), result in a functionally active modified substrate protein as described herein. Naturally occurring allelic variants can be identified by using well-known molecular biology techniques such as, for example, polymerase chain reaction (PCR) and hybridization techniques. Variant nucleotide sequences also can include synthetically derived sequences, such as those generated, for example, by site-directed mutagenesis but which still provide a functionally active modified substrate protein. Generally, variants of a nucleotide sequence of the recombinant nucleic acid molecules as described herein will have at least 70%,

75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the nucleotide sequence of the recombinant nucleic acid molecules as determined by sequence alignment programs and parameters as described elsewhere herein.

[0059] When making recombinant nucleic acid molecules as described herein and variants thereof, one of skill in the art can be further guided by knowledge of redundancy in the genetic code as shown below in Table 1.

Table 1: Redundancy in Genetic Code.

Residue	**Triplet Codons Encoding the Residue**
Ala (A)	GCU, GCC, GCA, GCG
Arg (R)	CGU, CGC, CGA, CGG, AGA, AGG
Asn (N)	AAU, AAC
Asp (D)	GAU, GAC
Cys (C)	UGU, UGC
Gln (Q)	CAA, CAG
Glu (E)	GAA, GAG
Gly (G)	GGU, GGC, GGA, GGG
His (H)	CAU, CAC
Ile (I)	AUU, AUC, AUA
Leu (L)	UUA, UUG, CUU, CUC, CUA, CUG
Lys (K)	AAA, AAG
Met (M)	AUG
Phe (F)	UUU, UUC
Pro (P)	CCU, CCC, CCA, CCG
Ser (S)	UCU, UCC, UCA, UCG, AGU, AGC
Thr (T)	ACU, ACC, ACA, ACG
Trp (W)	UGG
Tyr (Y)	UAU, UAC
Val (V)	GUU, GUC, GUA, GUG
START	AUG
STOP	UAG, UGA, UAA

[0060] Deletions, insertions and/or substitutions of the nucleotide sequence of the recombinant nucleic acid molecules are not expected to produce radical changes in their characteristics. However, when it is difficult to predict the exact effect of the substitution, deletion or insertion in advance of doing so, one of skill in the art will appreciate that the effect can be evaluated by expression assays.

[0061] Variant nucleic acid molecules also encompass nucleotide sequences derived from a mutagenic and recombinogenic procedure such as DNA shuffling. With such a procedure, the

nucleotide sequences of the recombinant nucleic acid molecules described herein can be manipulated to create a new nucleic acid molecule possessing the desired properties. In this manner, libraries of recombinant nucleic acid molecules can be generated from a population of related nucleic acid molecules comprising sequence regions that have substantial sequence identity and can be homologously recombined *in vitro* or *in vivo*. For example, using this approach, sequence motifs encoding a domain of interest can be shuffled between the nucleic acid molecules described herein and other known promoters to obtain a new nucleic acid molecule with an improved property such as increased promoter activity.

[0062] Methods of mutating and altering nucleotide sequences, as well as DNA shuffling, are well known in the art. *See*, Crameri *et al.* (1997) *Nature Biotech.* 15:436-438; Crameri *et al.* (1998) *Nature* 391:288-291; Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel *et al.* (1987) *Methods in Enzymol.* 154:367-382; Moore *et al.* (1997) *J. Mol. Biol.* 272:336-347; Stemmer (1994) *Proc. Natl. Acad. Sci. USA* 91:10747-10751; Stemmer (1994) *Nature* 370:389-391; Zhang *et al.* (1997) *Proc. Natl. Acad. Sci. USA* 94:4504-4509; and *Techniques in Molecular Biology* (Walker & Gaastra eds., MacMillan Publishing Co. 1983) and the references cited therein; as well as US Patent Nos. 4,873,192; 5,605,793 and 5,837,458. As such, the nucleic acid molecules as described herein can have many modifications.

[0063] Variants of the recombinant nucleic acid molecules described herein also can be evaluated by comparing the percent sequence identity between the polypeptide encoded by a variant and the polypeptide encoded by a reference nucleic acid molecule. Thus, for example, an isolated nucleic acid molecule can be one that encodes a polypeptide with a given percent sequence identity to the polypeptide of interest. Percent sequence identity between any two polypeptides can be calculated using sequence alignment programs and parameters described elsewhere herein. Where any given pair of polynucleotides of the present disclosure is evaluated by comparison of the percent sequence identity shared by the two polypeptides they encode, the percent sequence identity between the two encoded polypeptides can be at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity.

[0064] Determining percent sequence identity between any two sequences can be accomplished using a mathematical algorithm. Non-limiting examples of such mathematical algorithms include, but are not limited to, the algorithm of Myers & Miller (1988) *CABIOS* 4:11-17; the local alignment algorithm of Smith *et al.* (1981) *Adv. Appl. Math.* 2:482-489; the global alignment algorithm of Needleman & Wunsch (1970) *J. Mol. Biol.* 48:443-453; the search-for-

local alignment method of Pearson & Lipman (1988) *Proc. Natl. Acad. Sci. USA* 85:2444-2448; the algorithm of Karlin & Altschul (1990) *Proc. Natl. Acad. Sci. USA* 87:2264-2268, modified as in Karlin & Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877.

[0065] The present disclosure therefore includes recombinant nucleic acid molecules having a nucleotide sequence that encodes a modified substrate protein of a pathogen-specific protease, where the modified substrate protein has a heterologous protease recognition sequence and can be incorporated into nucleic acid constructs such as expression cassettes and vectors.

Nucleic Acid Constructs

[0066] Compositions of the present disclosure also include nucleic acid constructs, such as expression cassettes or vectors, having plant promoters operably linked with a nucleic acid molecule that encodes a substrate protein of a pathogen-specific protease and a heterologous pathogen-specific protease recognition sequence for use in transforming plant cells, plant parts and plants. In addition, the constructs can include a nucleic acid molecule that encodes a NB-LRR protein, particularly when such NB-LRR protein is not native/not endogenous to the plant cell, plant part or plant to be transformed.

[0067] As used herein, "nucleic acid construct" means an oligonucleotide or polynucleotide composed of deoxyribonucleotides, ribonucleotides or combinations thereof having incorporated therein the nucleotide sequences described herein. The nucleotide construct can be used for transforming organisms such as plants. In this manner, plant promoters operably linked to a nucleotide sequence for a modified substrate protein of a pathogen-specific protease as described herein are provided in nucleic acid constructs for expression in a plant cell, plant part or plant.

[0068] As used herein, "expression cassette" means a nucleic acid molecule having at least a control sequence operably linked to a coding sequence.

[0069] As used herein, "operably linked" means that the elements of the expression cassette are configured so as to perform their usual function. Thus, control sequences (*i.e.*, promoters) operably linked to a coding sequence are capable of effecting expression of the coding sequence. The control sequences need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated, yet transcribed, sequences can be present between a promoter and a coding sequence, and the promoter sequence still can be considered "operably linked" to the coding sequence.

[0070] As used herein, a "coding sequence" or "coding sequences" means a sequence that encodes a particular polypeptide, and is a nucleotide sequence that is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at a 5' (amino) terminus and a translation stop codon at a 3' (carboxy) terminus. A coding sequence can include, but is not limited to, viral nucleic acid sequences, cDNA from prokaryotic or eukaryotic mRNA, genomic DNA sequences from prokaryotic or eukaryotic DNA, and even synthetic DNA sequences. A transcription termination sequence will usually be located 3' to the coding sequence. Examples of coding sequences for use herein include nucleotide sequence that encodes a modified substrate protein of a pathogen-specific protease, a NB-LRR protein or both.

[0071] As used herein, "control sequence" or "control sequences" means promoters, polyadenylation signals, transcription and translation termination sequences, upstream regulatory domains, origins of replication, internal ribosome entry sites ("IRES"), enhancers, and the like, which collectively provide for replication, transcription and translation of a coding sequence in a recipient host cell. Not all of these control sequences need always be present so long as the selected coding sequence is capable of being replicated, transcribed and translated in an appropriate host cell.

[0072] As used herein, a "promoter" means a nucleotide region comprising a nucleic acid (*i.e.*, DNA) regulatory sequence, wherein the regulatory sequence is derived from a gene or synthetically created that is capable of binding RNA polymerase and initiating transcription of a downstream (3'-direction) coding sequence. A number of promoters can be used in the expression cassette, including the native promoter of the modified substrate protein or NB-LRR protein.

[0073] Alternatively, promoters can be selected based upon a desired outcome. Such promoters include, but are not limited to, "constitutive promoters" (where expression of a polynucleotide sequence operably linked to the promoter is unregulated and therefore continuous), "inducible promoters" (where expression of a polynucleotide sequence operably linked to the promoter is induced by an analyte, cofactor, regulatory protein, *etc.*), and "repressible promoters" (where expression of a polynucleotide sequence operably linked to the promoter is repressed by an analyte, cofactor, regulatory protein, *etc.*).

[0074] As used herein, "plant promoter" means a promoter that drives expression in a plant such as a constitutive, inducible (*e.g.*, chemical-, environmental-, pathogen- or wound-inducible), repressible, tissue-preferred or other promoter for use in plants.

[0075] Examples of constitutive promoters include, but are not limited to, the rice actin 1 promoter (Wang *et al.* (1992) *Mol. Cell. Biol.* 12:3399-3406; and US Patent No. 5,641,876), the CaMV 19S promoter (Lawton *et al.* (1987) *Plant Mol. Biol.* 9:315-324), the CaMV 35S promoter (Odell *et al.* (1985) *Nature* 313:810-812), the nos promoter (Ebert *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:5754-5749), the Adh promoter (Walker *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:6624-6628), the sucrose synthase promoter (Yang & Russell (1990) *Proc. Natl. Acad. Sci. USA* 87:4144-4148), the ubiquitin promoters, and the like. *See also*, US Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; 5,608,142 and 6,177,611.

[0076] Examples of chemical-inducible promoters include, but are not limited to, the maize Tn2-2 promoter, which is activated by benzenesulfonamide herbicide safeners; the maize GST promoter, which is activated by hydrophobic electrophilic compounds that are used as pre-emergent herbicides; and the tobacco PR-1a promoter, which is activated by salicylic acid. Other chemical-inducible promoters of interest include steroid-responsive promoters (*e.g.*, the glucocorticoid-inducible promoters in Aoyama & Chua (1997) *Plant J.* 11:605-612; McNellis *et al.* (1998) *Plant J.* 14:247-257; and Schena *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:10421-10425); tetracycline-inducible and tetracycline-repressible promoters (Gatz *et al.* (1991) *Mol. Gen. Genet.* 227:229-237; as well as US Patent Nos. 5,814,618 and 5,789,156); ABA- and turgor-inducible promoters, the auxin-binding protein gene promoter (Schwob *et al.* (1993) *Plant J.* 4:423-432), the UDP glucose flavonoid glycosyl-transferase gene promoter (Ralston *et al.* (1988) *Genetics* 119:185-187), the MPI proteinase inhibitor promoter (Cordero *et al.* (1994) *Plant J.* 6:141-150), and the glyceraldehyde-3-phosphate dehydrogenase gene promoter (Kohler *et al.* (1995) *Plant Mol. Biol.* 29:1293-1298; Martinez *et al.* (1989) *J. Mol. Biol.* 208:551-565; and Quigley *et al.* (1989) *J. Mol. Evol.* 29:412-421). Also included are the benzene sulphonamide-inducible (US. Patent No. 5,364,780) and alcohol-inducible (Int'l Patent Application Publication Nos. WO 97/06269 and WO 97/06268) systems and glutathione S-transferase promoters. Chemical-inducible promoters therefore can be used to modulate the expression of a nucleotide sequence of interest in a plant by applying an exogenous chemical regulator. Depending upon the objective, the promoter can be a chemical-inducible promoter,

whereby application of the chemical induces gene expression, or a chemical-repressible promoter, whereby application of the chemical represses gene expression. *See also*, Gatz (1997) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48:89.

[0077] Other inducible promoters include promoters from genes inducibly regulated in response to environmental stress or stimuli such as drought, pathogens, salinity and wounds. *See*, Graham *et al.* (1985) *J. Biol. Chem.* 260:6555-6560; Graham *et al.* (1985) *J. Biol. Chem.* 260:6561-6564; and Smith *et al.* (1986) *Planta* 168:94-100. Wound-inducible promoters include the metallocarboxypeptidase-inhibitor protein promoter (Graham *et al.* (1981) *Biochem. Biophys. Res. Comm.* 101:1164-1170).

[0078] Examples of tissue-preferred promoters include, but are not limited to, the rbcS promoter, the ocs, nos and mas promoters that have higher activity in roots or wounded leaf tissue, a truncated (–90 to +8) 35S promoter that directs enhanced expression in roots, an α-tubulin gene promoter that directs expression in roots, as well as promoters derived from zein storage protein genes that direct expression in endosperm. Additional examples of tissue-preferred promoters include, but are not limited to, the promoters of genes encoding the seed storage proteins (*e.g.*, β-conglycinin, cruciferin, napin and phaseolin), zein or oil body proteins (*e.g.*, oleosin), or promoters of genes involved in fatty acid biosynthesis (*e.g.*, acyl carrier protein, stearoyl-ACP desaturase and fatty acid desaturases (*e.g.*, fad 2-1)), and promoters of other genes expressed during embryo development (*e.g.*, Bce4; Kridl *et al.* (1991) *Seed Sci. Res.* 1:209-219). Further examples of tissue-specific promoters include, but are not limited to, the lectin promoter (Lindstrom *et al.* (1990) *Dev. Genet.* 11:160-167; and Vodkin (1983) *Prog. Clin. Biol. Res.* 138:87-98), the corn alcohol dehydrogenase 1 promoter (Dennis *et al.* (1984) *Nucleic Acids Res.* 12:3983-4000; and Vogel *et al.* (1989) *J. Cell. Biochem.* 13:Part D, M350 (Abstract)), corn light harvesting complex (Bansal *et al.* (1992) *Proc. Natl. Acad. Sci.* USA 89:3654-3658; and Simpson (1986) *Science* 233:34-380), corn heat shock protein (Odell *et al.* (1985) *Nature* 313:810-812; and Rochester *et al.* (1986) *EMBO J.* 5:451-458), the pea small subunit RuBP carboxylase promoter (Cashmore, "Nuclear genes encoding the small subunit of ribulose-1,5-bisphosphate carboxylase" 29-38 In: Gen. Eng. of Plants (Plenum Press 1983); and Poulsen *et al.* (1986) *Mol. Gen. Genet.* 205:193-200), the Ti plasmid mannopine synthase promoter (Langridge *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:3219-3223), the Ti plasmid nopaline synthase promoter (Langridge *et al.* (1989), *supra*), the petunia chalcone isomerase promoter (van Tunen *et al.* (1988) *EMBO J.* 7:1257-1263), the bean glycine rich protein 1 promoter

(Keller *et al.* (1989) *Genes Dev.* 3:1639-1646), the truncated CaMV 35s promoter (Odell *et al.* (1985), *supra*), the potato patatin promoter (Wenzler *et al.* (1989) *Plant Mol. Biol.* 13:347-354), the root cell promoter (Yamamoto *et al.* (1990) *Nucleic Acids Res.* 18:7449), the maize zein promoter (Langridge *et al.* (1983) *Cell* 34:1015-1022; Kriz *et al.* (1987) *Mol. Gen. Genet.* 207:90-98; Reina *et al.* (1990) *Nucleic Acids Res.* 18:6425; Reina *et al.* (1990) *Nucleic Acids Res.* 18:7449; and Wandelt *et al.* (1989) *Nucleic Acids Res.* 17:2354), the globulin-1 gene (Belanger *et al.* (1991) *Genetics* 129:863-872), the α-tubulin, cab promoter (Sullivan *et al.* (1989) *Mol. Gen. Genet.* 215:431-440), the PEPCase promoter (Hudspeth & Grula (1989) *Plant Mol. Biol.* 12:579-589), the R gene complex-associated promoters (Chandler *et al.* (1989) *Plant Cell* 1:1175-1183), and the chalcone synthase promoters (Franken *et al.* (1991) *EMBO J.* 10:2605-2612). *See also*, Canevascini *et al.* (1996) *Plant Physiol.* 112:513-524; Guevara-Garcia *et al.* (1993) *Plant J.* 4:495-505; Hansen *et al.* (1997) *Mol. Gen. Genet.* 254:337-343; Kawamata *et al.* (1997) *Plant Cell Physiol.* 38:792-803; Lam (1994) *Results Probl. Cell Differ.* 20:181-196; Matsuoka *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:9586-9590; Orozco *et al.* (1993) *Plant Mol. Biol.* 23:1129-1138; Rinehart *et al.* (1996) *Plant Physiol.* 112:1331-1341; Russell *et al.* (1997) *Transgenic Res.* 6:157-168; Van Camp *et al.* (1996) *Plant Physiol.* 112:525-535; Yamamoto *et al.* (1994) *Plant Cell Physiol.* 35:773-778; and Yamamoto *et al.* (1997) *Plant J.* 12:255-265.

[0079] In some instances, the tissue-preferred promoter can be a leaf-preferred promoter. *See*, Gan *et al.* (1995) *Science* 270:1986-1988; Gotor *et al.* (1993) *Plant J.* 3:509-518; Kwon *et al.* (1994) *Plant Physiol.* 105:357-367; Matsuoka *et al.* (1993), *supra*; Orozco *et al.* (1993), *supra*; Yamamoto *et al.* (1994), *supra*; and Yamamoto *et al.* (1997), *supra*.

[0080] In some instances, the tissue-preferred promoter can be a root-preferred promoter. *See*, Capana *et al.* (1994) *Plant Mol. Biol.* 25:681-691 (rolB promoter); Hire *et al.* (1992) *Plant Mol. Biol.* 20:207-218 (soybean root-specific glutamine synthetase gene); Keller & Baumgartner (1991) *Plant Cell* 3:1051-1061 (root-specific control element in the GRP 1.8 gene of French bean); Kuster *et al.* (1995) *Plant Mol. Biol.* 29:759-772 (VfENOD-GRP3 gene promoter) Miao *et al.* (1991) *Plant Cell* 3:11-22 (full-length cDNA clone encoding cytosolic glutamine synthetase (GS), which is expressed in roots and root nodules of soybean); and Sanger *et al.* (1990) *Plant Mol. Biol.* 14:433-443 (root-specific promoter of the mannopine synthase (MAS) gene of *A. tumefaciens*); *see also*, US Patent Nos. 5,837,876; 5,750,386; 5,633,363; 5,459,252; 5,401,836; 5,110,732; and 5,023,179. Likewise, Bogusz *et al.* (1990) *Plant Cell* 2:633-641

describes two root-specific promoters isolated from hemoglobin genes from the nitrogen-fixing nonlegume *Parasponia andersonii* and the related non-nitrogen-fixing nonlegume *Trema tomentosa*. Leach & Aoyagi (1991) *Plant Sci.* 79:69-76 describes an analysis of the promoters of the highly expressed rolC and rolD root-inducing genes of *Agrobacterium rhizogenes*. Teeri *et al.* (1989) *EMBO J.* 8:343-335 describes a gene fusion to lacZ to show that the Agrobacterium T-DNA gene encoding octopine synthase is especially active in the epidermis of the root tip and that the TR2' gene is root specific in the intact plant and stimulated by wounding in leaf tissue.

[0081] In some instances, the tissue-preferred promoter can be a seed-preferred promoter, which includes both "seed-specific" promoters (*i.e.*, promoters active during seed development such as promoters of seed storage proteins) and "seed-germinating" promoters (*i.e.*, promoters active during seed germination). *See*, Thompson *et al.* (1989) *BioEssays* 10:108-113. Examples of seed-preferred promoters include, but are not limited to, the Cim1 promoter (cytokinin-induced message); the cZ19B1 promoter (maize 19 kDa zein); the myo-inositol-1-phosphate synthase (milps) promoter (Int'l Patent Application Publication No. WO 00/11177; and US Patent No. 6,225,529); the γ-zein promoter; and the globulin 1 (Glb-1) promoter. For monocots, seed-specific promoters include, but are not limited to, promoters from maize 15 kDa zein, 22 kDa zein, 27 kDa zein, γ-zein, waxy, shrunken 1, shrunken 2 and Glb-1. *See also*, Int'l Patent Application Publication No. WO 00/12733, which discloses seed-preferred promoters from end1 and end2 genes. For dicots, seed-specific promoters include, but are not limited to, promoters from bean β-phaseolin, napin, β-conglycinin, soybean lectin, cruciferin and pea vicilin (Czako *et al.* (1992) *Mol. Gen. Genet.* 235:33-40). *See also*, US Patent No. 5,625,136.

[0082] In some instances, the tissue-preferred promoter can be a stalk-preferred promoter. Examples of stalk-preferred promoters include, but are not limited to, the maize MS8-15 gene promoter (Int'l Patent Application Publication No. WO 98/00533; and US Patent No. 5,986,174), and the promoters disclosed in Graham *et al.* (1997) *Plant Mol. Biol.* 33:729-735.

[0083] In some instances, the tissue-preferred promoter can be a vascular tissue-preferred promoter. For example, a vascular tissue-preferred promoter can be used to express the modified substrate protein in polypexylem and phloem tissue. Examples of vascular tissue-preferred promoters include, but are not limited to, the *Prunus serotina* prunasin hydrolase gene promoter (Int'l Patent Application Publication No. WO 03/006651), and the promoters disclosed in US Patent No. 6,921,815.

[0084] As an alternative to the promoters listed above, in some instances a low level of expression is desired and can be achieved by using a weak promoter. As used herein, "weak promoter" means a promoter that drives expression of a coding sequence at a low level. As used herein, "low level" means at levels of about 1/1000 transcripts to about 1/100,000 transcripts to about 1/500,000 transcripts. Alternatively, it is recognized that weak promoter also encompasses promoters that are expressed in only a few cells and not in others to give a total low level of expression. Where a promoter is expressed at unacceptably high levels, portions of the promoter sequence can be deleted or modified to decrease expression levels.

[0085] Examples of weak constitutive promoters include, but are not limited to, the core promoter of the Rsyn7 promoter (Int'l Patent Application Publication No. WO 99/43838 and US Patent No. 6,072,050), the core 35S CaMV promoter, and the like. Other exemplary weak constitutive promoters are described, for example, in US Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; 5,608,142 and 6,177,611.

[0086] Weak promoters can be used when designing expression cassettes for NB-LRR proteins, as NB-LRR genes preferably are constitutively expressed at low levels because high levels can lead to cell death in the absence of pathogens.

[0087] The expression cassette can include other control sequences 5' to the coding sequence. For example, the expression cassette can include a 5' leader sequence, which can act to enhance translation. Examples of 5' leader sequences include, but are not limited to, picornavirus leaders (*e.g.*, encephalomyocarditis virus (EMCV) leader; Elroy-Stein *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:6126-6130); potyvirus leaders (*e.g.*, tobacco etch virus (TEV) leader; Gallie *et al.* (1995) *Gene* 165:233-238); maize dwarf mosaic virus (MDMV) leader (Allison *et al.* (1986) *Virology* 154:9-20); human immunoglobulin heavy-chain binding protein (BiP; Macejak *et al.* (1991) *Nature* 353:90-94); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 94; Jobling *et al.* (1987) *Nature* 325:622-625); tobacco mosaic virus (TMV) leader (Gallie *et al.*, "Eukaryotic viral 5'-leader sequences act as translational enhancers in eukaryotes and prokaryotes" 237-256 In: Molecular Biology of RNA (Cech ed., Liss 1989)); and maize chlorotic mottle virus (MCMV) leader (Lommel *et al.* (1991) *Virology* 81:382-385). *See also*, Della-Cioppa *et al.* (1987) *Plant Physiol.* 84:965-968; and Gallie (1996) *Plant Mol. Biol.* 32:145-158. Other methods or sequences known to enhance translation also can be used, for example, introns, and the like.

[0088] The expression cassette also can include a coding sequence for the modified substrate protein of the pathogen-specific protease and/or NB-LRR protein. As discussed above, the modified substrate protein includes a heterologous protease recognition sequence. The heterologous protease recognition sequence can be located within, for example, an exposed loop of the substrate protein. As noted above, nucleic and amino acid sequences are well known in the art for many protease recognition sequences that can be inserted into the substrate protein such as PBS1. In addition, nucleic and amino acid sequences are known in the art for various NB-LRR proteins. These sequences can be used when constructing the expression cassette(s).

[0089] For example, the coding sequence can be SEQ ID NO:9 (modified PBS1 having an AvrRpt2 protease recognition sequence) operably linked to the native PBS1 promoter. Likewise, the coding sequence can include a NB-LRR protein such as RPS5 when the modified substrate protein is based upon PBS1.

[0090] The control sequence(s) and/or the coding sequence therefore can be native/analogous to the host cell or to each other. Alternatively, the control sequence(s) and/or coding sequence can be heterologous to the host cell or to each other. As used herein, "heterologous" means a sequence that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. For example, a promoter operably linked to a heterologous polynucleotide is from a species different from the species from which the polynucleotide was derived, or, if from the same/analogous species, one or both are substantially modified from their original form and/or genomic locus, or the promoter is not the native promoter for the operably linked polynucleotide.

[0091] The expression cassette also can include a transcriptional and/or translational termination region that is functional in plants. The termination region can be native with the transcriptional initiation region (*i.e.*, promoter), can be native with the operably linked coding sequence, can be native with the plant of interest, or can be derived from another source (*i.e.*, foreign or heterologous to the promoter, the coding sequence, the plant host cell, or any combination thereof). Termination regions are typically located downstream (3'-direction) from the coding sequence. Termination regions include, but are not limited to, the potato proteinase inhibitor (PinII) gene or the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. *See e.g.*, Ballas *et al.* (1989) *Nucleic Acids Res.* 17:7891-7903; Guerineau *et al.* (1991) *Mol. Gen. Genet.* 262:141-144; Joshi *et al.* (1987) *Nucleic Acid*

Res. 15:9627-9639; Mogen *et al.* (1990) *Plant Cell* 2:1261-1272; Munroe *et al.* (1990) *Gene* 91:151-158; Proudfoot (1991) *Cell* 64:671-674; and Sanfacon *et al.* (1991) *Genes Dev.* 5:141-149.

[0092] The expression cassette also can include one or more linkers. As used herein, "linker" means a nucleotide sequence that functions to link one element of the expression cassette with another without otherwise contributing to the transcription or translation of a nucleotide sequence of interest when present in the expression cassette. The linker can include plasmid sequences, restriction sequences and/or sequences of a 5'-untranslated region (5'-UTR). Alternatively, the linker further can include nucleotide sequences encoding the additional amino acid residues that naturally flank the heterologous protease recognition sequence in the substrate protein from which it was isolated. The length and sequence of the linker can vary and can be about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000 nucleotides or greater in length.

[0093] Just as expression of the modified substrate protein and/or NB-LRR protein can be targeted to specific tissues or cell types by appropriate use of promoters, it also can be targeted to different locations within a cell of a plant host by appropriate use of signal and/or targeting peptide sequences. Unlike a promoter, which acts at the transcriptional level, signal and/or targeting peptide sequences are part of the initial translation product. Therefore, the expression cassette also can include a signal and/or targeting peptide sequence. Examples of such sequences include, but are not limited to, the transit peptide for the acyl carrier protein, the small subunit of RUBISCO, plant EPSP synthase, and the like. *See*, Archer *et al.* (1990) *J. Bioenerg. Biomemb.* 22:789-810; Clark *et al.* (1989) *J. Biol. Chem.* 264:17544-17550; Daniell (1999) *Nat. Biotech.* 17:855-856; de Castro Silva Filho *et al.* (1996) *Plant Mol. Biol.* 30:769-780; Della-Cioppa *et al.* (1987) *Plant Physiol.* 84:965-968; Lamppa *et al.* (1988) *J. Biol. Chem.* 263:14996-14999; Lawrence *et al.* (1997) *J. Biol. Chem.* 272:20357-20363; Romer *et al.* (1993) *Biochem. Biophys. Res. Commun.* 196:1414-1421; Schmidt *et al.* (1993) *J. Biol. Chem.* 268:27447-27457; Schnell *et al.* (1991) *J. Biol. Chem.* 266:3335-3342; Shah *et al.* (1986) *Science* 233:478-481; Von Heijne *et al.* (1991) *Plant Mol. Biol. Rep.* 9:104-126; and Zhao *et al.* (1995) *J. Biol. Chem.* 270:6081-6087; as well as US Patent No. 6,338,168.

[0094] It may be desirable to locate the modified substrate protein and/or NB-LRR protein on specific plant membranes such as the plasma membrane or tonoplast membrane. This can be

accomplished, for example, by adding specific amino acid sequences to the N-terminus of these proteins by adding specific sequences to the expression cassette as described in Raikhel & Chrispeels, "Protein sorting and vesicle traffic" In: Biochemistry and Molecular Biology of Plants (Buchanan *et al.* eds., American Society of Plant Physiologists 2000). *See also*, Denecke *et al.* (1992) *EMBO J.* 11:2345-2355; Denecke *et al.* (1993) *J. Exp. Bot.* 44:213-221; Gomord *et al.* (1996) *Plant Physiol. Biochem.* 34:165-181; Lehmann *et al.* (2001) *Plant Physiol.* 127:436-449; Munro & Pelham (1986) *Cell* 46:291-300; Munro & Pelham (1987) *Cell* 48:899-907; Vitale *et al.* (1993) *J. Exp. Bot.* 44:1417-1444; and Wandelt *et al.* (1992) *Plant J.* 2:181-192.

[0095] Additional guidance on subcellular targeting of proteins in plants can be found, for example, in Bruce (2001) *Biochim Biophys Acta* 1541:2-21; Emanuelsson *et al.* (2000) *J. Mol. Biol.* 300:1005-1016; Emanuelsson & von Heijne (2001) *Biochim Biophys Acta* 1541:114-119; Hadlington & Denecke (2000) *Curr. Opin. Plant Biol.* 3:461-468; Nicchitta (2002) *Curr. Opin. Cell Biol.* 14:412-416; and Silva-Filho (2003) *Curr. Opin. Plant Biol.* 6:589-595.

[0096] The expression cassette also can include nucleotide sequences encoding agronomic and pesticidal polypeptides, and the like. Such sequences can be stacked with any combination of nucleotide sequences to create plant cells, plants parts and plants with a desired phenotype. For example, the nucleic acid molecule encoding modified substrate protein and/or NB-LRR protein can be stacked with nucleotide sequences encoding a pesticidal polypeptide such as a δ-endotoxin. The combinations generated also can include multiple copies of any one of the nucleotide sequences of interest. Examples of other nucleotide sequences of interest include, but are not limited to, sequences encoding for high oil (US Patent No. 6,232,529); balanced amino acids (hordothionins; US Patent Nos. 5,703,409; 5,885,801; 5,885,802 and 5,990,389); barley high lysine (Williamson *et al.* (1987) *Eur. J. Biochem.* 165:99-106; and Int'l Patent Application Publication No. WO 98/20122); high methionine proteins (Pedersen *et al.* (1986) *J. Biol. Chem.* 261:6279-6284; Kirihara *et al.* (1988) *Gene* 71:359-370; and Musumura *et al.* (1989) *Plant Mol. Biol.* 12:123-130); increased digestibility (modified storage proteins; US Patent No. 6,858,778); and thioredoxins (US Patent No. 7,009,087).

[0097] The nucleotide sequence encoding the modified substrate protein and/or NB-LRR disease resistance protein also can be stacked with nucleotide sequences encoding polypeptides for herbicide resistance (*e.g.*, glyphosate or HPPD resistance; *see*, *e.g.*, EPSPS genes, GAT genes (Int'l Patent Application Publication Nos. WO 02/36782 and WO 03/092360; and US Patent Application Publication No. 2004/0082770); lectins (Van Damme *et al.* (1994) *Plant Mol. Biol.*

24:825-830); fumonisin detoxification (US Patent No. 5,792,931); acetolactate synthase (ALS) mutants that lead to herbicide resistance such as the S4 and/or Hra mutations; inhibitors of glutamine synthase such as phosphinothricin or basta (*e.g.*, bar gene); modified starches (ADPG pyrophosphorylases (AGPase), starch synthases (SS), starch branching enzymes (SBE) and starch debranching enzymes (SDBE)); and polymers or bioplastics (US Patent No. 5,602,321); beta-ketothiolase, polyhydroxybutyrate synthase and acetoacetyl-CoA reductase (Schubert *et al.* (1988) *J. Bacteriol.* 170:5837-5847).

[0098] The nucleotide sequence encoding the modified substrate protein and/or NB-LRR disease resistance protein also can be stacked with nucleotide sequences encoding for agronomic traits such as male sterility (US Patent No. 5,583,210), stalk strength, flowering time or transformation technology traits such as cell cycle regulation or gene targeting (Int'l Patent Application Publication Nos. and WO 99/25821; WO 99/61619 and WO 00/17364).

[0099] These stacked combinations can be created by any method including, but not limited, to cross breeding plants by any conventional or TOPCROSS™ methodology (DuPont Specialty Grains; Des Moines, IA), zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) or other genetic transformation. If the traits are stacked by genetically transforming the plants, the nucleotide sequences of interest can be combined at any time and in any order. For example, a transgenic plant comprising one or more desired traits can be used as the target to introduce further traits by subsequent transformation. The traits can be introduced simultaneously in a co-transformation protocol with the polynucleotides of interest provided by any combination of transformation cassettes. For example, if two sequences will be introduced, the two sequences can be contained in separate expression cassettes (trans) or contained on the same transformation cassette (cis). Expression of the sequences can be driven by the same promoter or by different promoters. In certain instances, it may be desirable to introduce an expression cassette that will suppress the expression of the polynucleotide of interest. This may be combined with any combination of other suppression cassettes or overexpression cassettes to generate the desired combination of traits in the plant. It is further recognized that polynucleotide sequences can be stacked at a desired genomic location using a site-specific recombination system. *See*, Int'l Patent Application Publication Nos. WO 99/25821; WO 99/25840; WO 99/25853; WO 99/25854 and WO 99/25855.

[0100] In addition to the above, it is contemplated that the nucleic acid constructs can be used in the form of a system, particularly when used in plant cells, plant parts and plants that

lack a substrate protein of a pathogen-specific protease and NB-LRR protein pair. Such systems can include one or more nucleic acid constructs, such as expression cassettes or vectors, having a promoter that drives expression in a plant, plant part or plant cell operably linked to a coding sequence for a modified substrate protein of a pathogen-specific protease, where the substrate protein has a heterologous protease recognition sequence, and a sequence for a promoter that drives expression in a plant, plant part or plant cell operably linked to a coding sequence for a NB-LRR protein. The promoters can be the same or can be distinct. For example, the first promoter can be an inducible promoter and the second promoter can be a constitutive promoter, especially a weak constitutive promoter. Alternatively, both the first and second promoters can be inducible, repressible or constitutive. The NB-LRR protein can associate with, and can be activated by, the modified substrate. Such systems therefore can be used to provide the protein pair to a plant cell, plant part or plant that does not natively express the protein pair.

[0101] Alternatively, the system can include a first nucleic acid construct having nucleotide sequence for a promoter that drives expression in a plant cell, plant part or plant operably linked to a coding sequence for a modified substrate protein of a pathogen-specific protease as described herein, and a second nucleic acid construct having a nucleotide sequence for a promoter that drives expression in a plant cell, plant part or plant operably linked to a coding sequence for a NB-LRR protein.

[0102] Additional nucleic acid constructs also can be included in the system, where each construct has a nucleotide sequence that encodes a distinct modified substrate protein, each having a heterologous recognition sequence for a separate pathogen-specific protease. Although each modified substrate protein has a heterologous recognition sequence distinct from one another, each can associate with, and can activate, the NB-LRR protein.

[0103] Regardless of whether used as individual nucleic acid constructs or systems, and where appropriate, the nucleotide sequences can be optimized for increased expression in plants. That is, the nucleotide sequences can be synthesized using plant-preferred codons for improved expression. Methods for optimizing nucleotide sequences for expression in plants are well known in the art. *See*, Campbell & Gowri (1990) *Plant Physiol.* 92:1-11; Murray *et al.* (1989) *Nucleic Acids Res.* 17:477-498; and Wada *et al.* (1990) *Nucl. Acids Res.* 18:2367-2411; as well as US Patent Nos. 5,096,825; 5,380,831; 5,436,391; 5,625,136; 5,670,356 and 5,874,304.

[0104] Likewise, additional sequence modifications are known to enhance nucleotide sequence expression in plants. These include elimination of sequences encoding spurious polyadenylation signals, exon-intron splice site signals, transposon-like repeats, and other such well-characterized sequences that may be deleterious to gene expression. The G-C content of the sequence can be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host plant. When possible, the nucleotide sequence can be modified to avoid predicted hairpin secondary mRNA structures.

[0105] Suitable methods of constructing expression cassettes are well known in the art and can be found, for example, in Balbás & Lorence, *Recombinant Gene Expression: Reviews and Protocols*, 2nd ed. (Humana Press 2004); Davis *et al.*, *Basic Methods in Molecular Biology* (Elsevier Press 1986); Sambrook & Russell (2001), *supra*; Tijssen, *Laboratory Techniques in Biochemistry and Molecular Biology – Hybridization with Nucleic Acid Probes* (Elsevier 1993); Ausubel *et al.*(1995), *supra*; as well as US Patent Nos. 6,664,387; 7,060,491; 7,345,216 and 7,494,805.

[0106] The expression cassette therefore can include at least, in the direction of transcription (*i.e.*, 5' to 3' direction), a plant promoter that is functional in a plant cell, plant part or plant operably linked to a nucleotide sequence encoding a modified substrate protein having a heterologous protease recognition sequence. In some instances, the expression cassette also can include a nucleotide sequence encoding a NB-LRR disease resistance protein.

[0107] To assist in introducing the nucleotide sequences of interest into the appropriate host cells, the expression cassette can be incorporated or ligated into a vector. As used herein, "vector" means a replicon, such as a plasmid, phage or cosmid, to which another nucleic acid segment may be attached so as to bring about the replication of the attached segment. A vector is capable of transferring nucleic acid molecules to the host cells. Bacterial vectors typically can be of plasmid or phage origin.

[0108] Typically, the terms "vector construct," "expression vector," "gene expression vector," "gene delivery vector," "gene transfer vector," and "expression cassette" all refer to an assembly that is capable of directing the expression of a sequence or gene of interest. Thus, the terms include cloning and expression vehicles.

[0109] Vectors typically contain one or a small number of restriction endonuclease recognition sites where a nucleic acid molecule of interest can be inserted in a determinable

fashion without loss of essential biological function of the vector, as well as a selectable marker that can be used for identifying and selecting cells transformed with the vector.

[0110] A vector therefore can be capable of transferring nucleic acid molecule to target cells (*e.g.*, bacterial plasmid vectors, particulate carriers and liposomes). The selection of vector will depend upon the preferred transformation technique and the target species for transformation. The most commonly used plant transformation vectors are binary vectors because of their ability to replicate in intermediate host cells such as *E. coli* and *A. tumefaciens*. The intermediate host cells allow one to increase the copy number of the cloning vector and/or to mediate transformation of a different host cell. With an increased copy number, the vector containing the expression cassette of interest can be isolated in significant quantities for introduction into the desired plant. General descriptions of plant vectors can be found, for example, in Gruber *et al.*, "Vectors for plant transformation" 89-119 In: Methods in Plant Molecular Biology & Biotechnology (Glich *et al.* eds., CRC Press 1993). Examples of vectors for use with *A. tumefaciens* can be found, for example, in US Patent No. 7,102,057.

[0111] Restriction enzymes can be used to introduce cuts into the target nucleic acid molecule (*e.g.*, nucleotide sequence encoding a modified substrate protein and/or NB-LRR protein) and the plasmid to facilitate insertion of the target into the vector such as a plasmid. Moreover, restriction enzyme adapters such as EcoRI/NotI adapters can be added to the target mRNA when the desired restriction enzyme sites are not present within it. Methods of adding restriction enzyme adapters are well known in the art. *See*, Krebs *et al.* (2006) *Anal. Biochem.* 350:313-315; and Lönneborg *et al.* (1995), *supra*. Likewise, kits for adding restriction enzyme sites are commercially available, for example, from Invitrogen (Carlsbad, CA).

[0112] Alternatively, viruses such as bacteriophages can be used as the vector to deliver the target mRNA to competent host cells. Vectors can be constructed using standard molecular biology techniques as described, for example, in Sambrook & Russell (2001), *supra*.

[0113] As noted above, selectable markers can be used to identify and select transformed plants, plant parts or plant host cells. Selectable markers include, but are not limited to, nucleotide sequences encoding antibiotic resistance, such as those encoding neomycin phosphotransferase II (NEO), hygromycin phosphotransferase (HPT), as well as nucleotide sequences encoding resistance to ampicillin, kanamycin, spectinomycin or tetracycline, and even

nucleotide sequences encoding herbicidal compounds such as glufosinate ammonium, bromoxynil, imidazolinones and 2,4-dichlorophenoxyacetate (2,4-D).

[0114] Additional selectable markers can include phenotypic markers such as nucleic acid sequences encoding β-galactosidase, β-glucoronidase (GUS; Jefferson (1987) *Plant Mol. Biol. Rep.* 5:387-405); luciferase (Teeri *et al.* (1989) *EMBO J.* 8:343-350); anthocyanin production (Ludwig *et al.* (1990) *Science* 247:449-450), and fluorescent proteins such as green fluorescent protein (GFP; Chalfie *et al.* (1994) *Science* 263:802-805; Fetter *et al.* (2004) *Plant Cell* 16:215-228; and Su *et al.* (2004) *Biotechnol. Bioeng.* 85:610-619); cyan fluorescent protein (CYP; Bolte *et al.* (2004) *J. Cell Science* 117:943-954; and Kato *et al.* (2002) *Plant Physiol.* 129:913-942), and yellow fluorescent protein (PhiYFP™, available from Evrogen (Moscow, Russia); Bolte *et al.* (2004) *J. Cell Science* 117:943-954). For additional selectable markers, Baim *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:5072-5076; Barkley & Bourgeois, "Repressor recognition of operator and effectors" 177-120 In: The Operon (Miller & Reznikoff eds., Cold Spring Harbor Laboratory Press 1980); Bonin (1993) Ph.D. Thesis, University of Heidelberg; Brown *et al.* (1987) *Cell* 49:603-612; Christopherson *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:6314-6318; Degenkolb *et al.* (1991) *Antimicrob. Agents Chemother.* 35:1591-1595; Deuschle *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:5400-5404; Deuschle *et al.* (1990) *Science* 248:480-483; Figge *et al.* (1988) *Cell* 52:713-722; Fuerst *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:2549-2553; Gill *et al.* (1988) *Nature* 334:721-724; Gossen *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:5547-5551; Gossen (1993) Ph.D. Thesis, University of Heidelberg; Hillenand-Wissman (1989) *Topics Mol. Struc. Biol.* 10:143-162; Hlavka *et al.*, *Handbook of Experimental Pharmacology*, Vol. 78 (Springer-Verlag 1985); Hu *et al.* (1987) *Cell* 48:555-566; Kleinschnidt *et al.* (1988) *Biochemistry* 27:1094-1104; Labow *et al.* (1990) *Mol. Cell. Biol.* 10:3343-3356; Oliva *et al.* (1992) *Antimicrob. Agents Chemother.* 36:913-919; Reines *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:1917-1921; Reznikoff (1992) *Mol. Microbiol.* 6:2419-2422; Yao *et al.* (1992) *Cell* 71:63-72; Yarranton (1992) *Curr. Opin. Biotech.* 3:506-511; Wyborski *et al.* (1991) *Nucleic Acids Res.* 19:4647-4653; and Zambretti *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:3952-3956. The above list of selectable markers is not intended to be limiting, as any selectable marker can be used.

[0115] The vector therefore can be selected to allow introduction of the expression cassette into the appropriate host cell such as a plant host cell. Bacterial vectors are typically of plasmid or phage origin. Appropriate bacterial cells are infected with phage vector particles or transfected

with naked phage vector DNA. If a plasmid vector is used, the cells are transfected with the plasmid vector DNA.

[0116] The present disclosure therefore includes nucleotide constructs such as expression cassettes and vectors having a nucleotide sequence encoding a modified substrate protein of a pathogen-specific protease and a heterologous protease recognition sequence. In addition, the nucleic acid constructs can include a nucleotide sequence encoding a NB-LRR protein. The nucleic acid constructs can be introduced into an organism such as a plant to confer resistance to plant pathogens expressing specific proteases.

Recombinant Peptides, Polypeptides and Proteins

[0117] Compositions of the present disclosure also include isolated or purified, modified substrate proteins of a pathogen-specific protease, where the substrate proteins have heterologous protease recognition sequences, as well as fragments and/or variants thereof. Methods for producing peptide, polypeptides and proteins in plant cells, plant parts and plants are discussed elsewhere herein.

[0118] Methods of isolating or purifying peptides, polypeptides and proteins are well known in the art. *See*, Ehle & Horn (1990) *Bioseparation* 1:97-110; Hengen (1995) T*rends Biochem Sci.* 20:285-286; *Basic Methods in Protein Purification and Analysis: A Laboratory Manual* (Simpson *et al.* eds., Cold Spring Harbor Laboratory Press 2008); Regnier (1983) *Science* 222:245-252; Shaw, "Peptide purification by reverse-phase HPLC" 257-287 In: Methods in Molecular Biology, Vol. 32 (Walker ed., Humana Press 1994); as well as US Patent Application Publication No. 2009/0239262; and US Patent Nos. 5,612,454; 7,083,948; 7,122,641; 7,220,356 and 7,476,722.

[0119] As used herein, "peptide," "polypeptide" and "protein" are used interchangeably to mean a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residues is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers.

[0120] As used herein, "residue," "amino acid residue" and "amino acid" are used interchangeably to mean an amino acid that is incorporated into a molecule such as a peptide, polypeptide or protein. The amino acid can be a naturally occurring amino acid and, unless

otherwise limited, may encompass known analogues of natural amino acids that can function in a similar manner as naturally occurring amino acids.

[0121] As used herein, "recombinant," when used in connection with a peptide, polypeptide or protein, means a molecule that has been created or modified through deliberate human intervention such as by protein engineering. For example, a recombinant polypeptide is one having an amino acid sequence that has been modified to include an artificial amino acid sequence or to include some other amino acid sequence that is not present within its native/endogenous/non-recombinant form.

[0122] Further, a recombinant peptide, polypeptide or protein has a structure that is not identical to that of any naturally occurring peptide, polypeptide or protein. As such, a recombinant peptide, polypeptide or protein can be prepared by synthetic methods such as those known to one of skill in the art.

[0123] If, and when, modified substrate proteins are to be isolated, complete purification is not required. For example, the modified substrate proteins described herein can be isolated and purified from normally associated material in conventional ways, such that in the purified preparation, the proteins are the predominant species in the preparation. At the very least, the degree of purification is such that extraneous material in the preparation does not interfere with use of the proteins in the manner disclosed herein. The peptide, polypeptide or protein can be at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% pure. Alternatively stated, the polypeptide is substantially free of cellular material such that preparations of the polypeptide can contain less than 30%, 25%, 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% (dry weight) of contaminating protein. When the polypeptide or an active variant or fragment thereof is recombinantly produced, culture medium represents less than 30%, 25%, 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% (dry weight) of chemical precursors or non-protein-of-interest chemicals.

[0124] It is known in the art that amino acids within the same conservative group can typically substitute for one another without substantially affecting the function of a protein. For the purpose of the present disclosure, such conservative groups are set forth in Table 2 and are based on shared properties. *See also*, Alberts *et al.*, "Small molecules, energy, and biosynthesis" 56-57 In: Molecular Biology of the Cell (Garland Publishing Inc. 3rd ed. 1994).

Table 2. Amino Acid Conservative Substitutions.

Residue	Side Chain Polarity	Side Chain pH	Hydropathy Index	Preferred Conservative Substitution
Ala (A)	Non-polar	Neutral	1.8	Ser
Arg (R)	Polar	Basic (strongly)	-4.5	Lys, Gln
Asn (N)	Polar	Neutral	-3.5	Gln, His
Asp (D)	Polar	Acidic	-3.5	Glu
Cys (C)	Non-polar	Neutral	2.5	Ser
Gln (Q)	Polar	Neutral	-3.5	Asn, Lys
Glu (E)	Polar	Acidic	-3.5	Asp
Gly (G)	Non-polar	Neutral	-0.4	Pro
His (H)	Polar	Basic (weakly)	-3.2	Asn, Gln
Ile (I)	Non-polar	Neutral	4.5	Leu, Val
Leu (L)	Non-polar	Neutral	3.8	Ile, Val
Lys (K)	Polar	Basic	-3.9	Arg, Gln
Met (M)	Non-polar	Neutral	1.9	Leu, Ile
Phe (F)	Non-polar	Neutral	2.8	Met, Leu, Tyr
Pro (P)	Non-polar	Neutral	-1.6	Gly
Ser (S)	Polar	Neutral	-0.8	Thr
Thr (T)	Polar	Neutral	-0.7	Ser
Trp (W)	Non-polar	Neutral	-0.9	Tyr
Tyr (Y)	Polar	Neutral	-1.3	Trp, Phe
Val (V)	Non-polar	Neutral	4.2	Ile, Leu

[0125] The following six groups each contain amino acids that are typical, but not necessarily exclusive, conservative substitutions for one another: 1. Alanine (A), Serine (S), Threonine (T); 2. Aspartic acid (D), Glutamic acid (E); 3. Asparagine (N), Glutamine (Q); 4. Arginine (R), Lysine (K); 5. Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and 6. Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

[0126] Substantial changes in function of a peptide, polypeptide or protein can be made by selecting substitutions that are less conservative than those listed in the table above, that is, by selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of substitution, (b) the charge or hydrophobicity of the polypeptide at the target site, or (c) the bulk of a side chain. The substitutions that in general can be expected to produce the greatest changes in the polypeptide's properties will be those in which (a) a hydrophilic residue, for example, seryl or threonyl, is substituted by a hydrophobic residue, for example, leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted by any other residue; (c) a residue having an electropositive side chain, for example,

lysyl, arginyl or histidyl, is substituted by an electronegative side chain, for example, glutamyl or aspartyl; (d) a residue having a bulky side chain, for example, phenylalanyl, is substituted by a residue not having a side chain, for example, glycyl; or (e) by increasing the number of sulfation or glycosylation.

[0127] In one aspect, the present disclosure is directed to an isolated polypeptide encoded by the recombinant nucleic acid molecule comprising about 90% identity to an amino acid sequence selected from SEQ ID NO:10, SEQ ID NO:12, and SEQ ID NO:14, wherein the polypeptide is a substrate protein of a plant pathogen-specific protease. In another embodiment, the isolated polypeptide can comprise about 95% identity to an amino acid sequence selected from SEQ ID NO:10, SEQ ID NO:12, and SEQ ID NO:14, wherein the polypeptide is a substrate protein of a plant pathogen-specific protease. In other embodiments, the isolated polypeptide can comprise about 96% identity, about 97% identity, about 98% identity, about 99% identity, and even 100% identity to an amino acid sequence selected from SEQ ID NO:10, SEQ ID NO:12, and SEQ ID NO:14, wherein the polypeptide is a substrate protein of a plant pathogen-specific protease.

[0128] An example of a fusion protein (that is, a modified substrate protein of a pathogen-specific protease) therefore includes that of SEQ ID NO:10, SEQ ID NO:12, or SEQ ID NO:14, and including polypeptides comprising about 95% identity, about 96% identity, about 97% identity, about 98% identity, about 99% identity and even 100% identity to an amino acid sequence selected from SEQ ID NO:10, SEQ ID NO:12, and SEQ ID NO:14.

[0129] In addition to the full-length amino acid sequence of the modified substrate protein of the pathogen-specific protease, it is intended that the modified substrate protein can be a fragment or variant thereof that is capable of being recognized by the plant pathogen protease and/or its corresponding NB-LRR protein. For amino acid sequences, "fragment" means a portion of the amino acid sequence of a reference polypeptide or protein. Fragments of an amino acid sequence may retain the biological activity of the reference polypeptide or protein. For example, less than the entire amino acid sequence of the modified substrate protein can be used and may have substrate protein activity and/or NB-LRR protein binding activity. Thus, fragments of the reference polypeptide or protein can be at least 150, 200, 250, 300, 350, 400 or 450 amino acid residues, or up to the number of amino acid residues present in a full-length modified substrate protein. For example, about 80 amino acids can be deleted from the N-terminus of PBS1 while retaining function. *See*, DeYoung *et al.* (2012), *supra*. Alternatively, about 100 amino acids can be deleted from the C-terminus of PBS1 while retaining function. *Id.*

[0130] Likewise, a "variant" peptide, polypeptide or protein means a substantially similar amino acid sequence to the amino acid sequence of a reference peptide, polypeptide or protein. For amino acid sequences, a variant comprises an amino acid sequence derived from a reference peptide, polypeptide or protein by deletion (so-called truncation) of one or more amino acids at the N-terminal and/or C-terminal end of the amino acid sequence of the reference; deletion and/or addition of one or more amino acids at one or more internal sites in the amino acid sequence of the reference; or substitution of one or more amino acids at one or more sites in the amino acid sequence of the reference. Variant peptides, polypeptides or proteins encompassed by the present disclosure are biologically active, that is, they continue to possess the desired biological activity of the reference peptide, polypeptide or protein as described herein. Such variants may result from, for example, genetic polymorphism or human manipulation. Biologically active variants will have at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the amino acid sequence of the reference peptide polypeptide or protein as determined by sequence alignment programs and parameters described above. For example, a biologically active variant of a modified substrate protein may differ by as few as 1-15 amino acid residues, as few as 1-10, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue.

[0131] Deletions, insertions and substitutions of the modified substrate proteins are not expected to produce radical changes in the characteristics of the polypeptides. However, when it is difficult to predict the exact effect of the substitution, deletion or insertion in advance of doing so, one of skill in the art will appreciate that the effect can be evaluated by routine activity assays as described herein.

[0132] As above, variant peptides, polypeptides and proteins also encompass sequences derived from a mutagenic and recombinogenic procedure such as DNA shuffling. With such a procedure, one or more nucleic acid molecules can be manipulated to encode new modified substrate proteins possessing the desired properties. In this manner, libraries of recombinant nucleic acid molecules can be generated from a population of related nucleic acid molecules comprising sequence regions that have substantial sequence identity and can be homologously recombined *in vitro* or *in vivo*. For example, using this approach, sequence motifs encoding a domain of interest can be shuffled between the nucleic acid molecules identified by the methods described herein and other known substrate protein-encoding nucleic acid molecules to obtain a new nucleic acid molecule that encodes a modified substrate protein with an improved property

such as increased activity or an expanded pH or temperature range. As such, a peptide, polypeptide or protein of the present disclosure can have many modifications.

[0133] The present disclosure therefore includes recombinant modified substrate proteins/fusion proteins, where the substrate proteins have heterologous protease recognition sequences, as well as active fragments or variants thereof.

Transformed Plant Cells, Plant Parts and Plants

[0134] Compositions of the present disclosure also include transformed plant cells, plant parts and plants (*i.e.*, subject plant cells, plant parts or plants) having a resistance to an increased number of plant pathogens when compared with control/native plant cells, plant parts or plants.

[0135] The transformed plant cells, plant parts or plants can have at least one nucleic acid molecule, nucleic acid construct, expression cassette or vector as described herein that encodes a modified substrate protein of a pathogen-specific protease, where the modified substrate protein has a heterologous protease recognition sequence.

[0136] As used herein, "subject plant cell," "subject plant part" or "subject plant" means one in which a genetic alteration, such as transformation, has been effected as to a nucleic acid molecule of interest, or is a plant cell, plant part or plant that descended from a plant cell, plant part or plant so altered and that comprises the alteration.

[0137] As used herein, "control plant cell," "control plant part" or "control plant" means a reference point for measuring changes in phenotype of the subject plant cell, plant part or plant. A control plant cell, plant part or plant can comprise, for example: (a) a wild-type plant cell, plant part or plant (*i.e.*, of the same genotype as the starting material for the genetic alteration that resulted in the subject plant cell, plant part or plant); (b) a plant cell, plant part or plant of the same genotype as the starting material, but which has been transformed with a null construct (*i.e.*, with a construct that has no known effect on the trait of interest, such as a construct comprising a marker gene); (c) a plant cell, plant part or plant that is a non-transformed segregant among progeny of a subject plant cell, plant part or plant; (d) a plant cell, plant part or plant genetically identical to the subject plant cell, plant part or plant, but which is not exposed to conditions or stimuli that would induce expression of the gene of interest; or (e) the subject plant cell, plant part or plant itself, under conditions in which the nucleic acid molecule/construct of interest is not expressed.

[0138] Methods of introducing nucleotide sequences into plants, plant parts or plant host cells are well known in the art and are discussed in greater detail below.

[0139] As used herein, "plant cell" or "plant cells" means a cell obtained from or found in seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen and microspores. Plant cell also includes modified cells, such as protoplasts, obtained from the aforementioned tissues, as well as plant cell tissue cultures from which plants can be regenerated, plant calli and plant clumps.

[0140] As used herein, "plant part" or "plant parts" means organs such as embryos, pollen, ovules, seeds, flowers, kernels, ears, cobs, leaves, husks, stalks, stems, roots, root tips, anthers, silk and the like.

[0141] As used herein, "plant" or "plants" means whole plants and their progeny. Progeny, variants and mutants of the regenerated plants also are included, provided that they comprise the introduced nucleic acid molecule.

[0142] As used herein, "grain" means mature seed produced by commercial growers for purposes other than growing or reproducing the species. The class of plants that can be used in the methods described herein is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous (monocots) and dicotyledonous (dicots) plants.

[0143] Examples of plant species of interest herein include, but are not limited to, corn (*Zea mays*), *Brassica* spp. (*e.g.*, *B. napus*, *B. rapa*, *B. juncea*), particularly those Brassica species useful as sources of seed oil, alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor*, *Sorghum vulgare*), millet (*e.g.*, pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), finger millet (*Eleusine coracana*)), sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium barbadense*, *Gossypium hirsutum*), sweet potato (*Ipomoea batatus*), cassava (*Manihot esculenta*), coffee (*Coffea* spp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*),

sugar beets (*Beta vulgaris*), sugarcane (*Saccharum* spp.), oats (*Avena sativa*), barley *(Hordeum vulgare)*, vegetables, ornamentals, and conifers.

[0144] Vegetables of interest include, but are not limited to, tomatoes (*Lycopersicon esculentum*), lettuce (*e.g.*, *Lactuca sativa*), green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (*Lathyrus* spp.), and members of the genus *Cucumis* such as cucumber (*C. sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*).

[0145] Ornamentals of interest include, but are not limited to, azalea (*Rhododendron* spp.), hydrangea (*Macrophylla hydrangea*), hibiscus (*Hibiscus rosasanensis*), roses (*Rosa* spp.), tulips (*Tulipa* spp.), daffodils (*Narcissus* spp.), petunias (*Petunia hybrida*), carnation (*Dianthus caryophyllus*), poinsettia (*Euphorbia pulcherrima*), and chrysanthemum.

[0146] Conifers of interest include, but are not limited to, pines such as loblolly pine (*Pinus taeda*), slash pine (*Pinus elliotii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), and Monterey pine (*Pinus radiata*); Douglas fir (*Pseudotsuga menziesii*); Western hemlock (*Tsuga canadensis*); Sitka spruce (*Picea glauca*); redwood (*Sequoia sempervirens*); true firs such as silver fir (*Abies amabilis*) and balsam fir (*Abies balsamea*); and cedars such as Western red cedar (*Thuja plicata*) and Alaska yellow cedar (*Chamaecyparis nootkatensis*).

[0147] In some instances, the plant cells, plant parts or plants of interest are crop plants (*e.g.*, corn, alfalfa, sunflower, Brassica, soybean, cotton, safflower, peanut, sorghum, wheat, millet, tobacco, *etc.*).

[0148] Other plants of interest include grain plants that provide seeds of interest, oil-seed plants, and leguminous plants. Seeds of interest include grain seeds, such as corn, wheat, barley, rice, sorghum, rye, *etc.* Oil-seed plants include cotton, soybean, safflower, sunflower, Brassica, maize, alfalfa, palm, coconut, *etc.* Leguminous plants include beans and peas. Beans include guar, locust bean, fenugreek, soybean, garden beans, cowpea, mungbean, lima bean, fava bean, lentils, chickpea, *etc.*

[0149] The present disclosure therefore includes transgenic plant cells, plant parts and plants having incorporated therein at least one nucleic acid molecule that encodes a modified substrate protein of a pathogen-specific protease, where the modified substrate protein has a heterologous protease sequence, to confer disease resistance to plant pathogens expressing specific proteases.

Methods

[0150] Methods of the present disclosure include introducing and expressing in a plant cell, plant part or plant a nucleic acid molecule or construct as described herein. As used herein, "introducing" means presenting to the plant cell, plant part or plant, a nucleic acid molecule or construct in such a manner that it gains access to the interior of a cell of the plant. The methods do not depend on the particular method for introducing the nucleic acid molecule or nucleic acid construct into the plant cell, plant part or plant, only that it gains access to the interior of at least one cell of the plant or plant part. Methods of introducing nucleotide sequences, selecting transformants and regenerating whole plants, which may require routine modification in respect of a particular plant species, are well known in the art. The methods include, but are not limited to, stable transformation methods, transient transformation methods, virus-mediated methods and sexual breeding. As such, the nucleic acid molecule or construct can be carried episomally or integrated into the genome of the host cell.

[0151] As used herein, "stable transformation" means that the nucleic acid molecule or construct of interest introduced into the plant integrates into the genome of the plant and is capable of being inherited by the progeny thereof. As used herein, "transient transformation" means that the nucleic acid molecule or construct of interest introduced into the plant is not inherited by progeny.

[0152] Methods of transforming plants and introducing a nucleotide sequence of interest into plants can and will vary depending on the type of plant, plant part or plant host cell (*i.e.*, monocotyledonous or dicotyledonous) targeted for transformation. Methods of introducing nucleotide sequences into plant host cells therefore include *Agrobacterium*-mediated transformation (*e.g.*, *A. rhizogenes* or *A. tumefaciens*; US Patent Nos. 5,563,055 and 5,981,840), calcium chloride, direct gene transfer (Paszkowski *et al.* (1984) *EMBO J.* 3:2717-2722), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:5602-5606), microinjection (Crossway *et al.* (1986) *Biotechniques* 4:320-334), microprojectile bombardment/particle acceleration (McCabe *et al.* (1988) *Biotechnology* 6:923-926; and Tomes *et al.*, "Direct DNA transfer into intact plant cells via microprojectile bombardment" In: Plant Cell, Tissue, and Organ Culture: Fundamental Methods (Gamborg & Phillips eds., Springer-Verlag 1995); as well as US Patent Nos. 4,945,050; 5,879,918; 5,886,244 and 5,932,782), polyethylene glycol (PEG), phage infection, viral infection, and other methods known in the art. *See also*, EP Patent Nos. 0 295 959 and 0 138 341.

[0153] A nucleic acid molecule or construct as described above herein can be introduced into the plant cell, plant part or plant using a variety of transient transformation methods. Methods of transiently transforming plant cells, plant parts or plants include, but are not limited to, Agrobacterium infection, microinjection or particle bombardment. *See*, Crossway *et al.* (1986) *Mol. Gen. Genet.* 202:179-185; Hepler *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:2176-2180; Hush *et al.* (1994) *J. Cell Sci.* 107:775-784; and Nomura *et al.* (1986) *Plant Sci.* 44:53-58. Alternatively, the plant cell, plant part or plant can be transformed by viral vector systems or by precipitation of the nucleic acid molecule or construct in a manner that precludes subsequent release of the DNA. Thus, transcription from the particle-bound nucleotide sequence can occur, but the frequency with which it is released to become integrated into the genome is greatly reduced. Such methods include the use of particles coated with polyethylimine (PEI; Sigma; St. Louis, MO).

[0154] Likewise, the nucleic acid molecules or constructs as described herein can be introduced into the plant cell, plant part or plant by contacting it with a virus or viral nucleic acids. Generally, such methods involve incorporating the nucleic acid molecule or construct within a viral DNA or RNA molecule. It is recognized that the nucleotide sequences can be initially synthesized as part of a viral polyprotein, which later can be processed by proteolysis *in vivo* or *in vitro* to produce the desired recombinant protein. Methods for introducing nucleotide sequences into plants and expressing the protein encoded therein, involving viral DNA or RNA molecules, are well known in the art. *See*, Porta *et al.* (1996) *Mol. Biotechnol.* 5:209-221; as well as US Patent Nos. 5,866,785; 5,889,190; 5,889,191 and 5,589,367.

[0155] By way of example, in one embodiment, the SMV protease cleavage site (SEQ ID NO:2) is inserted into a GmPBS1 polyprotein sequence so that the modified PBS1 protein is produced as part of the full-length modified GmPBS1 sequence (e.g., SEQ ID NOs:10, 12, and 14).

[0156] Methods also are known in the art for the targeted insertion of a nucleic acid molecule or construct at a specific location in the plant genome. In some instances, insertion of the nucleic acid molecule or construct at a desired genomic location can be achieved by using a site-specific recombination system. *See*, Int'l Patent Application Publication Nos. WO 99/025821, WO 99/025854, WO 99/025840, WO 99/025855 and WO 99/025853.

[0157] Transformation techniques for monocots therefore are well known in the art and include direct gene uptake of exogenous nucleic acid molecules or constructs by protoplasts or cells (*e.g.*, by PEG- or electroporation-mediated uptake, and particle bombardment into callus tissue). Transformation of monocots via *Agrobacterium* also has been described. *See*, Int'l Patent Application Publication No. WO 94/00977 and US Patent No. 5,591,616; *see also*, Christou *et al.* (1991) *Bio/Technology* 9:957-962; Datta *et al.* (1990) *Bio/Technology* 8:736-740; Fromm *et al.* (1990) *Biotechnology* 8:833-844; Gordon-Kamm *et al.* (1990) *Plant Cell* 2:603-618; Koziel *et al.* (1993) *Bio/Technology* 11:194-200; Murashige & Skoog (1962) *Physiologia Plantarum* 15:473-497; Shimamoto *et al.* (1989) *Nature* 338:274-276; Vasil *et al.* (1992) *Bio/Technology* 10:667-674; Vasil *et al.* (1993) *Bio/Technology* 11:1553-1558; Weeks *et al.* (1993) *Plant Physiol.* 102:1077-1084; and Zhang *et al.* (1988) *Plant Cell Rep.* 7:379-384; as well as EP Patent Application Nos. 0 292 435; 0 332 581 and 0 392 225; Int'l Patent Application Publication Nos. WO 93/07278 and WO 93/21335; and US Patent No. 7,102,057.

[0158] Transformation techniques for dicots also are well known in the art and include Agrobacterium-mediated techniques and techniques that do not require Agrobacterium. Non-Agrobacterium-mediated techniques include the direct uptake of exogenous nucleic acid molecules by protoplasts or cells (*e.g.*, by PEG- or electroporation-mediated uptake, particle bombardment, or microinjection). *See*, Klein *et al.* (1987) *Nature* 327:70-73; Paszkowski *et al.* (1984) *EMBO J.* 3:2717-2722; Potrykus *et al.* (1985) *Mol. Gen. Genet.* 199:169-177; and Reich *et al.* (1986) *Bio/Technology* 4:1001-10041; as well as US Patent No. 7,102,057.

[0159] Plant cells that have been transformed can be grown into plants by methods well known in the art. *See*, McCormick *et al.* (1986) *Plant Cell Rep.* 5:81-84. These plants then can be grown, and either pollinated with the same transformed strain or different strains, and the resulting progeny having the desired phenotypic characteristic identified. Two or more generations can be grown to ensure that expression of the desired phenotypic characteristic is stably maintained and inherited, and then seeds harvested to ensure expression of the desired phenotypic characteristic has been achieved.

[0160] It has been shown that the produced plant cells and plants have enhanced confer resistance to disease. By way of example, by introducing a fusion protein (e.g., SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14), including the modified GmPBS1 sequence into soybean, there is seen an enhanced resistance to infection of the soybean by soybean mosaic virus (SMV). Particularly, the excised PBS1 protein, although cleaved in its activation loop by NIa protease,

appears to still be able to activate a native soybean resistance protein, which then prevents spread of SMV through the plant.

[0161] The present disclosure therefore provides methods of introducing into plants, plant parts and plant host cells the nucleic acid constructs described herein, for example, an expression cassette of the present disclosure, which encode a modified substrate protein of a pathogen-specific protease, where the substrate protein has a heterologous protease recognition sequence.

EXAMPLES

[0162] The disclosure will be more fully understood upon consideration of the following non-limiting examples, which are offered for purposes of illustration, not limitation.

EXAMPLE 1

[0163] In this Example, modified GmPBS1 substrate proteins were generated and analyzed as substrates for soybean mosaic virus (SMV) protease.

[0164] PBS1 is one of the most widely conserved defense genes in flowering plants. Using a bioinformatics approach, three genes within the *Glycine max* genome that encode proteins with significant amino acid homology to Arabidopsis PBS1 (AtPBS1; At5g13160) (SEQ ID NO:16) were identified (see FIGS. 1A & 1B). These were designated GmPBS1a (Glyma08g47570) (SEQ ID NO:3), GmPBS1b (Glyma10g44580) (SEQ ID NO:5), and GmPBS1c (Glyma20g39370) (SEQ ID NO:7). Phylogenetic analysis showed that all three GmPBS1 proteins clustered together and were more closely related to AtPBS1 than the next most similar gene to PBS1, PBL27. The three GmPBS1 orthologs contained several conserved domains present in AtPBS1, including conservation of the AvrPphB recognition motif within the activation segment and putative palmitoylation and myristoylation motifs for plasma membrane localization.

[0165] Since the amino acids at the AvrPphB cleavage site are conserved between AtPBS1 and the soybean PBS1 orthologs, it was hypothesized that modified soybean PBS1 substrate proteins could be engineered to function as 'decoys' for the SMV NIa protease. As shown in FIG. 2, using site-directed mutagenesis, the AvrPphB recognition motif Gly-Asp-Lys-Ser-His-Val-Ser (GDKSHVS) (SEQ ID NO:1) was replaced with a SMV NIa protease recognition motif Glu-Ser-Val-Ser-Leu-Gln-Ser (ESVSLQS) (SEQ ID NO:2), generating GmPBS1SCS derivatives.

Transient coexpression of the modified GmPBS1 constructs along with the SMV NIa protease in *N. benthamiana* resulted in NIa-mediated cleavage of GmPBS1SCS. Collectively, these data suggested engineered modified soybean PBS1 proteins could function as substrates for the SMV NIa protease.

EXAMPLE 2

[0166] In this Example, recognition of AvrPphB in soybean was analyzed.

[0167] Engineering resistance to crop plant pathogens may not require transferring the modified Arabidopsis 'decoy' recognition system to a crop plant if the crop plant recognizes AvrPphB. To test whether soybean recognizes AvrPphB, *P. syringae* pv. *glycinea* expressing AvrPphB or AvrB::Ω (a non-functional effector used as an empty vector control) were infiltrated into a unifoliate leaf of soybean cultivar Flambeau. The leaf was removed from the plant 24 hours post-inoculation and the chlorophyll removed with hot 70% ethanol. *P. syringae* pv. *glycinea* expressing AvrPphB induced a hypersensitive response (HR) in soybean cv. flambeau, indicated by browning of the inoculated leaf panel (see FIGS. 3A & 3B). These data suggested soybean likely contains an endogenous R protein that recognizes cleavage of GmPBS1.

EXAMPLE 3

[0168] In this Example, the effect of AvrPphB-specific R protein activation on soybean mosaic virus (SMV) resistance was analyzed.

[0169] Soybean mosaic virus (SMV)-mediated expression of AvrPphB in soybean v. Flambeau triggers resistance to SMV. Green fluorescent protein (GFP), AvrPphB or AvrPphB (C98S) (an enzymatically-inactive derivative of AvrPphB) were transiently expressed in 10-12-day old seedlings by rub-inoculation, as previously described in Wang et al. (2006). Briefly, approximately three weeks post-inoculation (wpi), the third trifoliate leaflet was photographed and harvested. For immunoblot analysis, proteins (10 g) were fractionated on 4-20% SDS-PAGE gels and subjected to immunoblot analysis using α-GFP, α-AvrPphB or α–SMV-CP (SMV coat protein) specific antibodies.

[0170] As shown in FIGS. 4A & 4B, insertion of AvrPphB into the modified SMV genome blocked symptom development and detectable SMV-CP (coat protein) accumulation in the upper, non-inoculated leaflets (FIG. 4A). This recognition of AvrPphB is dependent upon the

protease activity because a protease inactive derivative of AvrPphB (AvrPphB(C98S)) failed to prevent systemic spread of SMV. These results demonstrated that activation of the soybean resistance protein that detects AvrPphB protease activity is sufficient to confer resistance to SMV.

EXAMPLE 4

[0171] In this Example, transgenic Arabidopsis expressing a modified derivative of PBS1 containing a TuMV NIa protease cleavage site was analyzed.

[0172] Prior work established that PBS1 (SEQ ID NO:16) could be engineered to function as a target for viral proteases, at least when transiently overexpressed in *N. benthamiana*. To test whether cleavage of modified PBS1 can activate RPS5 and initiate an effective immune response against TuMV, transgenic Arabidopsis was generated expressing a modified PBS1 substrate protein (PBS1TuMV) under the native promoter and terminator. The PBS1TuMV transgenic Arabidopsis was then infected with TuMV::GFP.

[0173] At 11 days post-infection, TuMV::GFP spread from the initial site of infection to newly emerging leaves of wild-type (nontransgenic) Arabidopsis. Interestingly, the PBS1TuMV transgenic Arabidopsis lines developed extensive chlorosis and necrosis (see FIGS. 5B & 5C). Systemic cell death in the PBS1TuMV transgenic lines correlated with a significant reduction in GFP fluorescence and TuMV accumulation. Collectively, these data suggested that RPS5 could be activated by cleavage of an engineered PBS1, and this activation significantly reduced virus accumulation.

EXAMPLE 5

[0174] In this Example, transgenic Arabidopsis expressing a modified derivative of PBS1 using a strong promoter was analyzed.

[0175] The method as used in Example 4 with the modification of placing the *PBS1* gene under control of a strong constitutive promoter (cauliflower mosaic virus 35S promoter), allowing the modified PBS1 protein to accumulate to higher levels, was used. As shown in FIGS. 6A & 6B, transgenic Arabidopsis plants expressing $PBS1^{TuMV}$ under a strong promoter displayed resistance to TuMV infection without trailing necrosis at 19 days after viral infection. Particularly, as shown in FIG. 6A, all plants were infected with a TuMV derivative that

expressed green fluorescence protein (GFP) fused to the viral 6K2p rotein. Green fluorescence in the leaves indicates viral spread. The transgenic wild-type Col-0 pants and *pbs1* null mutants ($PBS1^{KO}$) transformed with $PBS1^{TuMV}$ showed no visible virus spread, whereas *rps5* null mutants plants ($RPS5^{KO}$) showed systemic spread.

[0176] Additionally, total protein was isolated from the indicated transgenic lines and immunoblotted to assess levels of the $PBS1^{TuMV}$ decoy protein (FIG. 6B, top row) and the virus 6K2:GFP protein (FIG. 6B, middle row). Each lane represents an independent transgenic line. No virus protein was detected in the wild-type and *pbs1* mutant lines.

EXAMPLE 6

[0177] In this Example, it was investigated whether crop plants such as soybean, wheat and barley may already contain disease resistance proteins that are functionally equivalent to RPS5. If so, the PBS1 decoy system developed herein should be able to be modified to be used with these plants.

[0178] To assess whether wheat, barley and soybean contain disease resistance proteins functionally equivalent to RPS5, these species were analyzed for their ability to recognize the protease AvrPphB from *Pseudomonas syringae*. Hypersensitive resistance assays were performed by injecting leaves of the indicated plant varieties with *P. syringae* strains expressing wild-type AvrPphB or the protease inactive mutant of AvrPphB (C98S). The soybean leaf has been extracted with ethanol to remove chlorophyll, revealing brown phenolic deposits marking the zone of cell death.

[0179] As shown in FIG. 7A, it was found that most varieties (cultivars) of wheat, barley and soybean responded to AvrPphB with a strong cell death response (soybean and barley) or strong chlorotic response (wheat), both of which are indicative of defense activation, and these responses are dependent on protease activity as a protease inactive mutant of AvrPphB (C98S) did not induce cell death or chlorosis. For the barley leaves, infiltrated regions are marked with up and down brackets, with wild type AvrPphB indicated by a dot, and C98S marked with only the brackets.

[0180] Furthermore, PBS1 orthologs was isolated from soybean and barley. The indicated proteins were co-expressed in *Nicotiana benthamiana* and then analyzed using immunoblots and

the indicated antibodies. It was demonstrated that the encoded PBS1 proteins were cleaved by AvrPphB (FIG. 7B). The boxed bands indicate cleavage products of PBS1.

[0181] These findings indicate that it should be possible to modify PBS1 proteins from diverse crop species to engineer novel resistance traits.

[0182] All of the patents, patent applications, patent application publications and other publications recited herein are hereby incorporated by reference to the extent they are consistent herewith.

[0183] The present disclosure has been described in connection with what are presently considered to be the most practical and preferred embodiments. However, the present disclosure has been presented by way of illustration and is not intended to be limited to the disclosed embodiments. Accordingly, one of skill in the art will realize that the present disclosure is intended to encompass all modifications and alternative arrangements of the compositions and methods as set forth in the appended claims.

CLAIMS

What is claimed is:

1. A recombinant nucleic acid molecule comprising a nucleotide sequence that encodes a *Glycine max* AvrPphB susceptible 1 (GmPBS1) substrate protein and a heterologous pathogen-specific protease recognition sequence.

2. The recombinant nucleic acid molecule of claim 1, wherein the nucleotide sequence encodes the heterologous pathogen-specific protease recognition sequence of SEQ ID NO:2.

3. The recombination nucleic acid molecule of claim 1, wherein the nucleotide sequence comprises one of SEQ ID NO:9, SEQ ID NO:11, and SEQ ID NO:13.

4. A fusion protein comprising a *Glycine max* AvrPphB susceptible 1 (GmPBS1) substrate protein and a heterologous pathogen-specific protease recognition sequence.

5. The fusion protein of claim 4, wherein the heterologous pathogen-specific protease recognition sequence is SEQ ID NO:2.

6. The fusion protein of claim 4 comprising the amino acid sequence selected from the group consisting of SEQ ID NO:10, SEQ ID NO:12, and SEQ ID NO:14.

7. A vector comprising the recombinant nucleic acid molecule according to claim 1.

8. A transformed plant cell comprising the recombinant nucleic acid molecule according to claim 1.

9. The transformed plant cell of claim 8, wherein the plant cell is from a soybean plant.

10. A transformed plant comprising the recombinant nucleic acid molecule according to claim 1.

11. The transformed plant of claim 10, wherein the plant is a soybean plant.

12. A transgenic seed of the transformed plant according to claim 10.

13. A method of protecting a plant from infection by a plant pathogen that secretes at least one specific protease, the method comprising the step of:

introducing to the plant a nucleotide sequence that encodes a *Glycine max* AvrPphB susceptible 1 (GmPBS1) substrate protein and a heterologous pathogen-specific protease recognition sequence.

14. The method of claim 13, wherein the nucleotide sequence encodes the heterologous pathogen-specific protease recognition sequence of SEQ ID NO:2.

15. The method of claim 13, wherein the nucleotide sequence comprises one of SEQ ID NO:9, SEQ ID NO:11, and SEQ ID NO:13.

FIG. 1A

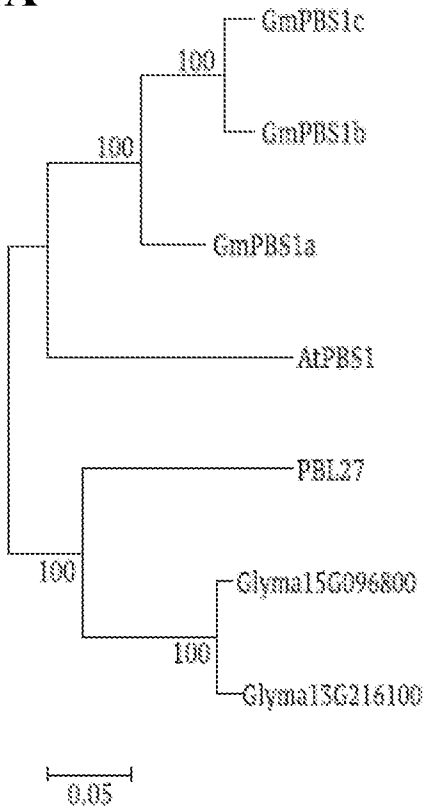
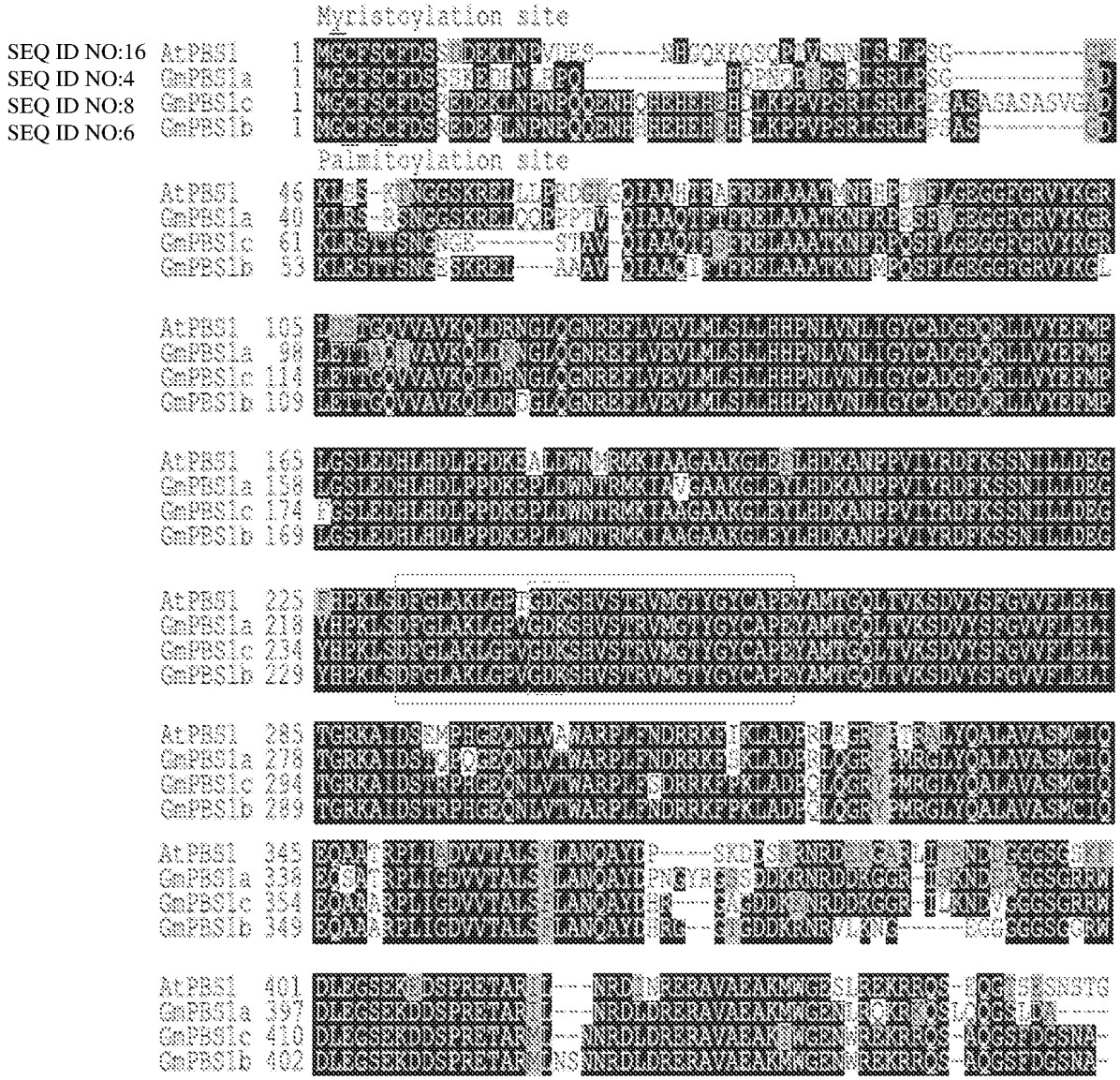

GmPBS1c
100
GmPBS1b
100
GmPBS1a
AtPBS1
PBL27
100
Glyma15G096800
100
Glyma13G216100
0.05

FIG. 1B


Myristoylation site
Palmitoylation site
SEQ ID NO:16 AtPBS1
SEQ ID NO:4 GmPBS1a
SEQ ID NO:8 GmPBS1c
SEQ ID NO:6 GmPBS1b

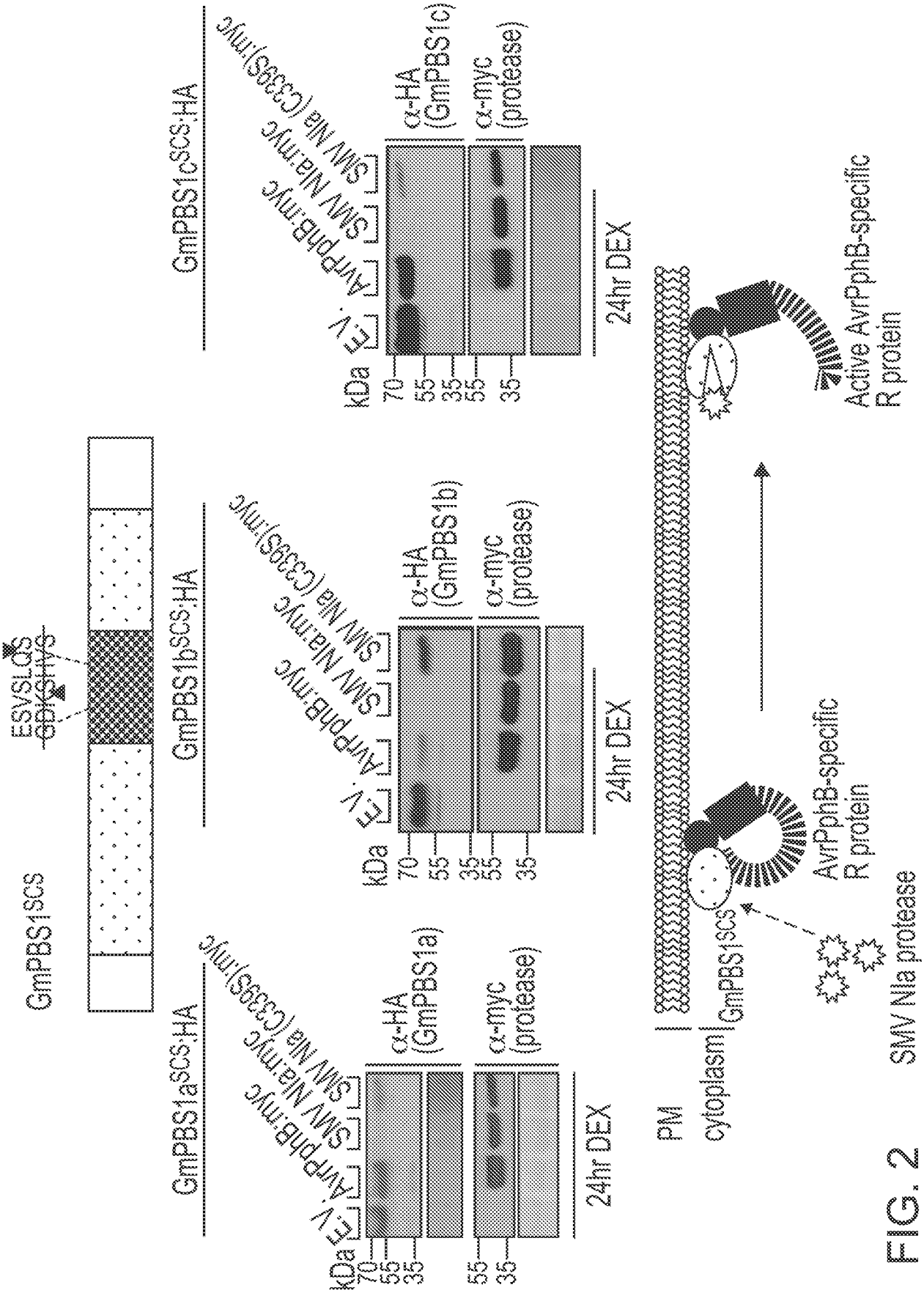

GmPBS1SCS
ESVSLQS
GDKSHYS
GmPBS1aSCS:HA
GmPBS1bSCS:HA
GmPBS1cSCS:HA
EV
AvrPphB:myc
SMV Nia:myc
SMV Nia (C339S):myc
kDa
70
55
35
α-HA (GmPBS1a)
α-myc (protease)
α-HA (GmPBS1b)
α-HA (GmPBS1c)
24hr DEX
PM
cytoplasm
GmPBS1SCS
AvrPphB-specific R protein
Active AvrPphB-specific R protein
SMV Nia protease

FIG. 2

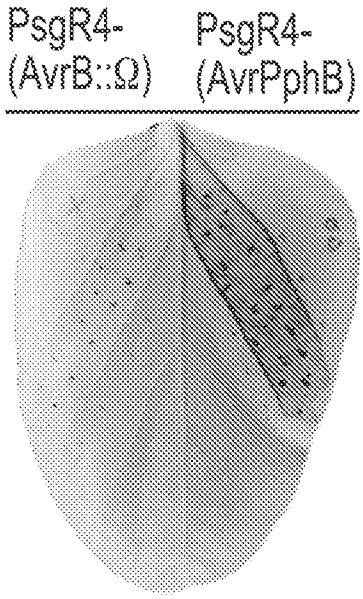

PsgR4-
(AvrB::Ω)
PsgR4-
(AvrPphB)

FIG. 3A

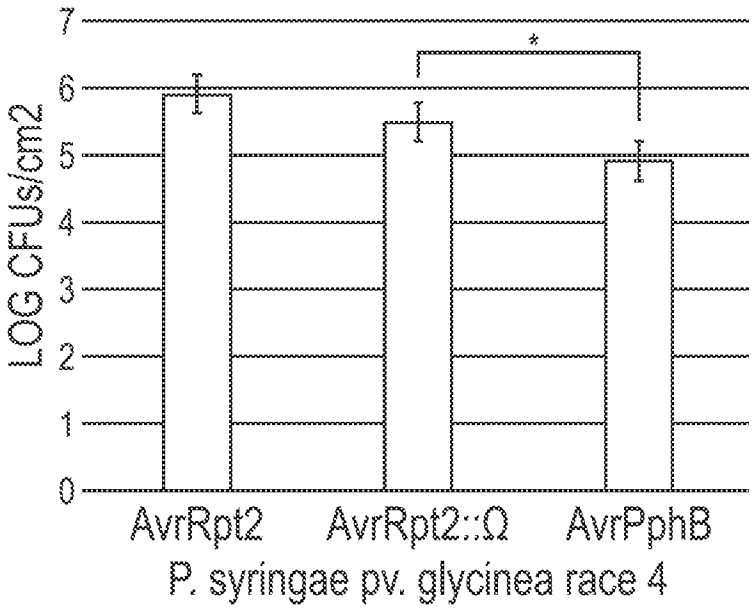

LOG CFUs/cm2
0
1
2
3
4
5
6
7
*
AvrRpt2
AvrRpt2::Ω
AvrPphB
P. syringae pv. glycinea race 4

FIG. 3B

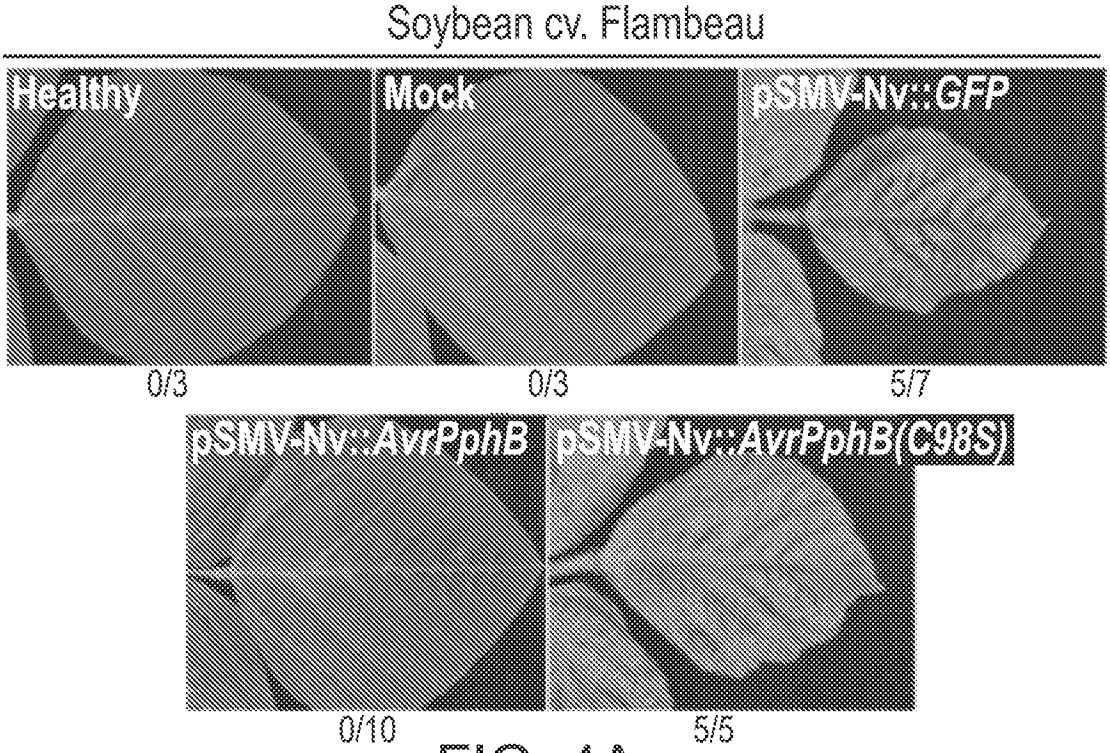

Soybean cv. Flambeau
Healthy
Mock
pSMV-Nv::GFP
0/3
0/3
5/7
pSMV-Nv::AvrPphB
pSMV-Nv::AvrPphB(C98S)
0/10
5/5

FIG. 4A

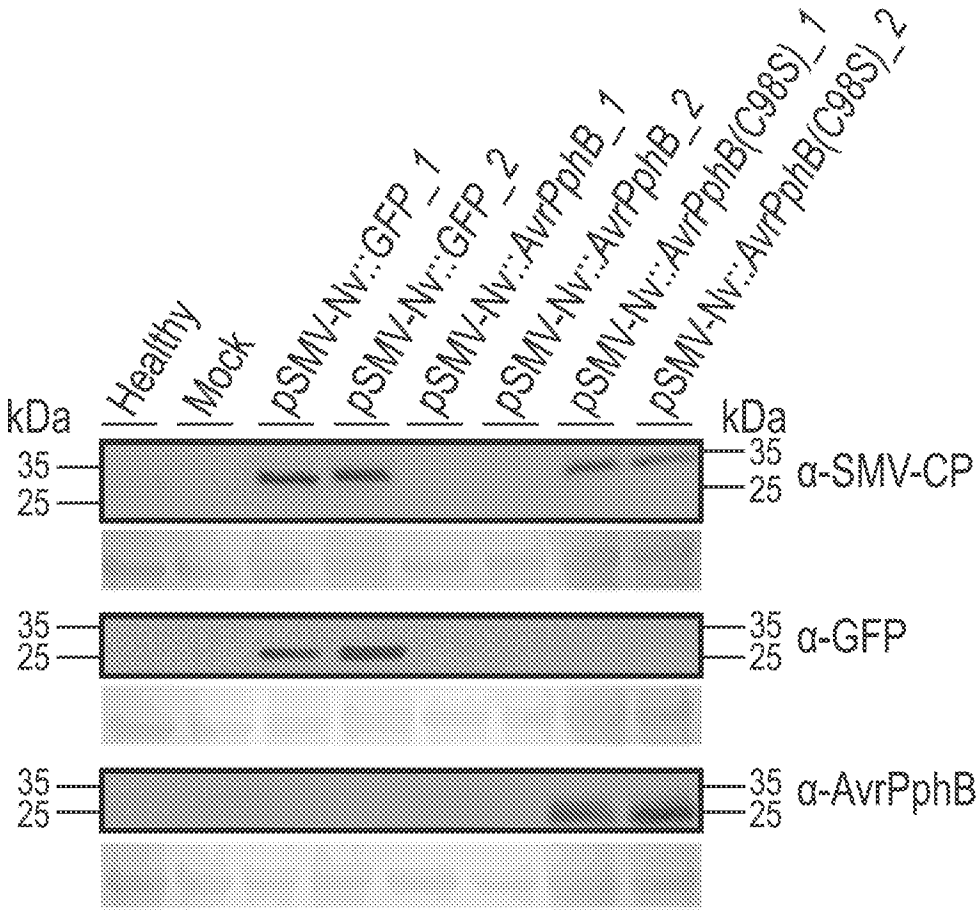

Healthy
Mock
pSMV-Nv::GFP_1
pSMV-Nv::GFP_2
pSMV-Nv::AvrPphB_1
pSMV-Nv::AvrPphB_2
pSMV-Nv::AvrPphB(C98S)_1
pSMV-Nv::AvrPphB(C98S)_2
kDa
kDa
35
25
α-SMV-CP
α-GFP
α-AvrPphB

FIG. 4B

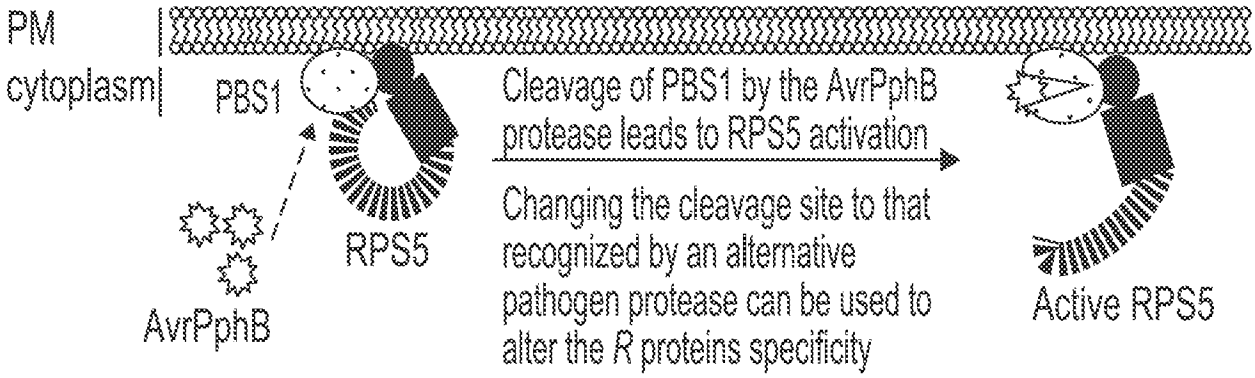


FIG. 5A

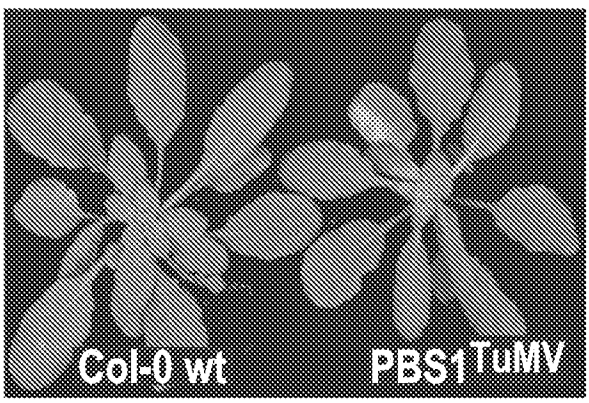


FIG. 5B

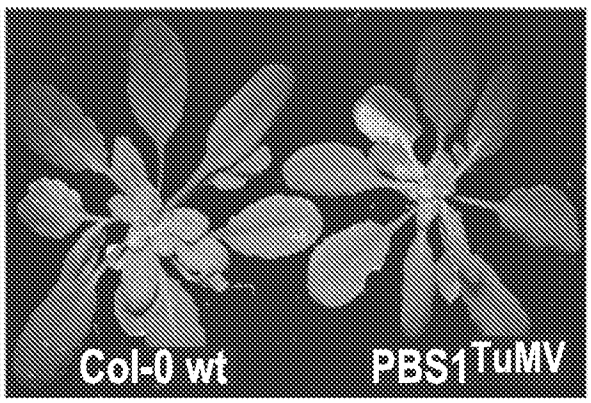


FIG. 5C

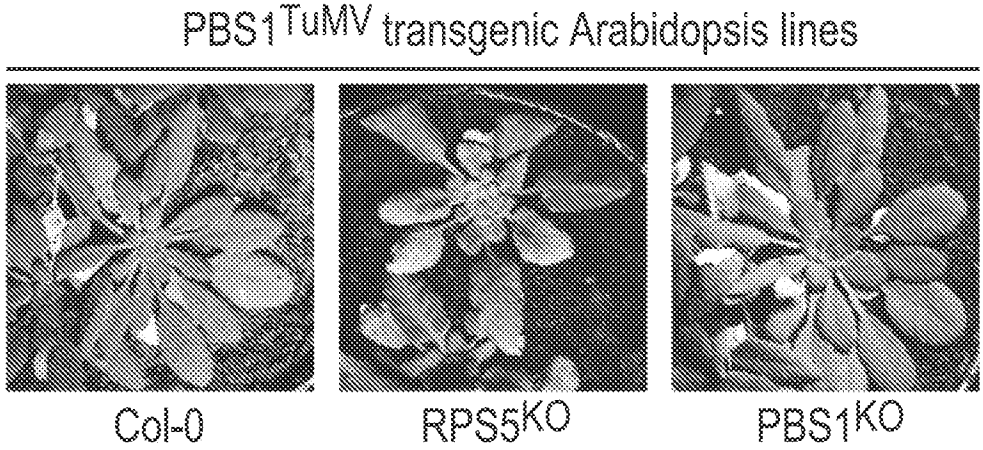

PBS1TuMV transgenic Arabidopsis lines
Col-0
RPS5KO
PBS1KO

FIG. 6A

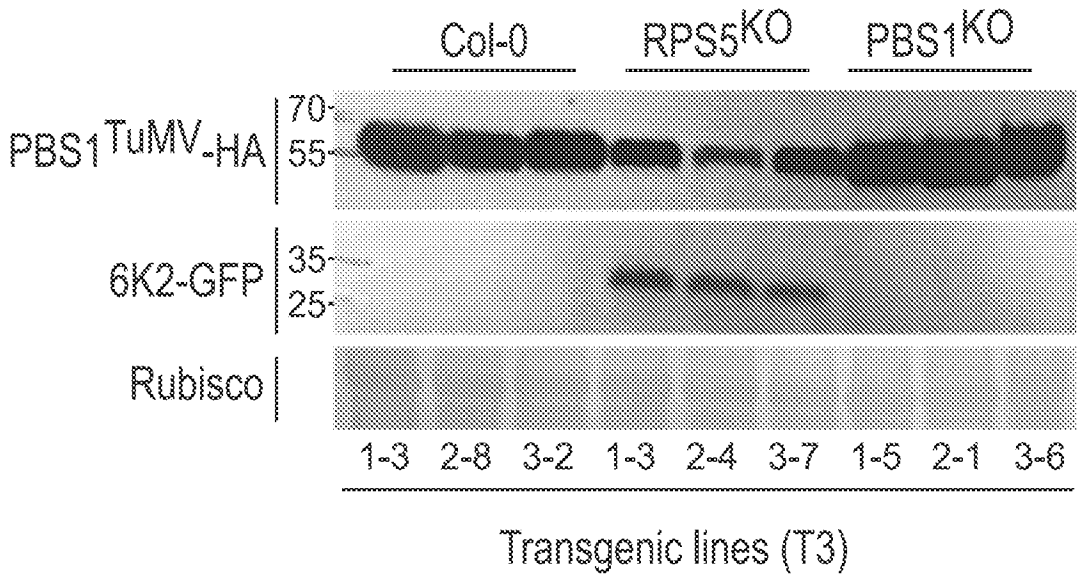

Col-0
RPS5KO
PBS1KO
PBS1TuMV-HA
70-
55-
6K2-GFP
35-
25-
Rubisco
1-3 2-8 3-2 1-3 2-4 3-7 1-5 2-1 3-6
Transgenic lines (T3)

FIG. 6B

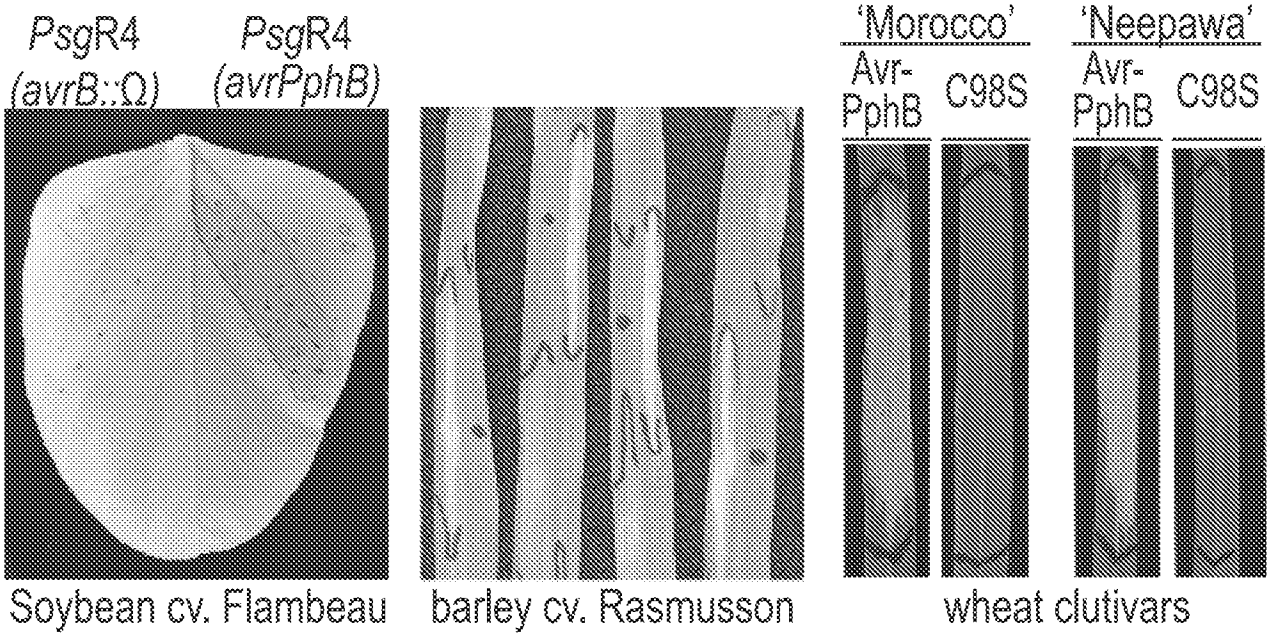

PsgR4 (avrB::Ω)
PsgR4 (avrPphB)
'Morocco'
Avr-PphB
C98S
'Neepawa'
Avr-PphB
C98S
Soybean cv. Flambeau
barley cv. Rasmusson
wheat clutivars

FIG. 7A

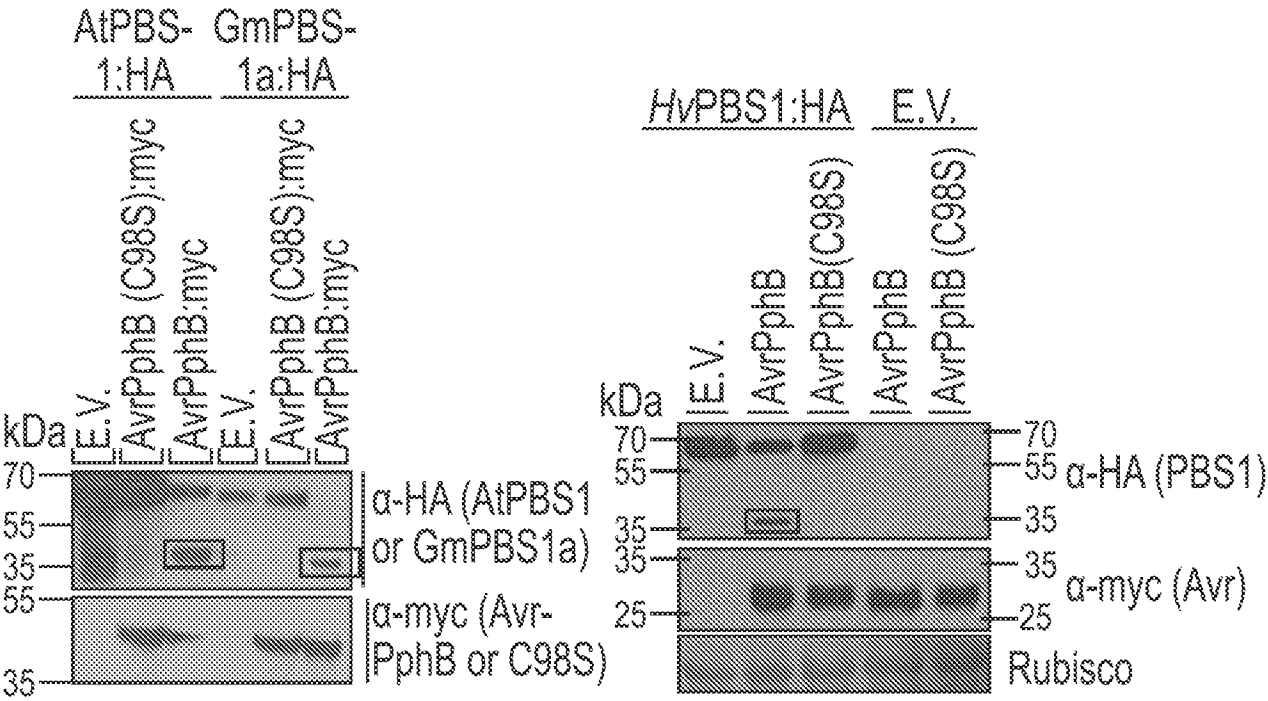

AtPBS-1:HA
GmPBS-1a:HA
E.V.
AvrPphB (C98S):myc
AvrPphB:myc
kDa
70
55
35
α-HA (AtPBS1 or GmPBS1a)
α-myc (Avr-PphB or C98S)
HvPBS1:HA
E.V.
AvrPphB
AvrPphB(C98S)
25
α-HA (PBS1)
α-myc (Avr)
Rubisco

FIG. 7B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/25511

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A01H 5/00, C07K 14/005, C07K 14/21 (2018.01)
CPC - C07K 14/21, C07K 14/005, C12N 15/8281

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y --- A	US 2015/0247163 A1 (INDIANA UNIVERSITY RESEARCH AND TECHNOLOGY CORPORATION) 03 September 2015 (03.09.2015); abstract, claim 1, claim 9, claim 14, para [0014], [0017], [0144], [0148], [0157], [0161]	1, 4, 7-12 --- 2, 5 --- 3, 6
Y	Uniprot Submission POLG_SBMVN, Soybean mosaic virus, 01 May 1991 [database online], [retrieved from the internet on 2018-06-11], Retrieved from the internet <URL: https://www.genome.jp/dbget-bin/www_bget?sp:POLG_SBMVN>	2, 5
A	Genbank Submission Accession No. FJ014791, Glycine max clone cw108 serine/threonine-protein kinase mRNA, 01 August 2009 [database online], [retrieved from the internet on 2018-06-11], Retrieved from the internet <URL: https://www.ncbi.nlm.nih.gov/nuccore/FJ014791>	3
A	Uniprot Submission Accession No. C6ZRV0, Serine/threonine-protein kinase, 22 September 2009 [database online], [retrieved from the internet on 2018-06-11], Retrieved from the internet <URL: http://www.uniprot.org/uniprot/C6ZRV0>	6

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 June 2018	07 AUG 2018

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/25511

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/25511

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

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

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

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

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

*****Continued in Supplemental Box*****

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

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

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

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-12 limited to GmPBS1 (SEQ ID NOs: 9 and 10)

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/25511

Continuation of Box No. III (Observations where unity of invention is lacking):

Group I+, Claims 1-12, directed to a recombinant nucleic acid molecule comprising a nucleotide sequence that encodes a Glycine max AvrPphB susceptible 1 (GmPBS1) substrate protein and a heterologous pathogen-specific protease recognition sequence, a fusion protein encoded by the same, a vector comprising the recombinant nucleic acid molecule, a transformed plant, plant cell or seed comprising the recombinant nucleic acid molecule. The recombinant nucleic acid molecule, and encoded fusion protein will be searched to the extent that the GmPBS1 substrate protein encompasses GmPBS1a (and corresponding nucleic acid sequence SEQ ID NO: 9 and amino acid sequence SEQ ID NO: 10). It is believed that claims 1-12 encompass this first named invention, and thus these claims will be searched without fee to the extent that the GmPBS1 substrate protein encompasses GmPBS1a (SEQ ID NOs: 9 and 10). Additional GmPBS1 substrate proteins will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected GmPBS1 substrate proteins. Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be recombinant nucleic acid comprising GmPBS1 substrate protein GmPBS1b (and corresponding nucleic acid sequence SEQ ID NO: 11 and amino acid sequence SEQ ID NO: 12) (claims 1-12).

Group II, claims 13-15, directed to a method of protecting a plant from infection by a plant pathogen that secretes at least one specific protease.

The inventions listed as Group I+ and II do not relate to a single special technical feature under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special technical features
Group I+ has the special technical feature of a recombinant nucleic acid molecule comprising a nucleotide sequence that encodes a Glycine max AvrPphB susceptible 1 (GmPBS1) substrate protein and a heterologous pathogen-specific protease recognition sequence, a fusion protein encoded by the same, a vector comprising the recombinant nucleic acid molecule, a transformed plant, plant cell or seed comprising the recombinant nucleic acid molecule, that is not required by Group II.

Group II has the special technical feature of introducing to a plant a nucleotide sequence, that is not required by Group I+.

The inventions of Group I+ each include the special technical feature of a unique GmPBS1 substrate protein and corresponding nucleic acid and amino acid sequences. Each GmPBS1 substrate protein is unique, and is considered a distinct technical feature.

Shared technical features

The inventions of Group I+ and Group II share the common technical feature of a plant comprising a nucleotide sequence that encodes a GmPBS1 substrate protein and a heterologous pathogen-specific protease recognition sequence.

No technical features are shared between the GmPBS1 substrate protein nucleic acid and amino acid sequences of Group I+ and, accordingly, these groups lack unity a priori.

Additionally, even if Group I+ were considered to share the technical features of including: a recombinant nucleic acid molecule comprising a nucleotide sequence that encodes a GmPBS1 substrate protein and a heterologous pathogen-specific protease recognition sequence, a vector comprising the recombinant nucleic acid molecule, a transformed plant, plant cell or seed comprising the recombinant nucleic acid molecule, and a fusion protein comprising a GmPBS1 substrate protein and a heterologous pathogen-specific protease recognition sequence, these shared technical features are previously disclosed by US 2015/0247163 A1 to Indiana University Research and Technology Corporation (hereinafter 'Indiana University').

Indiana University teaches a recombinant nucleic acid molecule comprising a nucleotide sequence (claim 1 -A recombinant nucleic acid molecule comprising a nucleotide sequence that encodes at least one substrate protein of a plant pathogen-specific protease having a heterologous pathogen-specific protease recognition sequence.) that encodes a Glycine max AvrPphB susceptible 1 (GmPBS 1) substrate protein (para [0017] -By way of example, the substrate protein of the pathogen-specific protease can be a PBS1 or a RIN4 homolog from Arabidopsis thaliana, and the NB-LRR protein can be a RPS5 or a RPS2 from A. thaliana, where the PBS1 or RIN4 homolog is modified to include a heterologous protease recognition sequence. As understood by those skilled in the art, "PBS1" refers to avrPphB susceptible 1; [0144] - It is contemplated that PBS1 orthologues from other plant species can be substituted for Arabidopsis PBS1, which typically have about 90% or higher identity.; [0157] -Examples of plant species of interest herein include, but are not limited to...Glycine max) and a heterologous pathogen-specific protease recognition sequence (claim 1 -A recombinant nucleic acid molecule comprising a nucleotide sequence that encodes at least one substrate protein of a plant pathogen-specific protease having a heterologous pathogen-specific protease recognition sequence.), a vector comprising the recombinant nucleic acid molecule (claim 9- A vector comprising the recombinant nucleic acid molecule according to claim 1.), a transformed plant and plant cell comprising the recombinant nucleic acid molecule (para [0014] -The compositions also include transformed plant cells, plant parts and plants having a nucleotide sequence...expression in a plant cell, plant part or plant; [0148] - transformed plant cells, plant parts and plants), and a transgenic seed of the transformed plant (claim 14- A seed of the transformed plant).

*****Continued in Supplemental Box*****

Continuation of Previosu page:

Indiana University further teaches a fusion protein comprising a Glycine max AvrPphB susceptible 1 (GmPBSI) substrate protein (claim 19 "An isolated polypeptide encoded by the recombinant nucleic acid molecule"; para [0017] -By way of example, the substrate protein of the pathogen-specific protease can be a PBS1 or a RIN4 homolog from Arabidopsis thaliana, and the NB-LRR protein can be a RPS5 or a RPS2 from A. thaliana, where the PBS1 or RIN4 homolog is modified to include a heterologous protease recognition sequence. As understood by those skilled in the art, "PBS1" refers to avrPphB susceptible 1; [0053] "a recombinant nucleic acid molecule having a nucleotide sequence that is part of a hybrid gene (i.e., a gene encoding a fusion protein)"; [0144] - It is contemplated that PBS1 orthologues from other plant species can be substituted for Arabidopsis PBS1, which typically have about 90% or higher identity.; [0157] -Examples of plant species of interest herein include, but are not limited to...Glycine max) and a heterologous pathogen-specific protease recognition sequence (claim 1 -A recombinant nucleic acid molecule comprising a nucleotide sequence that encodes at least one substrate protein of a plant pathogen-specific protease having a heterologous pathogen-specific protease recognition sequence"; [0053] "a recombinant nucleic acid molecule having a nucleotide sequence that is part of a hybrid gene (i.e., a gene encoding a fusion protein)").

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Therefore, Group I+ and II inventions lack unity under PCT Rule 13 because they do not share the same or corresponding special technical feature.