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(54) **Title:** ANTI-MICROBIAL PROTEINS

(57) **Abstract:** The disclosure provides polynucleotide molecules encoding novel defensins conferring increased pest tolerance and/or pesticidal activity when expressed in a plant, and recombinant DNA constructs and vectors comprising these molecules. Methods of making transgenic plants comprising recombinant defensin-encoding polynucleotide molecules and constructs, and transgenic plants, plant parts and seeds produced by these methods are provided. Compositions comprising one or more novel defensins of the disclosure are also provided having pesticidal and/or anti-microbial activity, as well as methods of their use.



ANTI-MICROBIAL PROTEINS

REFERENCE TO RELATED APPLICATION

[001] This application claims the benefit of United States Provisional Application No. 62/280,597, filed January 19, 2016, which is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

[002] The invention relates to the field of agricultural biotechnology. More specifically, the invention relates to nucleotide and polypeptide molecules, DNA constructs, and methods for producing plants with improved microbial pathogen or pest tolerance, as well as transgenic plants with improved pesticidal and/or fungicidal activity. The invention further relates to pesticidal and/or fungicidal compositions.

INCORPORATION OF SEQUENCE LISTING

[003] A sequence listing contained in the file named "MONS388WO_ST25.txt" which is 592 KB (measured in MS-Windows®) and created on January 18, 2017, is filed electronically herewith and incorporated by reference in its entirety.

BACKGROUND

[004] Control of fungal pathogens and other disease-causing microbes that affect plants is one of the major difficulties facing the agricultural and horticultural industries. Although chemical agents have been successfully employed, a range of environmental and regulatory concerns is associated with the continued use of chemical approaches to control plant pests. Furthermore, with increased use of chemical pesticides comes increased resistance to these chemicals in pathogen and pest populations. Thus, further investigation to discover alternative mechanisms of providing resistance in plants to pathogens, such as insects, nematodes, microorganisms, fungi, bacteria and viruses, is needed.

SUMMARY

[005] In one aspect, the invention provides a recombinant DNA construct comprising a nucleic acid sequence encoding a multi-domain defensin polypeptide comprising a first defensin region connected to a second defensin region by a linker region, the first defensin region and the second

defensin region each comprising a gamma-thionin domain, wherein the nucleic acid sequence encoding the defensin polypeptide is operably linked to a promoter functional in a plant cell.

[006] In another aspect, the invention provides a recombinant DNA construct comprising a nucleic acid sequence encoding a single domain defensin polypeptide.

[007] For the multi-domain defensin, in some embodiments, the first defensin region is heterologous with respect to the second defensin region or the linker region, while in other embodiments the first defensin region or the second defensin region is heterologous with respect to the linker region. In certain embodiments, the first defensin region is identical to the second defensin region. In other embodiments, the first defensin region is different from the second defensin region. In certain embodiments, the promoter may be a heterologous promoter. In further embodiments, each defensin domain comprises at least 6 cysteine residues. The multi-domain defensin may be at least 90 amino acids in length. Each defensin domain may comprise: (a) between 5 and 10 cysteine residues; (b) a Pfam gamma-thionin domain (PF00304) with an E-value cutoff of $1e^{-3}$; or (c) a Cys-stabilized $\alpha\beta$ (CS $\alpha\beta$) motif.

[008] In certain embodiments, the first defensin region or the second defensin region of a multi-domain defensin comprises a polypeptide having at least 80% identity, at least 90% identity, at least 95% identity, or at least 97% identity to an amino acid sequence selected from the group consisting of: SEQ ID NOs: 559-662, or a polypeptide having an amino acid sequence selected from the group consisting of: SEQ ID NOs: 559-662. In some embodiments, the linker region comprises a polypeptide having at least 80% identity, at least 90% identity, at least 95% identity, or at least 97% identity to an amino acid sequence selected from the group consisting of: SEQ ID NOs: 153-202, or a polypeptide comprising an amino acid sequence selected from the group consisting of: SEQ ID NOs: 153-202. In other embodiments, the multi-domain defensin polypeptide comprises an amino acid sequence having at least 80% identity, at least 90% identity, at least 95% identity, or at least 97% identity to an amino acid sequence selected from the group consisting of: SEQ ID NOs: 52-102, 329-454, and 1156, or a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 52-102, 329-454 and 1156. In further embodiments, the first defensin region or the second defensin region of a multi-domain defensin comprises an amino acid sequence having at least 80% identity, at least 90% identity, at least 95% identity, or at least 97% identity to a defensin sequence portion of an

amino acid sequence selected from the group consisting of: SEQ ID NOs: 1054-1152 and 1154, or an amino acid sequence selected from the group consisting of SEQ ID NOs: 1054-1152 and 1154. In further embodiments, the multi-domain defensin polypeptide encoded by nucleic acid sequence further comprises an N-terminal transit signal sequence having at least 80% identity, at least 90% identity, at least 95% identity, or at least 97% identity to an amino acid sequence selected from the group consisting of SEQ ID NOs: 809-954.

[009] In certain embodiments, the defensin polypeptide comprises an amino acid sequence having at least 80% identity, at least 90% identity, at least 95% identity, or at least 97% identity to a defensin sequence portion of an amino acid sequence selected from the group consisting of: SEQ ID NOs: 1054-1152 and 1154, or an amino acid sequence selected from the group consisting of: SEQ ID NOs: 1054-1152 and 1154. In further embodiments, the multi-domain defensin polypeptide encoded by nucleic acid sequence further comprises an N-terminal transit signal sequence having at least 80% identity, at least 90% identity, at least 95% identity, or at least 97% identity to an amino acid sequence selected from the group consisting of: SEQ ID NOs: 809-954.

[010] In another aspect, the invention provides a synthetic promoter as set forth in SEQ ID NO: 1158 to express single domain defensin or multi-domain defensin in plant cells.

[011] In another aspect, the invention provides a plant, seed, plant tissue, plant part, or cell comprising a recombinant DNA construct provided herein or comprising the multi-domain defensin polypeptide encoded by a recombinant DNA construct provided herein. The plant, seed, plant tissue, plant part, or cell may exhibit tolerance or activity against at least one plant fungal pathogen species within one or more of the following genera of fungi: *Fusarium*, *Collectotrichum*, *Stenocarpella*, and/or *Phakopsora*. The plant, seed, plant tissue, plant part, or cell may exhibit tolerance or activity against one or more of the following fungal species: *Fusarium graminearum*, *Fusarium verticilloides*, *Collectotrichum graminicola*, *Stenocarpella maydis*, and/or *Phakopsora pachyrhizi*.

[012] In another aspect, the invention provides a microorganism comprising a recombinant DNA construct provided herein, or a DNA molecule or vector comprising a recombinant DNA construct provided herein. The DNA molecule or vector may comprise a polynucleotide sequence having at least 70% identity, at least 85% identity, at least 90% identity, at least 95%

identity, or at least 97% identity to a nucleotide sequence selected from the group consisting of: SEQ ID NOs: 1-51, 203-328, 955-1053 and 1155, or a polynucleotide sequence having a nucleotide sequence selected from the group consisting of: SEQ ID NOs: 1-51, 203-328, 955-1053, and 1155.

[013] The invention further provides a method for conferring fungal pathogen tolerance or resistance to a plant, seed, cell, or plant part comprising expressing in said plant, seed, cell, or plant part the single domain defensin or the multi-domain defensin polypeptide encoded by the recombinant DNA construct disclosed herein. The invention further provides methods for expressing the recombinant DNA constructs of the invention in a plant cell or microorganism to produce a multi-domain defensin polypeptide, for example such that the multi-domain defensin polypeptide accumulates in a plant cell at a higher level relative to a single domain (1D) defensin control. The invention further provides a method for producing a transgenic plant with resistance or tolerance to a fungal pathogen comprising transforming a plant cell or tissue with the recombinant DNA molecule or vector provided herein, and regenerating a transgenic plant.

[014] In another aspect, the invention provides a recombinant DNA construct comprising a nucleic acid sequence encoding a defensin having at least 80% identity to an amino acid sequence selected from the group consisting of SEQ ID NOs: 1054-1152, wherein the nucleic acid sequence encoding the defensin polypeptide is operably linked to a promoter functional in a plant cell. In certain embodiments, the defensin polypeptide has an amino acid sequence selected from the group consisting of SEQ ID NOs: 1054-1152. In further embodiments, the promoter comprises a nucleotide sequence as set for in SEQ ID NO: 1158.

[015] The invention further provides a plant, seed, plant tissue, plant part, or cell comprising a recombinant DNA construct described herein. In some embodiments, the plant, seed, plant tissue, plant part, or cell has tolerance or activity against at least one plant fungal pathogen selected from the group consisting of *Fusarium*, *Collectotrichum*, *Stenocarpella*, and *Phakopsora*, for example at least one fungal species selected from the group consisting of *Fusarium graminearum*, *Fusarium verticilloides*, *Collectotrichum graminicola*, *Stenocarpella maydis*, and *Phakopsora pachyrhizi*.

[016] In a further aspect, the invention provides methods of producing a transgenic plant with tolerance to a fungal pathogen comprising transforming a plant cell or tissue with the recombinant DNA molecule or vector described herein, and regenerating a transgenic plant.

BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NOs: 1-51: Nucleotide sequences of native multi-domain defensins.

SEQ ID NOs: 52-102: Polypeptide sequences of native multi-domain defensins.

SEQ ID NOs: 103-152: Nucleotide sequences of linker regions from multi-domain defensins.

SEQ ID NOs: 153-202: Polypeptide sequences of linker regions from multi-domain defensins.

SEQ ID NOs: 203-328: Nucleotide sequences of several synthetic multi-domain defensins.

SEQ ID NOs: 329-454: Polypeptide sequences of several synthetic multi-domain defensins.

SEQ ID NOs: 455-558: Nucleotide sequences of defensin regions from multi-domain defensins.

SEQ ID NOs: 559-662: Polypeptide sequences of defensin regions from multi-domain defensins.

SEQ ID NOs: 663-808: Nucleotide sequences of transit signals (TS) of multi-domain defensins.

SEQ ID NOs: 809-954: Polypeptide sequences of TS of multi-domain defensins.

SEQ ID NOs: 955-1053: Nucleotide sequences of 1D defensins.

SEQ ID NOs: 1054-1152: Polypeptide sequences of 1D defensins.

SEQ ID NO: 1153: Nucleotide sequence of MEDsa.AFPm1.

SEQ ID NO: 1154: Polypeptide sequence of MEDsa.AFPm1.

SEQ ID NO: 1155: Nucleotide sequence of a synthetic heterodimeric defensin.

SEQ ID NO: 1156: Polypeptide sequence of a synthetic heterodimeric defensin.

SEQ ID NO: 1157: Nucleotide sequence of codon optimized PINSY.AFP1.

SEQ ID NO: 1158: Nucleotide sequence of a synthetic regulatory element for controlling transgene expression.

BRIEF DESCRIPTION OF THE DRAWINGS

[017] FIG. 1 shows: (Top) a diagram depicting the domain configuration of two single domain (1D) defensins and four homodimeric synthetic two-domain (2D) defensins derived from the naturally occurring single domain defensins Coix22 and MtDef4. L1 and L2 represent different 2D linker regions described herein. The N-terminal transit signal (TS) sequences are also shown. (Bottom) a diagram depicting the domain configuration of a heterodimeric synthetic 2D defensin derived from Coix22 and AMAr.AFP10. L3 represent a 2D linker region described herein.

[018] FIG. 2 shows: Relative RNA and protein expression levels of several homodimeric 2D defensins as compared to those levels of the corresponding 1D defensin.

DETAILED DESCRIPTION

[019] Plant diseases caused by fungal or other microbial pathogens can severely impact yield in crop plants, resulting in millions of tons of grain loss annually. Fungal plant diseases can result from a combination of several pathogens, and nearly every field of crop plants may experience fungal disease pressure to some extent. The development of effective methods of fungal control has been hindered by a lack of available agents with activity against different fungal pathogens, and/or agents which can be effectively combined with existing methods for fungal control. Yield loss due to fungal disease in agricultural plants therefore remains a significant problem.

[020] The invention provides novel anti-microbial peptides (AMPs) comprising defensin or defensin-like proteins, including multi-domain defensin proteins, capable of conferring pest resistance or tolerance and/or fungicidal activity to plants. Novel polynucleotide molecules and sequences encoding defensin or defensin-like proteins, as well as recombinant DNA constructs comprising these novel defensin-encoding polynucleotide sequences, are also provided. In addition, methods are provided for producing plants with increased pest control or pesticidal activity by expressing in a plant a polynucleotide of the invention encoding a defensin or defensin-like protein, such as by transforming a plant or plant cell with a recombinant DNA construct comprising a polynucleotide sequence encoding a defensin or defensin-like protein, and transgenic plants or plant cells produced by these methods and comprising a defensin-encoding DNA construct of the invention. Pesticidal and plant health compositions and methods

are also contemplated for administering or applying a defensin or defensin-like protein(s) of the invention to a plant, a plant growth medium or soil associated with the plant, or a plant part or seed.

[021] The invention provides novel polynucleotide and polypeptide sequences of defensins or defensin-like protein molecules including multi-domain defensins, and the use of these sequences and molecules for generating pest resistance or tolerance in plants. As used herein, "tolerance" or "improved tolerance" in a plant to a pest or pathogen is an indication that the plant is less affected by the pest or pathogen with respect to yield, survivability and/or other relevant agronomic measures, compared to a less resistant, more "susceptible" plant. "Resistance" or "improved resistance" in a plant to a pest or pathogen is an indication that the plant is more able to reduce the effect of the pest or pathogen than a non-resistant or less resistant plant. The defensins described herein may be introduced into various plant species to confer anti-fungal and/or anti-microbial activity, and thus resistance or tolerance to one or more plant pests or pathogens. The defensins and defensin-like molecules disclosed herein may be introduced and expressed in a plant or plant cell to confer the anti-fungal and/or anti-microbial activity to the plant or plant cell. According to embodiments of the invention, polynucleotides, constructs and proteins of the invention may confer resistance or tolerance to, and activity against, one or more fungal pathogens, including a *Fusarium*, *Colletotrichum*, *Stenocarpella*, and/or *Phakopsora* species, such as *Fusarium graminearum*, *Fusarium verticilloides*, *Colletotrichum graminicola*, *Stenocarpella maydis*, and/or *Phakopsora pachyrhizi*. In addition to having anti-fungal activity, constructs and proteins of the invention may confer resistance or tolerance to, and activity against, other microbial plant pathogen(s) and/or plant pest(s), such as oomycetes, bacteria, insects, nematodes, etc.

[022] Defensins or defensin-like proteins or polypeptides, hereinafter referred to jointly as defensins, are cysteine-rich cationic peptides, many of which exhibit inhibitory activity against a variety of microbial plant pathogens and agricultural pests. Single domain (1D) defensins are small globular proteins or peptide molecules, typically comprising approximately 50 amino acid (aa) residues that may be highly variable in sequence. Despite this variability, defensin peptides do share a gamma-thionin core consensus sequence (a gamma-thionin domain) and contain about 6-8 cysteine residues that may form disulfide bonds with protein folding. The three-dimensional structure of 1D defensins has been described as a Cys-stabilized $\alpha\beta$ motif (CS $\alpha\beta$) having three

anti-parallel β -sheets and one α -helix stabilized by multiple disulfide bridges formed by conserved cysteine residues (typically four disulfide bridges formed by eight conserved cysteine residues). See, e.g., Carvalho, AO et al., *Peptides* 30: 1007-1020 (2009); and Thomma, B et al., *Planta* 216: 193-202 (2002), the entire contents and disclosures of which are incorporated herein by reference. Defensins may further comprise an N-terminal transit signal (TS) sequence of variable length from only a few amino acids up to 20-30 amino acids, and/or a C-terminal extension sequence of variable length up to 35-30 amino acids when present. The N-terminal TS sequence on a defensin pro-protein may play a role in the targeting and/or export of the mature defensin protein into the apoplastic space or other sub-cellular compartment. However, the TS sequence may generally become cleaved and removed from the remainder of the defensin co/post-translationally to produce a mature defensin protein or peptide without the TS sequence. Some defensins may also have a C-terminal extension sequence that may also play a variety of roles in a plant cell and/or become cleaved from a mature defensin protein or peptide.

[023] In one aspect, the invention provides several multi-domain (MD) defensins from plants, including two-domain (2D) and four-domain (4D) defensins, comprising two or more defensin regions or domains connected or bridged together by one or more linker regions, in addition to N-terminal TS sequences and/or C-terminal extension sequences. Each of the defensin regions of a 2D or MD defensin may also be referred to as a “defensin component” of the 2D or MD defensin. Thus, polypeptide sequences of the invention may comprise one of these multi-domain defensin proteins or polypeptides. Examples of multi-domain defensins including 2D defensins that may be used according to embodiments of the invention include those provided herein as SEQ ID NOs: 52-102. Further provided are polynucleotide molecules and constructs encoding one of these multi-domain defensins, such as those provided as SEQ ID NOs: 1-51. These MD defensin-encoding polynucleotides may be used according to embodiments of the invention to confer pesticidal and/or anti-fungal activity when expressed in a plant. Multi-domain defensin proteins of the invention may also optionally comprise an N-terminal transit signal (TS) sequence, such as one of SEQ ID NOs: 809-954, and/or a C-terminal sequence, such as one identified by annotation in Table 2 below for SEQ ID NOs: 101 and 102.

[024] In one aspect, the invention provides several single domain (1D) defensins from plants that have pesticidal and/or anti-fungal activity, as well as polynucleotides encoding these 1D defensins. Thus, embodiments of the invention include polynucleotides and constructs encoding

these 1D defensins for expression in a plant, or at least comprising a defensin sequence portion of these 1D defensins as identified herein. Examples of 1D defensins that are used according to embodiments of the invention include one or more of those provided herein as SEQ ID NOs: 1054-1127 and 1129-1152, and examples of polynucleotide sequences encoding these 1D defensins include one or more of those provided herein as SEQ ID NOs: 955-1028 and 1030-1053. 1D defensins of the invention may further include those comprising a defensin component of a 2D or MD defensin identified herein, such as one or more of those provided herein as SEQ ID NOs: 559-662, as well as polynucleotide sequences encoding these defensin components, such as one or more of those provided herein as SEQ ID NOs: 455-558. It is further contemplated that 1D defensins of the invention may comprise polypeptide and polynucleotide sequences having a relaxed sequence identity relative to the above identified sequences. For example, a 1D defensin may have a polypeptide sequence that is at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100% identical to at least a defensin sequence portion of one of SEQ ID NOs: 1054-1127 or 1129-1152, and/or a 1D defensin may be encoded by a polynucleotide sequence that is at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100% identical to at least a defensin sequence portion of one of SEQ ID NOs: 955-1028 or 1030-1053. A 1D defensin may further comprise a fragment comprising at least 25, at least 50, at least 75, or at least 100, contiguous amino acids of a defensin sequence portion of one of SEQ ID NOs: 1054-1127 or 1129-1152, and/or a 1D defensin may be encoded by a polynucleotide sequence that is fragment comprising at least 25, at least 50, at least 75, or at least 100 contiguous nucleotides of a defensin sequence portion of one of SEQ ID NOs: 955-1028 or 1030-1053. In some embodiments, a fragment has the activity of the full-length defensin sequence portion of one of SEQ ID NOs: 1054-1127 or 1129-1152, or encoded by one of SEQ ID NOs: 955-1028 or 1030-1053. The 1D defensin-encoding polynucleotides and constructs may be used according to embodiments of the invention to confer pesticidal and/or anti-fungal activity when introduced and expressed in a plant.

[025] In addition to newly discovered single and multi-domain defensins from plants, methods and compositions are provided for the construction, synthesis and expression of synthetic multi-domain defensin proteins comprising heterologous or non-naturally occurring combinations of two or more defensin regions or domains and one or more linker region(s) connecting or

bridging the two or more defensin regions together, as well as polynucleotides encoding these synthetic multi-domain defensins. For example, each of the defensin regions of a synthetic multi-domain defensin may comprise a defensin sequence portion of a 1D defensin or a defensin component of a 2D or MD defensin, as identified herein. These synthetic multi-domain defensin proteins may thus be referred to as chimeric multi-domain defensins comprising defensin region(s) and/or linker region(s) that are heterologous in their origin. In addition to their novel structure, synthetic multi-domain defensins may also have one or more novel properties or characteristics, such as increased accumulation when expressed in a plant cell and/or new, altered or enhanced anti-fungal and/or pesticidal activity, relative to existing or known defensins, such as 1D defensins comprising one of their defensin components. A synthetic multi-domain defensin of the invention may comprise a heterologous or non-naturally occurring combination of a first defensin domain or region, a second defensin domain or region, and a linker region, wherein the first defensin region and the second defensin region are linked or connected to each other by the linker region. For example, a synthetic 2D defensin of the invention can comprise these regions in the following order (in the N-terminal to C-terminal direction): (i) first defensin region, (ii) linker region, and (iii) second defensin region. Synthetic multi-domain defensins may further comprise additional defensin region(s) connected by additional linker region(s). Furthermore, a 3D defensin may have the following order: (i) first defensin region, (ii) first linker region, (iii) second defensin region, (iv) second linker region, and (v) third defensin region, whereas a 4D defensin may additionally comprise a third linker region and a fourth defensin region with the third linker region being between the third and fourth defensin regions, and so on.

[026] Accordingly, synthetic multi-domain defensin proteins or polypeptides of the invention may comprise combinations of two or more defensin domains or regions, each of the defensin region(s) comprising of one of the sequences provided herein as SEQ ID NOs: 559-662 and/or a defensin sequence portion of one of SEQ ID NOs: 1054-1152 and/or 1154, wherein the two or more defensin regions are linked or joined together by linker region(s), such as one or more of those provided herein as SEQ ID NOs: 153-202. For example, the first defensin region of a synthetic 2D defensin may comprise one of SEQ ID NOs: 559-662, and/or a defensin sequence portion of one of SEQ ID NOs: 1054-1152 and/or 1154, the second defensin region may comprise one of SEQ ID NOs: 559-662 and/or a defensin sequence portion of one of SEQ ID

NOs: 1054-1152 and/or 1154, which may be the same as, or different than, the first defensin region, and the linker region connecting the first and second defensin regions may be one of SEQ ID NOs: 153-202.

[027] In one embodiment of the invention, a “defensin sequence portion” shall refer to the sequence portion of a 1D defensin or defensin-like protein that excludes the N-terminal TS sequence and the C-terminal extension sequence (if present), as well as a polynucleotide sequence encoding the defensin sequence portion of a defensin or defensin-like protein. See, e.g., Table 15 below providing annotation for the defensin sequence portion of 1D defensin sequences. However, according to some embodiments, a defensin region near the N-terminus of a synthetic 2D or MD protein may retain its native TS sequence, and/or a defensin region near the C-terminus of the synthetic 2D or MD protein may retain its native C-terminal extension. Synthetic multi-domain defensins of the invention may also optionally comprise an N-terminal transit signal (TS) sequence, such as one of SEQ ID NOs: 809-954, and/or a C-terminal sequence, such as one identified by annotation in Table 2 below for SEQ ID NOs: 101 and 102. For example, synthetic multi-domain defensins of the invention may comprise one or more of those provided herein as SEQ ID NOs: 329-454 and 1156.

[028] Polynucleotides of the invention may include sequences encoding synthetic multi-domain defensins. These polynucleotides may comprise combinations of two or more polynucleotide sequences encoding defensin domains or regions, each of these polynucleotide sequences encoding a defensin region may comprise a polynucleotide sequence provided herein as SEQ ID NOs: 455-558 or a defensin sequence portion of one of the polynucleotide sequences provided herein as SEQ ID NOs: 955-1053 and/or 1153, linked or joined together by a polynucleotide sequence(s) encoding a linker region(s), such as one or more of those provided herein as SEQ ID NOs: 103-152. For example, the sequence encoding the first defensin region of a synthetic 2D defensin may comprise one of SEQ ID NOs: 455-558 or a defensin sequence portion of one of the polynucleotide sequences provided herein as SEQ ID NOs: 955-1053 and/or 1153, the sequence encoding the second defensin region of the synthetic 2D defensin may comprise one of SEQ ID NOs: 455-558 or a defensin sequence portion of one of the polynucleotide sequences provided herein as SEQ ID NOs: 955-1053 and/or 1153, which may be the same as or different than the sequence encoding the first defensin region, and the sequence encoding the linker region of the synthetic 2D defensin connecting the first and second defensin regions may be one of SEQ

ID NOs: 103-152. Polynucleotides of the invention may also optionally comprise sequences encoding a N-terminal targeting signal (TS) sequence, such as one of SEQ ID NOs: 663-808, and/or a C-terminal sequence, such as one of the sequences identified by annotation in Table 1 herein for SEQ ID NOs: 50 and 51. For example, polynucleotides encoding synthetic multi-domain defensins of the invention may comprise one or more of those provided herein as SEQ ID NOs: 203-328 and 1155.

[029] Multi-domain defensin proteins of the invention may further include variants and homologues of the native and synthetic defensin sequences provided herein. According to some embodiments, multi-domain defensin variants may comprise one or more mutations, deletions, insertions, etc., relative to a native or synthetic defensin or multi-domain defensin sequence, or other engineered defensin-like sequences. Multi-domain defensin proteins or polypeptides of the invention may comprise combinations of two or more defensin domains or regions, wherein one or more of those defensin regions has a relaxed sequence identity relative to one or identity to one or more of SEQ ID NOs: 559-662, and/or a defensin sequence portion of one or more of SEQ ID NOs: 1054-1152 and/or 1154. Each defensin region of a multi-domain defensin may be at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 97% identical, at least 98% identical, at least 99% identical, or 100% identical to one of SEQ ID NOs: 559-662 and/or a defensin sequence portion of one of SEQ ID NOs: 1054-1152 and/or 1154. A defensin region of a multi-domain defensin may further comprise a fragment comprising at least 25, at least 50, at least 75, or at least 100, contiguous amino acids of one of SEQ ID NOs: 1054-1152 and/or 1154. In some embodiments, a fragment has the activity of the full-length defensin region of one of SEQ ID NOs: 1054- 1154. Each of the linker domain(s) or region(s) of a multi-domain defensin protein may also have a relaxed sequence identity relative to SEQ ID NOs: 153-202. Each linker region of a multi-domain defensin may be at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 97% identical, at least 98% identical, at least 99% identical, or 100% identical to one of SEQ ID NOs: 153-202. Indeed, multi-domain defensin proteins of the invention may comprise a polypeptide sequence that is at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 97% identical, at least 98% identical, at least 99% identical, or 100% identical to any one of SEQ ID NOs: 52-102, 329-454, or 1156. A multi-domain defensin protein may further comprise a fragment comprising at least 50, at least 75, at least 100, at least 125, at least

150, at least 175, or at least 200 contiguous amino acids of any one of SEQ ID NOs: 52-102, 329-454 or 1156. In some embodiments, a fragment has the activity of SEQ ID NOs: 52-102, 329-454, or 1156. However, a linker region of a multi-domain may also be highly variable in sequence and length with little or no sequence identity or similarity to SEQ ID NOs: 153-202. A linker region may also comprise two or more linker sequences arranged in tandem, wherein each of the linker sequences may comprise one of SEQ ID NOs: 153-202, a polypeptide sequence having a relaxed sequence identity relative to SEQ ID NOs: 153-202, or other sequence.

[030] The N-terminal targeting signal (TS) and/or a C-terminal sequences of a multi-domain defensin (if present) may each also have relaxed sequence identity relative to the sequences provided herein. The N-terminal targeting signal (TS) sequence may be at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 97% identical, at least 98% identical, at least 99% identical, or 100% identical to one of SEQ ID NOs: 809-954. The C-terminal sequence may be at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 97% identical, at least 98% identical, at least 99% identical, or 100% identical to one of the C-terminal sequences identified by annotation in Table 2 for SEQ ID NOs: 101 and 102. According to some embodiments, however, the N-terminal and/or C-terminal sequences of a multi-domain defensin may instead be dissimilar or have a lower sequence identity relative to the sequences provided herein.

[031] Polynucleotides of the invention may comprise sequences encoding multi-domain defensin proteins with relaxed sequence identity relative to the sequences provided herein. Polynucleotides encoding these multi-domain defensins may comprise combinations of two or more sequences encoding defensin domains or regions, wherein one or more of the polynucleotide sequences encoding these defensin regions have a relaxed sequence identity relative to one or more of SEQ ID NOs: 455-558 and/or a defensin sequence portion of one or more of SEQ ID NOs: 955-1053 and/or 1153. Accordingly, polynucleotide sequences encoding each defensin region of a multi-domain defensin may be at least 70% identical, at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 97% identical, at least 98% identical, at least 99% identical, or 100% identical to one of SEQ ID NOs: 455-558 and/or a defensin sequence portion of one of SEQ ID NOs: 955-1053 and/or 1153. A defensin region of a multi-domain defensin may further comprise a fragment comprising at least 50, at least 75, at least 100, at least 125, at least 150, at least 175, or

at least 200 contiguous nucleotides of any one of SEQ ID NOs: 455-558 and/or a defensin sequence portion of one of SEQ ID NOs: 955-1053 and/or 1153. In some embodiments, a fragment encodes a protein having the activity of a protein encoded by SEQ ID NOs: 455-558 and/or a defensin sequence portion of one of SEQ ID NOs: 955-1053 and/or 1153. The polynucleotide sequences encoding the linker domain(s) or region(s) of a multi-domain defensin protein may also have a relaxed sequence identity relative to SEQ ID NOs: 103-152. Accordingly, the polynucleotide sequence encoding a linker region of a multi-domain defensin may be at least 70% identical, at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 97% identical, at least 98% identical, at least 99% identical, or 100% identical to one of SEQ ID NOs: 103-152. Indeed, polynucleotides encoding multi-domain defensin proteins of the invention may comprise a polynucleotide sequence that is at least 70% identical, at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 97% identical, at least 98% identical, at least 99% identical, or 100% identical to any one of SEQ ID NOs: 1-51, 203-328, or 1155. However, a polynucleotide sequence encoding the linker region(s) of a multi-domain may also be highly variable in sequence and length with little or no sequence identity of similarity to SEQ ID NOs: 103-152. As mentioned above, a linker region may also comprise two or more linker sequences arranged in tandem, wherein each of the linker sequences may comprise one of SEQ ID NOs: 103-152, a polynucleotide sequence having a relaxed sequence identity relative to SEQ ID NOs: 103-152, or other sequence.

[032] If present, polynucleotide sequences encoding the N-terminal targeting signal (TS) sequence and/or the C-terminal sequence of a multi-domain defensin may also have a relaxed sequence identity relative to sequences provided herein. The polynucleotide sequence encoding the N-terminal targeting signal (TS) sequence of a multi-domain defensin may be at least 70% identical, at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 97% identical, at least 98% identical, at least 99% identical, or 100% identical to one SEQ ID NOs: 663-808. The polynucleotide sequence encoding the C-terminal sequence of a multi-domain defensin may be at least 70% identical, at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 97% identical, at least 98% identical, at least 99% identical, or 100% identical to one of the C-terminal sequences identified in Table 1 for SEQ ID NOs: 50 and 51.

According to some embodiments, however, the N-terminal and/or C-terminal sequences of a multi-domain defensin may instead be dissimilar or have a lower sequence identity relative to the sequences provided herein.

[033] For purposes of the invention, the percent identity of two polynucleotide or polypeptide sequences may be determined by first optimally aligning the two sequences and then determining the percentage of nucleotide bases or amino acid residues that are the same between the two sequences over a comparison window, which may be over the full length of one of the two sequences. An optimal alignment is defined as a best fit or match of the two sequences with resistance or tolerance for any gaps in the alignment. A number of computerized programs and algorithms are known in the art for achieving an optimal alignment of two or more sequences (GAP, BESTFIT, FASTA, BLAST, Smith-Waterman). When optimally aligned, the percent identity of a subject sequence to a reference sequence is determined by taking the number of matched or identical bases or amino acids between the two sequences, dividing by the length of the reference sequence, and then multiplying the quotient by 100%.

[034] Multi-domain defensins of the invention may further have characteristic structural features, which may be related to their pesticidal and anti-fungal activity. These features may allow for the identification of additional defensins that may be used in designing novel multi-domain defensins. For example, multi-domain defensin proteins of the invention may be defined as comprising the following features: (a) a Pfam gamma-thionin domain (PF00304), as determined by a search of the Pfam protein families database using an E-value cutoff of $1e^{-3}$ (Finn, *et al. Nucleic Acids Research*, 2014, Database Issue 42:D222-D230); (b) polypeptides comprising more than 8 cysteine residues; and (c) polypeptides comprising two or more gamma-thionin domains (GXCX_nC) separated by a polypeptide linker sequence, wherein n is 3-22 amino acids in length, and X is any amino acid. These putative MD defensins may further have a protein length of at least 90 amino acids or greater and an absence of any premature stop codons in its coding sequence. Furthermore, each defensin region of a multi-domain defensin may be defined as having at least 6 cysteine residues, or at least 8 cysteine residues, and a predicted Cys-stabilized $\alpha\beta$ motif (CS $\alpha\beta$) structure when folded as described above. However, one or more of the defensin region(s) may each have fewer cysteines according to some embodiments, such as 4 or 5 cysteines in one or more of the defensin region(s). Thus, a multi-domain defensin may have

at least 8 cysteine residues, or at least 10 cysteine residues, at least 12 cysteine residues, or at least 14 cysteine residues, and/or two or more predicted CS α β structural motifs. Multi-domain defensins may also be defined functionally as having certain pesticidal or anti-fungal activity against one or more plant fungal pathogens.

[035] As introduced above, the invention provides synthetic 2D or other MD defensins comprising at least a first defensin region linked to a second defensin region by a linker region. The defensin regions of a multi-domain defensin protein may be homomeric (i.e., the defensin regions are the same) or heteromeric (i.e., the defensin regions are different). A 2D or MD defensin of the invention may comprise a native combination of defensin regions (i.e., comprising the same combination of defensin regions present in a native multi-domain defensin) or a heterologous combination of defensin regions (i.e., comprising defensin regions that do not exist together in a native multi-domain defensin and/or derived from different defensins). One or more of the defensin region(s) may be heterologous with respect to a linker region(s) of the MD defensin. Indeed, a multi-domain defensin of the disclosure may comprise a native combination of defensin regions that may be homomeric or heteromeric relative to each other but heterologous with respect to a linker region. Without being bound by any theory, synthetic multi-domain defensins having a linker region that is heterologous with respect to at least one defensin region may tend to accumulate to higher levels when expressed in a plant or plant cell and/or confer new or altered anti-fungal or anti-microbial activities.

[036] As further described above, embodiments of the disclosure provide nucleic acids and polynucleotides comprising one or more of SEQ ID NOs: 455-558 and/or a defensin sequence portion of one or more of 955-1053, such as SEQ ID NOs: 1-51, 203-328, or 1155 and defensin polypeptides or proteins encoded by these polynucleotide sequences. Defensin polypeptides or proteins may comprise one or more of SEQ ID NOs: 559-662 and/or a defensin sequence portion of one or more of 1054-1152, such as SEQ ID NOs: 52-102, 329-454, or 1156. Complements to any of the nucleic acid or polynucleotide sequences described herein are also provided. Polynucleotide molecules of the invention may encode a multi-domain defensin polypeptide comprising two or more defensin domains or regions, such as a 2D, 3D, 4D, or other MD defensin protein. Each defensin region of a multi-domain defensin is separated from all other defensin regions by one or more linker region(s) with at least one linker region being present between adjacent or neighboring defensin regions. In specific embodiments, a multi-domain

defensin protein may comprise two or more defensin domains each comprising 4, 5, 6, 7, 8, 9, 10, or more cysteine residues. In certain embodiments as explained above, a polynucleotide molecule provided herein may be defined as comprising one or more nucleotide sequences, each having at least 70%, at least 75%, 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%, at least 99%, or 100% sequence identity to the full length sequence of SEQ ID NOs: 1-51, 103-152, 203-328, 455-558, 663-808, 1155 and/or a defensin sequence portion of 955-1053 and/or 1153. Similarly, a polypeptide molecule provided herein may be defined as comprising one or more protein sequences, each having 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%, at least 99%, or 100% sequence identity to the full length sequence of SEQ ID NOs: 52-102, 153-202, 329-454, 559-662, 809-954, 1156, and/or a defensin sequence portion of 1054-1152 and/or 1154.

[037] Polynucleotide sequences of the disclosure may, when expressed in a plant, confer increased pesticidal or fungicidal activity and/or increased pest or fungal control to the plant. Indeed, polynucleotide constructs encoding multi-domain defensins of the disclosure may be used to generate transgenic plants with greater resistance or tolerance to one or more fungal pathogens. Such pesticidal or fungicidal activity may be effective against one or more of a *Fusarium*, *Colletotrichum*, *Stenocarpella*, and/or *Phakopsora* species, such as one or more of *Fusarium graminearum*, *Fusarium verticilloides*, *Colletotrichum graminicola*, *Stenocarpella maydis*, and/or *Phakopsora pachyrhizi*. Such pesticidal activity may be further effective against other microbial plant pathogen(s) and/or plant pest(s), such as oomycetes, bacteria, insects, nematodes, etc.

[038] The invention also provides recombinant DNA constructs comprising the polynucleotide sequences described herein, as well as plants, plant cells and seeds transformed therewith. In one embodiment, such DNA constructs may be used in expressing nucleotide sequences encoding 1D, 2D, or other MD defensins in plants for the purposes of protecting the plant from plant pests, such as one or more fungi. In another embodiment, such constructs may be of use in generating transgenic or recombinant plants with increased or enhanced resistance or tolerance to plant fungal pathogens. Thus, embodiments of the invention further comprise transformation vectors comprising a defensin-encoding DNA construct of the invention or a portion of such defensin-encoding DNA construct. According to some embodiments, defensin-encoding DNA constructs

and vectors may be used for generating 1D, 2D, or MD defensin transcripts and/or proteins in microorganisms such as bacteria or yeast.

[039] A recombinant DNA construct, molecule or vector of the invention may comprise a polynucleotide expression cassette comprising a coding sequence that encodes a 1D defensin or multi-domain defensin protein, wherein the coding sequence is operably linked to a promoter that is functional in a plant cell. Alternatively, the defensin-encoding polynucleotide sequence may be operably linked to a promoter suitable for expression of a defensin protein in a microorganism. Any suitable promoter known in the art may be used to express a defensin coding sequence of the invention in a plant, such as a constitutive, tissue-specific, tissue-enhanced or tissue-preferred, developmental, inducible, disease inducible, etc., promoter. Further, the plant promoter operably linked to the defensin coding sequence may be native, homologous or heterologous relative to the plant species to be transformed with the defensin-encoding DNA construct, or alternatively, the promoter may be chimeric or synthetic. A synthetic nucleotide sequence may be a nucleotide sequence that is not known to occur in nature or that is not naturally occurring. Generally, such a synthetic nucleotide sequence will comprise at least one nucleotide difference when compared to any other naturally occurring nucleotide sequence. It is recognized that a gene-regulatory element of the invention comprises a synthetic nucleotide sequence. Preferably, the synthetic nucleotide sequence shares little or no extended homology to natural sequences. Extended homology in this context generally refers to 100% sequence identity extending beyond about 25 nucleotides of contiguous sequence. A synthetic gene-regulatory element of the invention comprises a synthetic nucleotide sequence.

[040] The polynucleotide coding sequence for expressing the defensin protein may also be operatively linked to one or more additional regulatory element(s), such as an enhancer(s), leader, transcription start site (TSS), linker, 5' and 3' untranslated region(s), intron(s), polyadenylation signal, termination region or sequence, etc., that are suitable or necessary for regulating or allowing expression of the multi-domain defensin in a plant cell. Such additional regulatory element(s) may be optional and/or used to enhance or optimize expression of the defensin transgene or coding sequence. The term "operably linked" refers to a functional connection between the two sequences. A promoter or enhancer is "operably linked" to a transgene or coding sequence by affecting, causing, driving, promoting, etc., transcription and expression of the transgene or coding sequence.

[041] According to embodiments of the invention, the term “recombinant” in reference to a DNA molecule, construct, vector, etc., refers to a DNA molecule or sequence that is not found in nature and/or is present in a context in which it is not found in nature, including a DNA molecule, construct, etc., comprising a combination of DNA sequences that would not naturally occur contiguously or in close proximity together without human intervention, and/or a DNA molecule, construct, etc., comprising at least two DNA sequences that are heterologous with respect to each other. A recombinant DNA molecule, construct, etc., may comprise DNA sequence(s) that is/are separated from other polynucleotide sequence(s) that exist in proximity to such DNA sequence(s) in nature, and/or a DNA sequence that is adjacent to (or contiguous with) other polynucleotide sequence(s) that are not naturally in proximity with each other. A recombinant DNA molecule, construct, etc., may also refer to a DNA molecule or sequence that has been genetically engineered and constructed outside of a cell. For example, a recombinant DNA molecule may comprise any suitable plasmid, vector, etc., and may include a linear or circular DNA molecule. Such plasmids, vectors, etc., may contain various maintenance elements including a prokaryotic origin of replication and selectable marker, as well as a defensin expressing transgene or cassette perhaps in addition to a plant selectable marker gene, etc. The term “recombinant” may also further refer to proteins expressed or encoded by these recombinant DNA molecules, constructs, etc., if they comprise non-native sequences.

[042] As used herein, the term “isolated” in reference to a DNA molecule, construct, vector, etc., may refer to a DNA molecule or sequence that is not found in nature and/or is present in a context in which it is not found in nature. The term “isolated” may also refer to a nucleic acid molecule that has undergone at least one step towards being isolated or concentrated or enriched from a more complex solution or source. The term “isolated,” however, is in no way intended to limit the nucleic acid molecule to a particular location or state, and the invention extends to the nucleic acid molecule when introduced into the genome of a cell or when it is resident in progeny of cells into which the nucleic acid molecule has been introduced into its genome.

[043] According to another broad aspect of the invention, methods are provided for transforming a plant cell, tissue or explant with a recombinant DNA molecule or construct comprising a defensin transgene or coding sequence to produce a defensin containing transgenic plant. Numerous methods for transforming chromosomes in a plant cell with a recombinant DNA molecule are known in the art, which may be used according to methods of the invention to

produce a transgenic plant cell and plant. Any suitable method or technique for transformation of a plant cell known in the art may be used according to present methods. Effective methods for transformation of plants include bacterially mediated transformation, such as *Agrobacterium*-mediated or *Rhizobium*-mediated transformation, and microprojectile bombardment-mediated transformation. Other methods for plant transformation are also known in the art including, but not limited to, gene editing, site-directed integration, PEG-mediated transformation, protoplast transformation, electroporation, microinjection, agitation with silica/carbon fibers, virus-mediated or liposome-mediated transformation, etc. A variety of methods are known in the art for transforming explants with a transformation vector via bacterially mediated transformation or microprojectile bombardment and then subsequently culturing, etc, those explants to regenerate or develop transgenic plants. Methods are further provided for expressing a multi-domain defensin transgene of the invention in one or more plant cells or tissues under the control of a promoter operable in a plant cell. Such methods may be used to confer anti-fungal and pesticidal properties to a plant and combat a fungal infection.

[044] Transformation of a target plant material or explant may be practiced in tissue culture on nutrient media, for example a mixture of nutrients that allow cells to grow in vitro. Recipient cell targets or explants may include, but are not limited to, meristems, shoot tips, protoplasts, hypocotyls, calli, immature or mature embryos, shoots, buds, nodal sections, leaves, gametic cells such as microspores, pollen, sperm and egg cells, etc., or any suitable portions thereof. It is contemplated that any transformable cell or tissue from which a fertile plant can be regenerated or grown/developed may be used as a target for transformation. Transformed explants, cells or tissues may be subjected to additional culturing steps, such as callus induction, selection, regeneration, etc., as known in the art. Transformed cells, tissues or explants containing a recombinant DNA insertion may be grown, developed or regenerated into transgenic plants in culture, plugs or soil according to methods known in the art. Transgenic plants may be further crossed to themselves or other plants to produce transgenic seeds and progeny. A transgenic plant may also be prepared by crossing a first plant comprising the recombinant DNA sequence or transformation event with a second plant lacking the insertion. For example, a recombinant DNA sequence may be introduced into a first plant line that is amenable to transformation, which may then be crossed with a second plant line to introgress the recombinant DNA sequence into the second plant line. Progeny of these crosses can be further back crossed into the more

desirable line multiple times, such as through 6 to 8 generations or back crosses, to produce a progeny plant with substantially the same genotype as the original parental line but for the introduction of the recombinant DNA sequence.

[045] A recombinant DNA molecule or construct of the invention may be included within a DNA transformation vector for use in transformation of a target plant cell, tissue or explant. Such a transformation vector of the invention may generally comprise sequences or elements necessary or beneficial for effective transformation in addition to the defensin expressing transgene or expression cassette. For *Agrobacterium*-mediated transformation, the transformation vector may comprise an engineered transfer DNA (or T-DNA) segment or region having two border sequences, a left border (LB) and a right border (RB), flanking at least the defensin expressing transgene or cassette, such that insertion of the T-DNA into the plant genome will create a transformation event for the defensin transgene or cassette. In other words, the defensin transgene would be located between the left and right borders of the T-DNA, perhaps along with an additional transgene(s) or expression cassette(s), such as a plant selectable marker transgene and/or other gene(s) of agronomic interest that may confer a trait or phenotype of agronomic interest to a plant. In addition to protein encoding sequences, a gene of agronomic interest may further comprise a polynucleotide sequence encoding a RNA suppression element. According to alternative embodiments, the defensin-encoding transgene or cassette and the plant selectable marker transgene (or other gene of agronomic interest) may be present in separate T-DNA segments on the same or different recombinant DNA molecule(s), such as for co-transformation. A transformation vector or construct may further comprise prokaryotic maintenance elements, which for *Agrobacterium*-mediated transformation may be located in the vector backbone outside of the T-DNA region(s).

[046] A plant selectable marker transgene in a transformation vector or construct of the invention may be used to assist in the selection of transformed cells or tissue due to the presence of a selection agent, such as an antibiotic or herbicide, wherein the plant selectable marker transgene provides tolerance or resistance to the selection agent. Thus, the selection agent may bias or favor the survival, development, growth, proliferation, etc., of transformed cells expressing the plant selectable marker gene, such as to increase the proportion of transformed cells or tissues in the R₀ plant. Commonly used plant selectable marker genes include, for example, those conferring tolerance or resistance to antibiotics, such as kanamycin and

paromomycin (*nptII*), hygromycin B (*aph IV*), streptomycin or spectinomycin (*aadA*) and gentamycin (*aac3* and *aacC4*), or those conferring tolerance or resistance to herbicides such as glufosinate (*bar* or *pat*), dicamba (*DMO*) and glyphosate (*aroA* or *EPSPS*). Plant screenable marker genes may also be used, which provide an ability to visually screen for transformants, such as luciferase or green fluorescent protein (GFP), or a gene expressing a beta glucuronidase or *uidA* gene (GUS) for which various chromogenic substrates are known.

[047] Additionally provided herein are transgenic plants, plant parts, propagules and plant cells transformed with a recombinant DNA construct or vector of the invention having a polynucleotide sequence encoding a multi-domain defensin. Such a transgenic plant, plant part, or plant cell may comprise a transformation event or insertion of a defensin-encoding recombinant DNA construct or sequence of the invention into the genome of at least one plant cell thereof. A transgenic plant comprising the defensin-encoding DNA construct or sequence may be produced by any suitable transformation method, which may be followed by selection, culturing, regeneration, development, etc., as desired or needed to produce a transgenic R₀ plant, which may then be selfed or crossed to other plants to generate R₁ seed and subsequent progeny generations and seed through additional crosses, etc. Similarly, embodiments of the invention further include a plant cell, tissue, explant, etc., comprising one or more transgenic cells having a transformation event or genomic insertion of a recombinant DNA or polynucleotide sequence comprising the defensin-encoding transgene, construct or sequence. Transgenic plants comprising a defensin-encoding transgene may have increased resistance or tolerance to one or more plant pests or fungi and/or increased anti-fungal properties or activities, relative to a wild type or control plant not having the defensin-encoding transgene.

[048] For purposes of the invention, a “plant” may include an explant, embryo, seedling, plantlet or whole plant at any stage of regeneration or development. As used herein, a “transgenic plant” refers to a plant whose genome has been altered by the integration or insertion of a recombinant DNA molecule, construct or sequence. A transgenic plant includes an R₀ plant developed or regenerated from an originally transformed plant cell(s) as well as progeny transgenic plants in later generations or crosses from the R₀ transgenic plant. As used herein, a “plant part” may refer to any organ or intact tissue of a plant, such as a meristem, shoot organ/structure (e.g., leaf, stem and tuber), root, flower or floral organ/structure (e.g., bract, sepal, petal, stamen, carpel, anther, pollen and ovule), seed (e.g., embryo, endosperm, and seed

coat), fruit (e.g., the mature ovary), propagule, or other plant tissues (e.g., cuttings, vascular tissue, ground tissue, and the like), callus, protoplasts, or any portion thereof. Plant parts of the invention may be viable, nonviable, regenerable, and/or non-regenerable. A “propagule” may include any plant part that is capable of growing into an entire plant. As used herein, a “transgenic plant cell” simply refers to any plant cell that is transformed with a stably-integrated recombinant DNA molecule or sequence. A transgenic plant cell may include an originally-transformed plant cell, a transgenic plant cell of a regenerated or developed R₀ plant, or a transgenic plant cell from any progeny plant or offspring of the transformed R₀ plant, including cell(s) of a plant seed or embryo, or a cultured plant or callus cell, etc.

[049] The transformed plants may be analyzed for the presence of the defensin-encoding sequence or transgene and/or its expression level and/or profile in a plant or plant cell or tissue. Those of skill in the art are aware of numerous methods available for the analysis of transformed plants. For example, methods for plant analysis include, but are not limited to, Southern blots or northern blots, PCR-based approaches, biochemical analyses, phenotypic screening methods, and immunoblotting assays. The expression of a transcribable DNA molecule or transgene can be measured, for example, using TaqMan® (Applied Biosystems, Foster City, CA) reagents and methods as described by the manufacturer and PCR cycle times determined using the TaqMan® Testing Matrix. As an alternative example, the Invader® (Third Wave Technologies, Madison, WI) reagents and methods as described by the manufacturer can be used to evaluate transgene expression.

[050] The transgenic plants of the invention comprising a defensin-encoding polynucleotide sequence or construct can be any agricultural crop species. The species may be a monocotyledonous or dicotyledonous plant. Particularly useful plants may include but are not limited to wheat, carrot, sorghum rice, barley, soybean, potato, corn, Brassica, canola, tomato, alfalfa, peanut, sugarcane and cotton. The plant can be an R₀ transgenic plant (*i.e.*, a plant derived from the original transformed tissue). The transgenic plant can also be any generation of progeny plants derived from the original R₀ transgenic plant, such as by any known method of crossing, introgressing, converting or propagating plants.

[051] The invention also provides methods for producing a transgenic plant with increased pest resistance or tolerance and/or pesticidal activity comprising introducing or transforming into the

plant a recombinant DNA construct encoding a defensin protein as described herein. Such a method may further comprise: growing said plant to produce a further generation; and selecting at least one plant from said further generation comprising the recombinant DNA construct, wherein said plant has increased pest resistance or tolerance and/or anti-fungal activity relative to a control plant that does not comprise the recombinant DNA construct. Further provided is a method for producing plants with increased pest resistance or tolerance and/or pesticidal activity comprising crossing a transgenic plant of the invention with itself or a second plant to produce at least a first progeny plant, wherein said progeny plant comprises increased pest resistance or tolerance. Seed from plants comprising the recombinant DNA construct and having increased pesticidal activity and/or pest resistance or tolerance may be obtained from any number of sources.

[052] According to another aspect of the invention, pesticidal compositions are provided comprising a defensin polypeptide or protein of the invention having pesticidal or anti-fungal activity. Such pesticidal compositions may further comprise other compounds or active pesticidal molecules that may be effective against one or more insects, nematodes, microbes, fungi, nematodes, or viruses. These pesticidal compositions comprising one or more recombinant defensin(s) of the invention may be formulated as a solid or liquid and/or applied as a topical, foliar, soil, or granular application or treatment to prevent or inhibit fungal infections and/or other plant pest infestations. Examples of other types of plant pests include insects, nematodes, weeds, microbes, such as bacteria, fungi, and viruses, etc. Methods for formulating a pesticidal composition of the invention may be similar to methods known in the art for other pesticidal formulations. Ingredients or components for pesticidal composition of the invention may include one or more carriers, diluents, surfactants, or other formulation ingredients known in the art.

EXAMPLES

Example 1. Identification of Defensins with Two or More Defensin Regions

[053] Plant defensins are small polypeptides comprising an N-terminal signal peptide and a defensin region comprising approximately 50 amino acids and usually having 6 to 8 cysteines. The invention provides new multi-domain defensins comprising two defensin regions (2D

defensins) or multiple defensin regions (MD defensins) connected by a short linker region. Many of these 2D or MD defensins were further found to comprise an N-terminal transit signal (TS) sequence or region that may generally become cleaved from the remainder of the protein to produce a mature defensin protein. Some of the 2D or MD defensins were also found to comprise a C-terminal extension sequence.

[054] To identify novel multi-domain defensins, genomic and transcriptome sequences from 19 different plant species were mined and analyzed to identify longer polypeptide sequences having one or more structural or functional features characteristic of defensins. Polypeptides meeting the following criteria were identified as multi-domain defensins and subjected to further analysis: (a) Polypeptides comprising a Pfam gamma-thionin domain (PF00304), as determined by a search of the Pfam protein families database using an E-value cutoff of $1e^{-3}$ (Finn, *et al. Nucleic Acids Research*, 2014, Database Issue 42:D222-D230); (b) Polypeptides comprising at least 100 amino acids; (c) Polypeptides comprising at least 8 cysteine residues; and (d) Polypeptides comprising two or more extended gamma-thionin domains (GXCX_nC) separated by a short polypeptide linker sequence, wherein the n is 3-22 amino acids in length, and X is any amino acid. These putative multi-domain defensins were further identified by the absence of a premature stop codon in their coding sequence.

[055] A total of 51 multi-domain defensin proteins including mostly 2D defensins were identified (see below) by mining the genomes of the following 19 plant genomes: *Arabidopsis lyrata*, *Arabidopsis thaliana*, *Brassica rapa*, *Brassica napus*, *Carica papaya*, *Citrus clementina*, *Citrus sinensis*, *Cucumis melo*, *Cucumis sativus*, *Glycine max*, *Kochia scoparia*, *Malus domestica*, *Medicago truncatula*, *Portulaca oleracea*, *Prunus persica*, *Raphanus raphanistrum*, *Rosa blanda*, *Trifolium repens*, and *Zea mays*. The detection of mRNA transcripts for these multi-domain defensins and their discovery in many plant species supports the conclusion that these multi-domain defensins are expressed in plants.

Example 2. Annotation of Multi-domain Defensins

[056] Full-length multi-domain protein sequences identified by the above criteria were further analyzed using a combination of manual calls, multiple sequence alignments, and N-terminal cleavage site (SignalP) predictions to identify and locate the N-terminal TS or signal peptide

sequences, defensin regions, intervening linker regions, and C-terminal extensions. Linker regions were identified as being between the last cysteine residue of the defensin region on the N-terminal side and one or two amino acids upstream of the first cysteine residue in the defensin region on the C-terminal side. Determining the position of the C-terminal end of the linker region of an identified 2D or MD defensin is based on the expected position of the predicted cleavage site of the downstream defensin region on the C-terminal side of the linker region, although this site may generally not become cleaved, unlike the cleavage site between the N-terminal TS sequence and the adjacent defensin region.

[057] The 51 multi-domain defensin sequences identified from the 19 plant genomes are shown in Tables 1 and 2. SEQ ID NOs: 1-51 represent nucleotide coding sequences of the newly identified 2D or MD defensins, and SEQ ID NOs: 52-102 represent the corresponding polypeptide sequences of these identified 2D or MD defensins. For each of the identified 2D and MD defensins, the sequence boundaries (start and stop positions) for each of the defensin regions (D1, D2, etc.), linker region(s) (L1, L2, etc.), N-terminal transit signal (TS) sequence, and C-terminal (CT) extension sequence (if present; “NA” if not present) are shown for the DNA and protein sequences in Tables 1 and 2, respectively. The full coding sequence (CDS) is also provided in Table 1. The defensin components of these 2D or MD defensins correspond to each of the defensin regions annotated in Tables 1 and 2. For illustration, the defensin components of “ARAlly_AFP26” on a nucleotide level correspond to nucleotides positions 82-207 (D1) and 229-372 (D2) of SEQ ID NO: 1, whereas the defensin components of “ARAlly_AFP26” on a protein level correspond to amino acid positions 28-69 (D1) and 77-124 (D2) of SEQ ID NO: 52.

Table 1. Nucleotide start and end positions for each region within the identified MD or 2D defensin sequences.

Locus ID	DNA SEQ ID NO.	Protein SEQ ID NO.	TS		CDS		CT		D1		D2		D3		D4		L1		L2		L3	
			TS Start	TS End	CDS Start	CDS End	CT Start	CT End	D1 Start	D1 End	D2 Start	D2 End	D3 Start	D3 End	D4 Start	D4 End	Linker1 Start	Linker1 End	Linker2 Start	Linker2 End	Linker3 Start	Linker3 End
ARALY_AFP26	1	52	1	81	82	372	NA	NA	82	207	229	372					208	228				
At_AFP10	2	53	1	63	64	387	NA	NA	64	234	259	387					235	258				
At_AFP11	3	54	1	78	79	381	NA	NA	79	210	241	381					211	240				
At_AFP12	4	55	1	81	82	360	NA	NA	82	195	220	360					196	219				
At_AFP8	5	56	1	78	79	378	NA	NA	79	204	259	378					205	258				
At_AFP9	6	57	1	78	79	366	NA	NA	79	210	226	366					211	225				
BASsc_AFP21	7	58	1	21	22	354	NA	NA	22	162	193	354					163	192				
Bn_AFP35	8	59	1	99	100	402	NA	NA	100	228	259	402					229	258				
Bn_AFP36	9	60	1	18	19	330	NA	NA	19	150	187	330					151	186				
Bn_AFP47	10	61	1	78	79	366	NA	NA	79	204	238	366					205	237				
Bn_AFP74	11	62	1	78	79	381	NA	NA	79	210	238	381					211	237				
Bn_AFP75	12	63	1	45	46	357	NA	NA	46	180	214	357					181	213				
Bn_AFP76	13	64	1	78	79	381	NA	NA	79	210	238	381					211	237				
Bn_AFP79	14	65	1	78	79	366	NA	NA	79	192	223	366					193	222				
Bn_AFP80	15	66	1	78	79	366	NA	NA	79	192	223	366					193	222				

Locus ID	DNA SEQ ID NO.	Protein SEQ ID NO.	TS		CDS		CT		D1		D2		D3		D4		L1		L2		L3	
			TS Start	TS End	CDS Start	CDS End	CT Start	CT End	D1 Start	D1 End	D2 Start	D2 End	D3 Start	D3 End	D4 Start	D4 End	Linker1 Start	Linker1 End	Linker2 Start	Linker2 End	Linker3 Start	Linker3 End
Br_AFP1	16	67	1	42	43	333	NA	NA	43	177	241	333					178	240				
Br_AFP2	17	68	1	78	79	360	NA	NA	79	198	220	360					199	219				
CARpa_AFP1	18	69	1	45	46	402	NA	NA	46	192	217	402					193	216				
CARpa_AFP2	19	70	1	63	64	420	NA	NA	64	207	232	420					208	231				
CARpa_AFP3	20	71	1	69	70	384	NA	NA	70	192	202	384					193	201				
CITcl_AFP10	21	72	1	81	82	393	NA	NA	82	198	208	393					199	207				
CITcl_AFP11	22	73	1	66	67	402	NA	NA	67	195	220	402					196	219				
CITcl_AFP2	23	74	1	90	91	408	NA	NA	91	225	247	408					226	246				
CITcl_AFP3	24	75	1	75	76	390	NA	NA	76	216	250	390					217	249				
CITsi_AFP1	25	76	1	93	94	411	NA	NA	94	216	226	411					217	225				
CITsi_AFP2	26	77	1	54	55	369	NA	NA	55	195	226	369					196	225				
CUCme_AFP1	27	78	1	24	25	345	NA	NA	25	168	205	345					169	204				
CUCsa_AFP3	28	79	1	24	25	345	NA	NA	25	168	205	345					169	204				
Gm_AFP1	29	80	1	81	82	411	NA	NA	82	207	217	411					208	216				
Gm_AFP2	30	81	1	81	82	387	NA	NA	82	207	244	387					208	243				
MALdo_AFP11	31	82	1	87	88	408	NA	NA	88	219	250	408					220	249				

Locus ID	DNA SEQ ID NO.	Protein SEQ ID NO.	TS		CDS		CT		D1		D2		D3		D4		L1		L2		L3	
			TS Start	TS End	CDS Start	CDS End	CT Start	CT End	D1 Start	D1 End	D2 Start	D2 End	D3 Start	D3 End	D4 Start	D4 End	Linker1 Start	Linker1 End	Linker2 Start	Linker2 End	Linker3 Start	Linker3 End
Mt_AFP14	32	83	1	87	88	408	NA	NA	88	237	259	408					238	258				
Mt_AFP60	33	84	1	84	85	390	NA	NA	85	204	244	390					205	243				
Mt_AFP65	34	85	1	78	79	384	NA	NA	79	204	235	384					205	234				
Mt_AFP66	35	86	1	69	70	285	NA	NA	70	105	142	285					106	141				
Mt_AFP67	36	87	1	81	82	387	NA	NA	79	207	235	387					208	234				
Mt_AFP77	37	88	1	81	82	390	NA	NA	82	189	232	390					190	231				
Mt_AFP78	38	89	1	78	79	384	NA	NA	79	207	238	384					208	237				
PORol_AFP1	39	90	1	63	64	360	NA	NA	64	228	256	360					229	255				
PORol_AFP2	40	91	NA	NA	1	297	NA	NA	1	165	193	297					166	192				
PORol_AFP27	41	92	1	87	88	390	NA	NA	88	219	241	390					220	240				
PRUpe_AFP1	42	93	1	18	19	402	NA	NA	19	201	211	402					202	210				
RAPra_AFP23	43	94	1	96	97	411	NA	NA	97	228	265	411					229	264				
RAPra_AFP24	44	95	1	78	79	384	NA	NA	79	213	238	384					214	237				
RAPra_AFP25	45	96	1	27	28	345	NA	NA	28	165	199	345					166	198				
RAPra_AFP26	46	97	1	9	10	294	NA	NA	10	132	163	294					133	162				
RAPra_AFP27	47	98	1	78	79	363	NA	NA	79	201	232	363					202	231				

Locus ID	DNA SEQ ID NO.	Protein SEQ ID NO.	TS		CDS		CT		D1		D2		D3		D4		L1		L2		L3	
			TS Start	TS End	CDS Start	CDS End	CT Start	CT End	D1 Start	D1 End	D2 Start	D2 End	D3 Start	D3 End	D4 Start	D4 End	Linker1 Start	Linker1 End	Linker2 Start	Linker2 End	Linker3 Start	Linker3 End
ROSbl_AFP11	48	99	1	78	79	360	NA	NA	79	204	214	360					205	213				
TRlre_AFP5	49	100	1	84	85	387	NA	NA	85	204	241	387					205	240				
Zm_AFP101	50	101	1	24	25	420	421	474	25	120	283	420					121	282				
Zm_AFP100	51	102	1	162	163	966	967	1002	163	312	397	534	613	750	829	966	313	396	535	612	751	828

Table 2. Protein start and end positions for each region within the identified MD or 2D defensin sequences.

Locus ID	DNA SEQ ID NO.	Protein SEQ ID NO.	TS		CT		D1		D2		D3		D4		L1		L2		L3	
			TS Start	TS End	CT Start	CT End	D1 Start	D1 End	D2 Start	D2 End	D3 Start	D3 End	D4 Start	D4 End	Linker1 Start	Linker1 End	Linker2 Start	Linker2 End	Linker3 Start	Linker3 End
ARALY_AFP26	1	52	1	27	NA	NA	28	69	77	124					70	76				
At_AFP10	2	53	1	21	NA	NA	22	78	87	129					79	86				
At_AFP11	3	54	1	26	NA	NA	27	70	81	127					71	80				
At_AFP12	4	55	1	27	NA	NA	28	65	74	120					66	73				
At_AFP8	5	56	1	26	NA	NA	27	68	87	126					69	86				
At_AFP9	6	57	1	26	NA	NA	27	70	76	122					71	75				
BASsc_AFP21	7	58	1	7	NA	NA	8	54	65	118					55	64				
Bn_AFP35	8	59	1	33	NA	NA	34	76	87	134					77	86				
Bn_AFP36	9	60	1	6	NA	NA	7	50	63	110					51	62				
Bn_AFP47	10	61	1	26	NA	NA	27	68	80	122					69	79				
Bn_AFP74	11	62	1	26	NA	NA	27	70	80	127					71	79				
Bn_AFP75	12	63	1	15	NA	NA	16	60	72	119					61	71				
Bn_AFP76	13	64	1	26	NA	NA	27	70	80	127					71	79				
Bn_AFP79	14	65	1	26	NA	NA	27	64	75	122					65	74				
Bn_AFP80	15	66	1	26	NA	NA	27	64	75	122					65	74				

Locus ID	DNA SEQ ID NO.	Protein SEQ ID NO.	TS		CT		D1		D2		D3		D4		L1		L2		L3	
			TS Start	TS End	CT Start	CT End	D1 Start	D1 End	D2 Start	D2 End	D3 Start	D3 End	D4 Start	D4 End	Linker1 Start	Linker1 End	Linker2 Start	Linker2 End	Linker3 Start	Linker3 End
Br_AFP1	16	67	1	14	NA	NA	15	59	81	111					60	80				
Br_AFP2	17	68	1	26	NA	NA	27	66	74	120					67	73				
CARpa_AFP1	18	69	1	15	NA	NA	16	64	73	134					65	72				
CARpa_AFP2	19	70	1	21	NA	NA	22	69	78	140					70	77				
CARpa_AFP3	20	71	1	23	NA	NA	24	64	68	128					65	67				
CITcl_AFP10	21	72	1	27	NA	NA	28	66	70	131					67	69				
CITcl_AFP11	22	73	1	22	NA	NA	23	65	74	134					66	73				
CITcl_AFP2	23	74	1	30	NA	NA	31	75	83	136					76	82				
CITcl_AFP3	24	75	1	25	NA	NA	26	72	84	130					73	83				
CITsi_AFP1	25	76	1	31	NA	NA	32	72	76	137					73	75				
CITsi_AFP2	26	77	1	18	NA	NA	19	65	76	123					66	75				
CUCme_AFP1	27	78	1	8	NA	NA	9	56	69	115					57	68				
CUCsa_AFP3	28	79	1	8	NA	NA	9	56	69	115					57	68				
Gm_AFP1	29	80	1	27	NA	NA	28	69	73	137					70	72				
Gm_AFP2	30	81	1	27	NA	NA	28	69	82	129					70	81				
MALdo_AFP11	31	82	1	29	NA	NA	30	73	84	136					74	83				

Locus ID	DNA SEQ ID NO.	Protein SEQ ID NO.	TS		CT		D1		D2		D3		D4		L1		L2		L3	
			TS Start	TS End	CT Start	CT End	D1 Start	D1 End	D2 Start	D2 End	D3 Start	D3 End	D4 Start	D4 End	Linker1 Start	Linker1 End	Linker2 Start	Linker2 End	Linker3 Start	Linker3 End
Mt_AFP14	32	83	1	29	NA	NA	30	79	87	136					80	86				
Mt_AFP60	33	84	1	28	NA	NA	29	68	82	130					69	81				
Mt_AFP65	34	85	1	26	NA	NA	27	68	79	128					69	78				
Mt_AFP66	35	86	1	23	NA	NA	24	35	48	95					36	47				
Mt_AFP67	36	87	1	26	NA	NA	27	69	79	129					70	78				
Mt_AFP77	37	88	1	27	NA	NA	28	63	78	130					64	77				
Mt_AFP78	38	89	1	26	NA	NA	27	69	80	128					70	79				
PORol_AFP1	39	90	1	21	NA	NA	22	76	86	120					77	85				
PORol_AFP2	40	91	NA	NA	NA	NA	1	55	65	99					56	64				
PORol_AFP27	41	92	1	29	NA	NA	30	73	81	130					74	80				
PRUpe_AFP1	42	93	1	6	NA	NA	7	67	71	134					68	70				
RAPra_AFP23	43	94	1	32	NA	NA	33	76	89	137					77	88				
RAPra_AFP24	44	95	1	26	NA	NA	27	71	80	128					72	79				
RAPra_AFP25	45	96	1	9	NA	NA	10	55	67	115					56	66				
RAPra_AFP26	46	97	1	3	NA	NA	4	44	55	98					45	54				
RAPra_AFP27	47	98	1	26	NA	NA	27	67	78	121					68	77				

Locus ID	DNA SEQ ID NO.	Protein SEQ ID NO.	TS		CT		D1		D2		D3		D4		L1		L2		L3	
			TS Start	TS End	CT Start	CT End	D1 Start	D1 End	D2 Start	D2 End	D3 Start	D3 End	D4 Start	D4 End	Linker1 Start	Linker1 End	Linker2 Start	Linker2 End	Linker3 Start	Linker3 End
ROSbl_AFP11	48	99	1	26	NA	NA	27	68	72	120					69	71				
TRlre_AFP5	49	100	1	28	NA	NA	29	68	81	129					69	80				
Zm_AFP101	50	101	1	8	967	1002	9	40	95	140					41	94				
Zm_AFP100	51	102	1	54	421	474	55	104	133	178	205	250	277	322	105	132	179	204	251	276

Example 3. Identification of Linker Regions from Multi-Domain Defensins

[058] Upon examination of the discovered multi-domain plant defensins, linker regions were identified between the defensin regions. The unique linker regions identified from the 2D and MD defensins are shown in Table 3. SEQ ID NOs: 103-152 represent nucleotide sequences encoding these identified defensin linker regions, and SEQ ID NOs: 153-202 represent the corresponding polypeptide sequences of these defensin linker regions. As can be seen in Table 3, there were 50 unique linker region DNA and protein sequences identified from these MD defensins since two of the linker regions for the 4D defensin (Zm_AFP100) are the same (SEQ ID NOs: 152 and 202). N-terminal TS sequences and C-terminal extension sequences (when present) for each of these identified MD defensins are further shown by sequence position annotations in Tables 1 and 2 above. The lengths of the TS sequences (if present) were observed to vary from a few amino acids to over 30 amino acids, and the C-terminal extension sequence was generally absent from the identified 2D defensins but was present in a 2D defensin (Zm_AFP101) and a 4D defensin (Zm_AFP100) at a variable length of about 35-50 amino acids. Most of the linkers were found to be proline-rich, while others were enriched with glycine or charged amino acids. Many of the linkers were approximately 10-20 amino acids in length, but a small subset of linkers had either very short linkers of only a few amino acids or longer linker sequence lengths of 20-65 amino acids.

Table 3. 2D and MD Defensin Linker Regions.

Locus ID	Linker ID	DNA SEQ ID NO	Protein SEQ ID NO
ARAl _y _AFP26	ARAl _y 26L10	103	153
At_AFP10	At10L18	104	154
At_AFP11	At11L19	105	155
At_AFP12	At12L20	106	156
At_AFP8	At8L21	107	157
At_AFP9	At9L22	108	158
BASsc_AFP21	BASsc21L23	109	159
Bn_AFP35	Bn35L11	110	160
Bn_AFP36	Bn36L1	111	161

Locus ID	Linker ID	DNA SEQ ID NO	Protein SEQ ID NO
Bn_AFP47	Bn47L2	112	162
Bn_AFP74	Bn74L3	113	163
Bn_AFP75	Bn75L13	114	164
Bn_AFP76	Bn76L3	113	163
Bn_AFP79	Bn79L7	115	165
Bn_AFP80	Bn80L7	115	165
Br_AFP1	Br1L24	116	166
Br_AFP2	Br2L25	117	167
CARpa_AFP1	CARpa1L26	118	168
CARpa_AFP2	CARpa2L27	119	169
CARpa_AFP3	CARpa3L28	120	170
CITcl_AFP10	CITcl10L29	121	171
CITcl_AFP11	CITcl11L30	122	172
CITcl_AFP2	CITcl2L17	123	173
CITcl_AFP3	CITcl3L14	124	174
CITsi_AFP1	CITsi1L31	125	175
CITsi_AFP2	CITsi2L32	126	176
CUCme_AFP1	CUCme1L33	127	177
CUCsa_AFP3	CUCsa3L34	128	178
Gm_AFP1	Gm1L35	129	179
Gm_AFP2	Gm2L36	130	180
MALdo_AFP11	MALdo11L12	131	181
Mt_AFP14	Mt14L6	132	182
Mt_AFP60	Mt60L4	133	183
Mt_AFP65	Mt65L8	134	184
Mt_AFP66	Mt66L15	135	185
Mt_AFP67	Mt67L16	136	186
Mt_AFP77	Mt77L5	137	187
Mt_AFP78	Mt78L9	138	188

Locus ID	Linker ID	DNA SEQ ID NO	Protein SEQ ID NO
PORol_AFP1	PORol1L37	139	189
PORol_AFP2	PORol2L38	140	190
PORol_AFP27	PORol27L39	141	191
PRUpe_AFP1	PRUpe1L40	142	192
RAPra_AFP23	RAPra23L41	143	193
RAPra_AFP24	RAPra24L42	144	194
RAPra_AFP25	RAPra25L43	145	195
RAPra_AFP26	RAPra26L44	146	196
RAPra_AFP27	RAPra27L45	147	197
ROSbl_AFP11	ROSbl11L46	148	198
TRlre_AFP5	TRlre5L47	149	199
Zm_AFP101	Zm101L49	150	200
Zm_AFP100 L1	Zm100L48	151	201
Zm_AFP100 L2	Zm100L50	152	202
Zm_AFP100 L3	Zm100L50	152	202

Example 4. Design of Synthetic Multi-Domain Defensins

[059] In this example, synthetic homodimeric 2D defensins were designed and constructed to comprise two copies of a cognate single domain (1D) defensin, for example MtDef4 (nucleotide SEQ ID NO: 1029; polypeptide SEQ ID NO: 1128) or Coix22 (nucleotide SEQ ID NO: 990; polypeptide SEQ ID NO: 1089), connected by various linker regions derived from native 2D or MD defensins described herein. Synthetic multi-domain defensins comprising Coix22 defensin regions and having one of the nucleic acid sequences of SEQ ID NOs: 203-219 (encoding the protein sequences of SEQ ID NOs: 329-345, respectively) linked or connected by 17 different linker regions (identified in Table 4) were assessed in corn protoplasts. Synthetic multi-domain defensins comprising MtDef4 defensin regions and having nucleic acid sequences of SEQ ID NOs: 220-236 (encoding the protein sequences of SEQ ID NOs: 346-362, respectively) with the same linker regions as identified in Table 4 were also assessed in corn and soy protoplasts.

Further reference is made to Tables 3 and 5 for the sequence identifiers corresponding to these 17 linker regions.

[060] Also described in this example is the design of a synthetic heterodimeric defensin as set forth in nucleotide SEQ ID NO: 1155 and polypeptide SEQ ID NO: 1156 using Coix 22, linker Mt.AFP65 (nucleotide SEQ ID NO: 134; polypeptide SEQ ID NO: 184) and AMAr. AFP10 (nucleotide SEQ ID NO: 963; polypeptide SEQ ID NO: 1062). Stop codons were removed from the N-terminal defensin region of these synthetic multi-domain 2D defensins. The defensin N-terminal transit signal (TS) domain was maintained at the N-terminus of the N-terminal defensin region, but removed from the N-terminus of the C-terminal defensin region. Diagrammatic examples of these synthetic defensins are shown in FIG. 1 (L1, L2 and L3 refer to linkers derived from different 2D defensins identified herein).

Table 4. Homodimeric synthetic 2D using MtDef4 or Coix22 defensins

Nucleotide SEQ ID NO	Protein SEQ ID NO	Template ID	Linker ID	Protein TS Start	Protein TS End	Protein MP Start	Protein MP End	Protein Linker Start	Protein Linker End	Protein MP2 Start	Protein MP2 End	DNA TS Start	DNA TS End	DNA MP Start	DNA MP End	DNA Linker Start	DNA Linker End	DNA MP2 Start	DNA MP2 End
203	329	CI22FL_ARAly26L10_CI22D1	ARAly26L1	1	31	32	80	81	87	88	136	1	93	94	240	241	261	262	408
204	330	CI22FL_Bn35L11_CI22D1	Bn35L11	1	31	32	80	81	90	91	139	1	93	94	240	241	270	271	417
205	331	CI22FL_Bn36L1_CI22D1	Bn36L1	1	31	32	80	81	92	93	141	1	93	94	240	241	276	277	423
206	332	CI22FL_Bn47L2_CI22D1	Bn47L2	1	31	32	80	81	91	92	140	1	93	94	240	241	273	274	420
207	333	CI22FL_Bn74L3_CI22D1	Bn74L3	1	31	32	80	81	89	90	138	1	93	94	240	241	267	268	414
208	334	CI22FL_Bn75L13_CI22D1	Bn75L13	1	31	32	80	81	91	92	140	1	93	94	240	241	273	274	420
209	335	CI22FL_Bn79L7_CI22D1	Bn79L7	1	31	32	80	81	90	91	139	1	93	94	240	241	270	271	417
210	336	CI22FL_CITcl2L17_CI22D1	CITcl2L17	1	31	32	80	81	87	88	136	1	93	94	240	241	261	262	408
211	337	CI22FL_CITcl3L14_CI22D1	CITcl3L14	1	31	32	80	81	91	92	140	1	93	94	240	241	273	274	420
212	338	CI22FL_MALdo11L12_CI22D1	MALdo11L	1	31	32	80	81	90	91	139	1	93	94	240	241	270	271	417
213	339	CI22FL_Mt14L6_CI22D1	Mt14L6	1	31	32	80	81	87	88	136	1	93	94	240	241	261	262	408
214	340	CI22FL_Mt60L4_CI22D1	Mt60L4	1	31	32	80	81	93	94	142	1	93	94	240	241	279	280	426
215	341	CI22FL_Mt65L8_CI22D1	Mt65L8	1	31	32	80	81	90	91	139	1	93	94	240	241	270	271	417
216	342	CI22FL_Mt66L15_CI22D1	Mt66L15	1	31	32	80	81	92	93	141	1	93	94	240	241	276	277	423
217	343	CI22FL_Mt67L16_CI22D1	Mt67L16	1	31	32	80	81	89	90	138	1	93	94	240	241	267	268	414
218	344	CI22FL_Mt77L5_CI22D1	Mt77L5	1	31	32	80	81	94	95	143	1	93	94	240	241	282	283	429
219	345	CI22FL_Mt78L9_CI22D1	Mt78L9	1	31	32	80	81	90	91	139	1	93	94	240	241	270	271	417
220	346	MtDef4FL_ARAly26L10_MtDef4 D1	ARAly26L1 0	1	29	30	76	77	83	84	130	1	87	88	228	229	249	250	390

Nucleotide SEQ ID NO	Protein SEQ ID NO	Template ID	Linker ID	Protein TS Start	Protein TS End	Protein MP Start	Protein MP End	Protein Linker Start	Protein Linker End	Protein MP2 Start	Protein MP2 End	DNA TS Start	DNA TS End	DNA MP Start	DNA MP End	DNA Linker Start	DNA Linker End	DNA MP2 Start	DNA MP2 End
222	348	MtDef4FL_Bn36L1_MtDef4D1	Bn36L1	1	29	30	76	77	88	89	135	1	87	88	228	229	264	265	405
223	349	MtDef4FL_Bn47L2_MtDef4D1	Bn47L2	1	29	30	76	77	87	88	134	1	87	88	228	229	261	262	402
224	350	MtDef4FL_Bn74L3_MtDef4D1	Bn74L3	1	29	30	76	77	85	86	132	1	87	88	228	229	255	256	396
225	351	MtDef4FL_Bn75L13_MtDef4D1	Bn75L13	1	29	30	76	77	87	88	134	1	87	88	228	229	261	262	402
226	352	MtDef4FL_Bn79L7_MtDef4D1	Bn79L7	1	29	30	76	77	86	87	133	1	87	88	228	229	258	259	399
227	353	MtDef4FL_CITcl2L17_MtDef4D1	CITcl2L17	1	29	30	76	77	83	84	130	1	87	88	228	229	249	250	390
228	354	MtDef4FL_CITcl3L14_MtDef4D1	CITcl3L14	1	29	30	76	77	87	88	134	1	87	88	228	229	261	262	402
229	355	MtDef4FL_MALdo11L12_MtDef4D1	MALdo11L12	1	29	30	76	77	86	87	133	1	87	88	228	229	258	259	399
230	356	MtDef4FL_Mtl4L6_MtDef4D1	Mtl4L6	1	29	30	76	77	83	84	130	1	87	88	228	229	249	250	390
231	357	MtDef4FL_Mt60L4_MtDef4D1	Mt60L4	1	29	30	76	77	89	90	136	1	87	88	228	229	267	268	408
232	358	MtDef4FL_Mt65L8_MtDef4D1	Mt65L8	1	29	30	76	77	86	87	133	1	87	88	228	229	258	259	399
233	359	MtDef4FL_Mt66L15_MtDef4D1	Mt66L15	1	29	30	76	77	88	89	135	1	87	88	228	229	264	265	405
234	360	MtDef4FL_Mt67L16_MtDef4D1	Mt67L16	1	29	30	76	77	85	86	132	1	87	88	228	229	255	256	396
235	361	MtDef4FL_Mt77L5_MtDef4D1	Mt77L5	1	29	30	76	77	90	91	137	1	87	88	228	229	270	271	411
236	362	MtDef4FL_Mt78L9_MtDef4D1	Mt78L9	1	29	30	76	77	86	87	133	1	87	88	228	229	258	259	399

Example 5. Protein Accumulation of 1D versus 2D Defensins in Corn and Soy Plant Cells**A. Protein accumulation in corn protoplasts**

[061] Select synthetic homodimeric 2D defensins described in Example 4 were synthesized and delivered in a pUC57 cloning vector. Polymerase chain reaction (PCR) amplification and restriction digestion were used to amplify sequences encoding the synthetic 2D defensins from the pUC57 vectors, which were then ligated into a protoplast expression vector comprising a 35S promoter linked to the synthetic 2D defensin coding sequences. Protoplasts isolated from corn leaf mesophyll tissues were transformed with the protoplast expression plasmid vectors expressing native 1D Coix22 or synthetic 2D defensins comprising Coix22 defensin domains/regions connected by a linker region. For these synthetic 2D constructs, the homodimeric Coix22 defensin regions were heterologous with respect to the linker region. Following transformation, the protoplasts were lysed and the total proteins were subjected to standard Western immunoblotting using rabbit polyclonal antibodies raised against the native 1D Coix22. A goat anti-rabbit antibody conjugated to horseradish peroxidase (HRP) was used as secondary antibody and the blots were visualized with the Super Signal West Femto kit (Thermo Fisher Scientific, Waltham, MA). Following Western blotting, the membranes were subjected to Coomassie staining to confirm equal sample loading. Dilutions of a purified full length CI_AFP22 were western blotted and the resulting bands were subject to densitometry imaging (Imager Lab 5.1) and used to create a standard curve. The standard curve was then used as a basis for comparison to quantify the 1D and 2D CI_AFP22 proteins that were expressed in corn protoplasts.

[062] Protein accumulation of homodimeric Coix22 domains in combination with various heterologous linker sequences (see Table 4) were compared with protein accumulation for Coix22 1D defensin components. In these experiments, many of the synthetic 2D defensin proteins appear to accumulate to greater levels than their 1D defensin counterparts. Although one of the synthetic 2D defensins tested, homodimeric Coix22 2D linked by Mt77L5 (nucleic acid SEQ ID NO: 218; protein SEQ ID NO: 344), was cleaved in both soy and corn protoplasts, no cleavage product was observed for the remainder of the synthetic 2D Coix22 defensins tested. Accordingly, it is proposed that linker regions may be leveraged to enhance accumulation of 2D and MD defensins in plants to enable more effective control of plant pathogens and/or pests.

Table 5. 2D Defensin Protein Accumulation in Corn Protoplasts.

Western Lane	GOI Description	defensin SEQ ID NO:	Linker SEQ ID NO:	Amount Loaded	Protein MW (Dal.)	Quantification (ng)	Quantification of full-length protein (fmol)	Quantification of ID Defensin (fmol)	2D of interest amount/PB68	Conclusion
1	MW Prestain	/	/							/
2	CL_AFP22	990/1085	/	2.5 ng	5427.26	2.5	461	461		/
3	CL_AFP22	990/1085	/	1.25 ng	5427.26	1.25	230	230		/
	CL_AFP22	990/1085	/	0.625 ng	5427.26	0.625	115	115		/
5	CL_AFP22	990/1085	/	1x (15000 cells)	5427.26	0.55	101	101		/
6	CL_AFP22	990/1085	/	2x*	5427.26	0.54	99.5	99.5		/
7	CL_AFP22+CITc12L17+CL_AFP22	210/336	123	2.5 ng	11549.32	2.5	216	432		/
8	CL_AFP22+CITc12L17+CL_AFP22	210/336	123	1.25 ng	11549.32	1.25	108	216		/
9	CL_AFP22+CITc12L17+CL_AFP22	210/336	123	0.625 ng	11549.32	0.625	54	108		/
10	N/A			1x (15000 cells)	N/A	N/A	N/A	N/A		/
11	CL_AFP22+Bn35L1+CL_AFP22	204/330	160	1x (15000 cells)	11860.68	1.55	131	262	2.59	Enhanced Accumulation
12	CL_AFP22+Bn36L1+CL_AFP22	205/331	161	1x (15000 cells)	12098.93	1.411	117	234	2.32	Enhanced Accumulation
13	CL_AFP22+Bn47L2+CL_AFP22	206/332	162	1x (15000 cells)	12005.81	1.418	118	236	2.34	Enhanced Accumulation

Western Lane	GOI Description	defensin SEQ ID NO:	Linker SEQ ID NO:	Amount Loaded	Protein MW (Dal.)	Quantification (ng)	Quantification of full-length protein (fmoles)	Quantification of ID Defensin (fmoles)	2D of interest amount/pPB68	Conclusion
14	CL_AFP22+MALdo11L12+CL_AFP22	211/337	131	1x (15000 cells)	11880.67	1.398	118	236	2.34	Enhanced Accumulation
15	CL_AFP22+Bn75L13+CL_AFP22	208/334	164	1x (15000 cells)	12009.79	1.236	103	206	2.04	Enhanced Accumulation
16	CL_AFP22+Mr60L4+CL_AFP22	214/340	133	1x (15000 cells)	12178.03	1.489	122	244	2.42	Enhanced Accumulation
17	CL_AFP22+Mr77L5+CL_AFP22	218/344	137	1x (15000 cells)	12502.41	0.762	60.94	121.88	1.21	Linker is cleaved; Accumulation drops in 1D configuration - 2D configuration enhances stability; Increased accumulation of defensin domain
18	CL_AFP22+Mr77L5+CL_AFP22	218/344	137	1x (15000 cells)	12502.41	0.644	51.51	103.02	1.02	Linker is cleaved; Accumulation drops in 1D configuration - 2D configuration enhances stability; Increased accumulation of defensin domain
19	CL_AFP22+CITe13L14+CL_AFP22	210/336	124	1x (15000 cells)	11991.82	1.243	104	208	2.06	Enhanced Accumulation

Western Lane	GOI Description	defensin SEQ ID NO:	Linker SEQ ID NO:	Amount Loaded	Protein MW (Dal.)	Quantification (ng)	Quantification of full-length protein (fmoles)	Quantification of ID Defensin (fmoles)	2D of interest amount/PFB68	Conclusion
				cells)						
20	Cl_AFP22+Mr14L6+Cl_AFP22	213/339	132	1x (15000 cells)	11622.44	1.55	133	266	2.63	Enhanced Accumulation
21	Cl_AFP22+Bn79L7+Cl_AFP22	209/335	165	1x (15000 cells)	11858.54	1.545	130	260	2.57	Enhanced Accumulation
22	Cl_AFP22+Mt66L15+Cl_AFP22	216/342	135	1x (15000 cells)	12066.93	0.742	62	124	1.23	
23	Cl_AFP22+Mt66L15+Cl_AFP22	215/341	134	1x (15000 cells)	11795.66	1.343	114	228	2.26	Enhanced Accumulation
24	Cl_AFP22+Mt67L16+Cl_AFP22	217/343	136	1x (15000 cells)	11693.52	1.182	101	202	2	
25	Cl_AFP22+Mr78L9+Cl_AFP22	219/345	138	1x (15000 cells)	11828.6	1.445	122	244	2.42	Enhanced Accumulation
26	Cl_AFP22+CITc12L17+Cl_AFP22	210/336	123	1x (15000 cells)	11585.34	1.213	104	208	2.06	Enhanced Accumulation

B. Protein accumulation in transgenic soybean plants

[063] Transgenic soybean plants expressing Coix22 and Six Coix22 homodimeric 2D defensins with different heterologous linkers were established, and samples were collected at V2 and lyophilized prior to RNA and protein quantification. Protein quantification was performed using an indirect ELISA with a rabbit IgG primary antibody raised against the protein of interest and an anti-rabbit goat secondary antibody. Two concentrations of standard protein were used to calculate protein detected which is then normalized by total protein extracted. The QuantiGene technology measures RNA directly via a nucleic acid hybridization platform in which target RNAs are captured through cooperative binding of multiple oligonucleotide probes that are conjugated to magnetic microbeads. Cooperative binding of the multiple oligonucleotide probes with specificity for the target sequence results in exceptionally high assay specificity. Detection of this oligonucleotide complex occurs through amplification of a branched DNA amplifier and fluorescent signal, which is counted digitally by high-throughput flow cytometry sorting of the microbead. The results of RNA and Protein levels are shown in Figure 2. While the variance in RNA is not significant between the 1D coix22 and the homodimeric Coix22 2D defensins, three of the five 2D proteins expressed at higher levels than the single domain, even after normalizing for the difference in protein size (2.25-fold increase for the 2D defensin using linker Bn36L1, 2-fold for the 2D defensin using linker Bn35L1, and 1.6-fold for the 2D defensin using linker CITcl2L17). These results demonstrate that multiple domains may help in protein accumulation through increased stability in transgenic soybean plants.

Example 6. Methods for Assessing Activity of Multi-Domain Defensins

[064] In this example, synthetic 2D defensins and their 1D defensin counterparts were tested for their ability to inhibit growth of several fungal species. The defensins were expressed in *Pichia* yeast cells and purified for use in fungal plate assays as described below.

Defensin Production using Pichia cells

[065] Constructs designed for *Pichia* and containing a *Pichia* signal peptide were synthesized by Bio Basic Inc. For clarity, this “*Pichia* signal peptide” used for these experiments is different than the N-terminal TS sequence of defensin proteins and may be present in addition to the TS sequence. The *Pichia* signal peptide is used to enable secretion of the synthesized protein from

the Pichia cells to facilitate collection and protein purification. Approximately 5 µg of DNA was linearized, purified, and transformed into electrocompetent PichiaPink™ cells (Thermo Scientific) by electroporation. After recovery, the cells were plated onto Pichia Adenine Dropout (PAD) selection plates and incubated at 29 °C for 3 days. One colony from each transformation was cultured for 3 days for outgrowth. The cell pellets were then collected and induced with media containing methanol. The cells were cultured and supplemented with methanol daily for 3 additional days. The cell pellets were collected and the media used for the purification. Methods for purification of the Pichia-expressed defensin proteins depended on their expected isoelectric point (pI).

[066] *Purification of Defensins Having pI >7 Using Cation Exchange.* Media was diluted at a ratio of 8:1 with 50 mM sodium acetate at pH 5.0 and passed over SP sepharose resin (GE Healthcare #17-0729-01) using a vacuum manifold. The resin was washed with 50 mM sodium acetate at pH 5.0 and eluted in twice the resin volume with 50 mM sodium acetate at pH 5.0 containing 1M NaCl.

[067] *Purification of Defensins Having pI <7 Using Anion Exchange.* Media was diluted at a ratio of 8:1 with 50 mM CHES at pH 9.0 and passed over Q sepharose resin (GE Healthcare #17-0510-01) using a vacuum manifold. The resin was washed with 50 mM CHES at pH 9.0 and eluted in twice the resin volume with 50 mM CHES at pH 9.0 containing 1M NaCl.

[068] *Buffer Exchange and Concentration.* The purified protein was dialyzed overnight into 8 mM Tris HCl at pH 8 using Pura-A-Lyzer Maxi 3500 Dialysis tubes (Sigma #PURX35050). The resulting solution was concentrated by drying in the Pura-A-Lyzer tubes until a final volume of 400-450 µl was achieved.

Fungal growth conditions

[069] Fungi were first grown according to the conditions in Table 5 for 3-4 weeks or less depending on fungal species as indicated.

Table 6. Fungal Growth Conditions.

Fungus	Media	Lighting and Temperature Conditions	Time before use
<i>Fusarium graminearum</i> (Gibberella stalk rot)	0.25x Potato Dextrose Agar	12 hour diurnal cycle at 19 °C in the dark and 23 °C with 100 μ mol light	21-28 days
<i>Fusarium verticilloides</i> (Fusarium stalk rot)	0.25x Potato Dextrose Agar	12 hour diurnal cycle at 19 °C in the dark and 23 °C with 100 μ mol light	21-28 days
<i>Colletotrichum graminicola</i> (Anthracnose stalk rot)	1x oatmeal agar	12 hour diurnal cycle at 19 °C in the dark and 23 °C with 100 μ mol light	21-28 days
<i>Stenocarpella maydis</i> (Diplodia ear rot)	1X PDA + Cefotaxime	12 hour diurnal cycle at 26 °C with 100 μ mol of light during the daytime period	21-28 days
<i>Phakopsora pachyrhizi</i> (Soy Asian rust)	Culture in planta	14 hour diurnal light cycle with 19 °C in the dark and 22 °C with 500 μ mol light during the daytime period, 75% relative humidity	At least 12 days after plant inoculation

Plate Functional Assay for Fungal Growth Inhibition

[070] Following expression and purification of the 1D and 2D defensin proteins and growth of the fungal species in culture, the ability of the individual defensins to inhibit fungal growth was tested in plate assays. On the day of the growth test assay, fungi were collected in test media, filtered, and resuspended to the appropriate titer as shown in Table 7.

Table 7. Conditions for Fungal Growth Inhibition Assay.

Fungus	Test media	Filter condition	Test media	Titer
<i>Fusarium graminearum</i> (Gibberella stalk rot)	0.05% Triton X100	100 μ m Steriflip filter	0.5x POTATO DEXTROSE BROTH	$4e^4$ spores/ml
<i>Fusarium verticilloides</i> (Fusarium stalk rot)	0.05% Triton X100	100 μ m Steriflip filter	0.5x POTATO DEXTROSE BROTH	$6.5e^4$ spores/ml
<i>Colletotrichum</i> <i>graminicola</i> (Anthracnose stalk rot)	0.05% Triton X100	100 μ m Steriflip filter	2x POTATO DEXTROSE BROTH	$1e^5$ spores/ml
<i>Stenocarpella maydis</i> (Diplodia ear rot)	0.05% Triton X100	100 μ m Steriflip filter	2x POTATO DEXTROSE BROTH	$6.5e^4$ spores/ml
<i>Phakopsora pachyrhizi</i> (Soy Asian rust)	0.05% Triton X100	20 μ m and 60 μ m Steriflip filter	0.5x POTATO DEXTROSE BROTH	$5e^4$ spores/ml

[071] 20 μ l of each fungal solution according to Table 7 was added to 20 μ l of the purified defensin protein to be tested in 20 mM Tris HCL, pH 8.0, in 96 well view plates (Perkin Elmer). Plates were sealed with parafilm and incubated in a clear humidity box for a predetermined period of time, temperature and conditions according to Table 8, before being imaged for fungal growth in the presence of the defensin.

Table 8. Conditions and Times for Incubation of Fungi with Defensins.

Fungus	Lighting and Temperature Conditions	Time
<i>Fusarium graminearum</i> (Gibberella stalk rot)	12 hour diurnal cycle at 23 °C with 100 μ mol of light during the daytime period	24 hours
<i>Fusarium verticilloides</i> (Fusarium stalk rot)	12 hour diurnal cycle at 23 °C with 100 μ mol of light during the daytime period	24 hours
<i>Colletotrichum graminicola</i> (Anthracnose stalk rot)	12 hour diurnal cycle at 26 °C with 100 μ mol of light during the daytime period	24 hours
<i>Stenocarpella maydis</i> (Diplodia ear rot)	12 hour diurnal cycle at 26 °C with 100 μ mol of light during the daytime period	24 hours
<i>Phakopsora pachyrhizi</i> (Soy Asian rust)	12 hour diurnal cycle at 23 °C with 100 μ mol of light during the daytime period	16 hours

Imaging Fungal Growth

[072] The plates were visually imaged using the Perkin Elmer Operetta High Content Imager, using brightfield settings and a 10x objective lens. Depending on the particular fungal species being tested, the stored images were scored according to the ratings or criteria provided in Table 9. Two replicates and up to 4 fields per replicates were averaged for each of the visual inhibition scores.

Table 9. Scoring of Inhibition of Fungal Growth.

Fungus	Scoring
<i>Fusarium graminearum</i> (Gibberella stalk rot)	0- Germinated, hyphal elongation and no activity 1- Germinated and hyphal elongation of $\geq 250 \mu\text{m}$ 2- Germinated and hyphal elongation of 100-250 μm 3- germinated and hyphal elongation of 50-100 μm 4- no germination >80% of spores; hyperbranching “starburst” phenotype with hyphal elongation <50 μm
<i>Fusarium verticilloides</i> (Fusarium stalk rot)	0- Germinated, hyphal elongation and no activity 1- Germinated and hyphal elongation of $\geq 400 \mu\text{m}$ 2- Germinated and hyphal elongation of 200-400 μm 3- germinated and hyphal elongation of 100-200 μm 4- no germination >50% of spores; hyperbranching “starburst” phenotype with hyphal elongation <100 μm
<i>Colletotrichum graminicola</i> (Anthracnose stalk rot)	0- Germinated and hyphal elongation $\geq 400 \mu\text{m}$ 1- Germinated and hyphal elongation of 200-400 μm 2- Germinated and hyphal elongation of 125-200 μm 3- germinated and hyphal elongation of <125 μm 4- no germination
<i>Stenocarpella maydis</i> (Diplodia ear rot)	0- Germinated and hyphal elongation $\geq 400 \mu\text{m}$ 1- Germinated and hyphal elongation of 200-400 μm 2- Germinated and hyphal elongation of 125-200 μm 3- germinated and hyphal elongation of <125 μm 4- activity with no germination >25% of spores; hyphal elongation <25 μm
<i>Phakopsora pachyrhizi</i> (Soy Asian rust)	0- 0% Inhabitation $\geq 330 \mu\text{m}$ 1- 25% Inhabitation 330-250 μm 2- 20-75% Inhabitation 100-250 μm 3- 75-100% Inhabitation 0-100 μm

Example 7. Fungal Inhibition Activity of Multi-Domain Defensins

A. 2D defensins inhibit fungal growth in plate assay

[073] Eighteen native 2D defensins of the invention were tested in the above *in vitro* plate assay and compared with their 1D defensin counterparts to determine whether the 2D defensins are active against the fungal pathogens listed in Table 9. These 19 2D defensins are identified in Table 10, and their sequences are provided above in Tables 1 and 2 (their linker sequences are also provided in Table 3; and corresponding protein sequences may be determined from the nucleotide sequences listed). The anti-fungal activities of the 2D defensins were also compared to their cognate or component 1D defensins to determine if their potency and spectrum of activity were enhanced relative to their 1D components. The 1D or 2D defensins tested were expressed in *Pichia* and purified as described above. The fungal inhibition scores for each defensin were calculated as an average of the reps performed.

Table 10. Fungal Inhibition Activity of 2D Defensins.

NUC SEQ ID NO	protein name	locus ID	Domain	Reps	<i>Colletotrichum graminicola</i>	<i>Fusarium graminearum</i>	<i>Fusarium verticillioides</i>	<i>Phakopsora pachyrhizi</i>	<i>Stenocarpella maydis</i>	>20 ppm
1	PHT001688	ARAl _y _AFP26	D1D2	2	0	1	0	0	1	not known
8	PHT002159	Bn_AFP35	D1D2	2	0	0	0	0	0	not known
9	PHT002230	Bn_AFP36	D1D2	2	0	0	0			not known
10	PHT002187	Bn_AFP47	D1D2	2	0	0	0	0	0.5	not known
11	PHT002172	Bn_AFP74	D1D2	2	0	0	0	0	0	not known
12	PHT002235	Bn_AFP75	D1D2	2	0	0	0		0	not known
13	PHT002236	Bn_AFP76	D1D2	2	0	0	0	0	0	not known
14	PHT002182	Bn_AFP79	D1D2	2	0	0	0	0	0.5	not

										known
15	PHT002219	Bn_AFP80	D1D2	2	0	0	0	0	0	not known
24	PHT002366	CITcl_AFP3	D1D2	2	0	0	0	0.5	1.5	not known
31	PHT002131	MALdo_AFP11	D1D2	2	0	1.5	0.5	1.5	1	yes
32	PHT002270	Mt_AFP14	D1D2	2	0	4	2.5		2	yes
33	PHT002295	Mt_AFP60	D1D2	2	0	0	0	0	0	not known
34	PHT002294	Mt_AFP65	D1D2	2	0	0.5	0	0	0	not known
35	PHT002293	Mt_AFP66	D1D2	2	0	0	0	0	0	not known
37	PHT002296	Mt_AFP77	D1D2	2	0	1	0	0	0.5	yes
38	PHT002297	Mt_AFP78	D1D2	2	0	0	0	0	1	not known
--	Empty Vector	negative control	N/A	4	0	0.25	0	0	1.5	no
1153	MEDsa.AFPm1	positive control	N/A	4	2.75	3.7	2.3	1.25	2.7	yes

[074] As shown in Table 10, some of these native 2D defensins were able to inhibit growth of fungal pathogens tested in this plate functional assay. Protein concentrations (ppm) of some of the defensin preparations used in the fungal growth assay were measured and compared. In this experiment, at least one of the different 2D defensins was found to have activity against each of the fungal species tested. In cases where the protein concentration was measured and determined to be greater than 20 ppm, inhibitory activity against one or more pathogens was observed for that defensin protein.

B. Transgenic corn plants expressing MALdo_AFP11 have enhanced disease resistance

[075] Transgenic corn plants expressing MALdo_AFP11 (nucleotide SEQ ID NO: 31 and polypeptide SEQ ID NO: 82) were generated and grown in the greenhouse. At the VT growth stage, two nodes were infected by wounding the stalk and injecting a suspension of *Colletotrichum graminicola* inoculum into the wound. Corn plants were evaluated ~2-3 weeks after inoculation. Stalks were harvested, leaves were removed and the stalks were split

longitudinally. Disease severity was reported as the percent necrosis of the cut surface and is compared to the percent necrosis of non-transformed plants. Five transgenic corn events out of 9 transgenic corn events tested were shown to have significant disease reduction at p-value <0.05 as compared to controls.

Example 8. Fungal Inhibition by Synthetic Multi-Domain Defensins

[076] Synthetic 2D defensins comprising two identical defensin regions connected or bridged together by a linker region were compared with 1D defensins. As shown in Example 5 above, many of these longer 2D defensins exhibited increased accumulation relative to their 1D counterparts when expressed in the corn and soy protoplast system, perhaps due to their increased size. The synthetic 2D defensins that accumulated to higher levels in protoplasts also tended to have enhanced or altered activity against fungal pathogens in the plate assays.

[077] Coix22 (PHT000006; nucleotide SEQ ID NO: 990; polypeptide SEQ ID NO: 1089) and MtDef4 (PHT000025; nucleotide SEQ ID NO: 1029; polypeptide SEQ ID NO: 1128) are 1D defensins shown to be active against fungal pathogens. These Coix22 and MtDef4 domains were fused with heterologous defensin linker regions to create novel synthetic 2D defensins similar to those expressed in Examples 4 and 5 above but modified for expression in *Pichia*. Seventeen unique defensin linkers from 2D defensins identified from different plant species were tested for their ability to create active 2D defensins. Each synthetic defensin was expressed from a *Pichia* expression vector, and proteins were purified and tested *in vitro* for activity as described above. The 1D Coix22 and MtDef4 defensins were used as controls. The fungal inhibition activities or scores for these synthetic 2D defensins are shown in Tables 11, 12, and 13. Tables 12 and 13 provide these fungal inhibition activities or scores standardized to 1 μ M or 0.5 μ M protein concentration. Standardization of these fungal inhibition scores allows for a more direct comparison of the intrinsic anti-fungal activities of these 2D and 1D defensins, apart from the effects of their variable protein accumulation levels.

Table 11. Fungal Inhibition Activity of Synthetic 2D defensins.

Defensin PRT SEQ ID NO	Protein name	linker	final assay conc (μM)	estimate of Pichia processing	<i>C. graminicola</i>	<i>F. graminearum</i>	<i>F. verticilloides</i>	<i>P. pachyrhizi</i>	<i>S. maydis</i>
---	Empty Vector	(Empty)	5	(Empty)	0	1.5	0.5	0	1
1154	MEDsa.AFPm1	(Empty)	3.72	(Empty)	4	3	3	3	3
1089	PHT000006	none	5	N/A	3	1	0	2	2.5
365	PHT002411	PPTPPSPPTRP	5	not cleaved	3	2.5	2.5	2	1.5
366	PHT002412	GPPSPTPPHHK	5	cleaved	3.5	1	2	1	2.5
373	PHT002416	APKKVEP	3.96	not cleaved	3	2	1	2	0.5
369	PHT002417	NNESASPASK	1.75	not cleaved	2.5	2	1	1	1
375	PHT002418	GGKAGKKAPK	5	not cleaved	2.5	4	3.5	2	1
363	PHT002420	ASFKPAK	2.86	cleaved	2	1	2	0.5	2
364	PHT002529	ATPPTTPPK	5	not cleaved	3	3	2.5	2	0.5
372	PHT002530	GPPSPPPYSK	5	cleaved	2	1.5	0.5	2	3
368	PHT002531	EPPLSTSTPLN	5	not cleaved	2	2.5	2	2	0.5
377	PHT002534	GGKPGKKAP	1.1	not cleaved	1	1.5	1	0	2.5
370	PHT002535	AGRGKK	5	not cleaved	2	1	1	0.5	2.5
---	Tris	(Empty)	(Empty)	(Empty)	0.9	0.7	0	0.3	0.4

Table 12. Fungal Inhibition Activity of Synthetic 2D Defensins Standardized to a Protein Concentration of 1 μ M.

Defensin PRT SEQ ID NO	Protein name	linker	final assay conc (μ M)	estimate of Pichia processing	C. graminicola	F. graminearum	F. verticilloides	P. pachyrhizi	S. maydis
---	Empty Vector	(Empty)	1	(Empty)	1.5	1	0	0	1
1154	MEDsa.AFPm1	(Empty)	1	(Empty)	2.5	3	2.5	0.5	3
1089	PHT000006	none	1	N/A	0.5	0	1	0.5	2
365	PHT002411	PPTPPSPPTRP	1	not cleaved	1.5	0	1	1	3
366	PHT002412	GPPSPTPPHHK	1	cleaved	1.5	0	1	1	2.5
373	PHT002416	APKKVEP	1	not cleaved	1	1	2	0.5	3
369	PHT002417	NNESASPASK	1	not cleaved	1.5	1	1	0	1.5
375	PHT002418	GGKAGKKAPK	1	not cleaved	2	1	1.5	0	2
363	PHT002420	ASFKPAK	1	cleaved	2	1	1.5	0.5	1.5
364	PHT002529	ATPPTPTPPK	1	not cleaved	2.5	0	1	1	2
372	PHT002530	GPPSPPPYSK	1	cleaved	1.5	1	0.5	0.5	1.5
368	PHT002531	EPPLSTSTPLN	1	not cleaved	1.5	0	1	1	1
377	PHT002534	GGKPGKKAP	1	not cleaved	0	1	0.5	0	0
370	PHT002535	AGRGDKK	1	not cleaved	1	0	0	0	1.5

Table 13. Fungal Inhibition Activity of Synthetic 2D Defensins Standardized to a Protein Concentration of 0.5 μ M.

Defensin PRT SEQ ID NO	Protein name	linker	final assay conc (μ M)	estimate of Pichia processing	C. graminicola	F. graminearum	F. verticilloides	P. pachyrhizi	S. maydis
---	Empty Vector	(Empty)	0.5	(Empty)	1	0	0	0	1.5
1154	MEDsa.AFPm1	(Empty)	0.5	(Empty)	1	3	1.5	1	2.5
1089	PHT000006	none	0.5	N/A	2.5	0.5	1	1	1
365	PHT002411	PPTPPSPPTRP	0.5	not cleaved	2.5	0	1	1	2
366	PHT002412	GPPSPTPPHHK	0.5	cleaved	2.5	0.5	1	0.5	2
373	PHT002416	APKKVEP	0.5	not cleaved	0.5	0	1.5	1	1
369	PHT002417	NNESASPASK	0.5	not cleaved	2.5	0	1	1.5	2.5
375	PHT002418	GGKAGKKAPK	0.5	not cleaved	1	0.5	1	1	2
363	PHT002420	ASFKPAK	0.5	cleaved	1	1	1.5	0.5	2.5
364	PHT002529	ATPPTPTPPK	0.5	not cleaved	2	1	0.5	1.5	2.5
372	PHT002530	GPPSPPPYSK	0.5	cleaved	0.5	0	1	1	2
368	PHT002531	EPPLSTSTPLN	0.5	not cleaved	0	0	1	0	2
377	PHT002534	GGKPGKKAP	0.5	not cleaved	0.5	1	1	0	1
370	PHT002535	AGRGDKK	0.5	not cleaved	1	1	0	0	2.5

Example 9. Fungal Inhibition by Synthetic Multi-Domain Defensins

[078] As demonstrated in Example 8, defensin regions can be connected by a linker region sequence to create a functionally active synthetic or chimeric 2D defensin and tested for anti-fungal activity. Seventeen linker regions from different 2D defensins were tested in combination with five native 2D defensin regions by replacing the native linker region with one of the 16 heterologous linkers (one of the 17 linkers was the native linker for that 2D defensin). The five native 2D defensins were chosen from a set of 17 2D defensins based on the cysteines in the 2 defensin regions. Two defensins, Mt_AFP14 and Bn_AFP79, represented the most common 8:8 class of 2D defensins. Bn_AFP79 and Mt_AFP65 represented the 8:10 class, and Mt_AFP60 represented the 7:9 class of defensins. See Tables 1 and 2 providing sequence identifiers for these native 2D proteins. For 2D defensins, this x:y nomenclature refers to the relative number of cysteines in the two defensin regions. For example, the 8:10 class refers to a 2D defensin having 8 cysteines in the first defensin region and 10 cysteines in the second defensin region. A total of 80 constructs (16 heterologous linker regions in 5 native 2D defensins) and three controls were tested for fungal inhibition activity in the plate assay. Data available for 57 of these constructs is shown in Table 14 below. Each of the five native 2D defensins were also tested (i.e., without the linker swap). The results are shown in Table 14. Proteins were expressed in *Pichia* and purified as described above.

Table 14. Fungal Inhibition Activity of Synthetic Chimeric 2D Defensins.

NUC SEQ ID NO	PRT SEQ ID NO	template_id	<i>Colletotrichum graminicola</i>	<i>Fusarium graminearum</i>	<i>Fusarium verticillioides</i>	<i>Stenocarpella maydis</i>	<i>Phakopsora pachyrhizi</i>	protein expression
14	65	BRANA.G1156000011.1	0	0	0	0	0	N/A
10	61	BRANA.G181000052.1	0	0	0	0.5	0	N/A
32	83	contig_21756_1.1	0	2.5	0	0	0	N/A
33	84	Medtr2g037250.1	0	1.5	0	0	0	N/A
34	85	Medtr2g037190.1	0	1	0	0	0	N/A
271	397	Bn47D1_Bn35L11_Bn47D2		0	0		0	band not

NUC SEQ ID NO	PRT SEQ ID NO	template_id	Colletotrichum graminicola	Fusarium graminearum	Fusarium verticillioides	Stenocarpella maydis	Phakopsora pachyrhizi	protein expression
								visible
272	398	Bn47D1_Bn36L1_Bn47D2	0	0	0	0	0	band not visible
273	399	Bn47D1_MALdo11L12_Bn4 7D2	0	0	0	0	0	band not visible
274	400	Bn47D1_Bn74L3_Bn47D2	0	0	0	0	0	band not visible
275	401	Bn47D1_Bn75L13_Bn47D2	0	0	0	2	0	band not visible
276	402	Bn47D1_Mt60L4_Bn47D2	0	0	0	0	0	band not visible
277	403	Bn47D1_Mt77L5_Bn47D2		0	0	0	0	band not visible
278	404	Bn47D1_CITcl3L14_Bn47D 2		0	0		0	+
279	405	Bn47D1_Mt14L6_Bn47D2	0	0	0	0	0	band not visible
280	406	Bn47D1_Mt66L15_Bn47D2	0	0	0	0	0	+
281	407	Bn47D1_Mt65L8_Bn47D2	0	0	0	0	0	band not visible
282	408	Bn47D1_Mt67L16_Bn47D2	0	0	0	0	0	+
283	409	Bn47D1_Mt78L9_Bn47D2	0	0	0	0	0	band not visible
284	410	Bn47D1_CITcl2L17_Bn47D 2	0	0	0	0	0	band not visible
285	411	Mt60D1_Bn36L1_Mt60D2	0	0	0	0	0	band not visible
286	412	Mt60D1_Bn47L2_Mt60D2	0	0	0	0	0	+

NUC SEQ ID NO	PRT SEQ ID NO	template_id	Colletotrichum graminicola	Fusarium graminearum	Fusarium verticillioides	Stenocarpella maydis	Phakopsora pachyrhizi	protein expression
287	413	Mt60D1_MALdo11L12_Mt60D2	0	0	0	0	0	+
288	414	Mt60D1_Bn75L13_Mt60D2	0	0	0	3	0	band not visible
289	415	Mt60D1_Mt77L5_Mt60D2	0	0	0	0	0	++
290	416	Mt60D1_CITcl3L14_Mt60D2	0	2	0	0	0	++
291	417	Mt60D1_Mt14L6_Mt60D2	0	0	0	0	0	band not visible
292	418	Mt60D1_Bn79L7_Mt60D2	0	0	0	0	0	band not visible
293	419	Mt60D1_Mt66L15_Mt60D2	0	0	0	0	0	++
294	420	Mt60D1_Mt65L8_Mt60D2	0	0.5	0	0	0	++
295	421	Mt60D1_Mt78L9_Mt60D2	0	0	0	0	0	+
296	422	Mt60D1_CITcl2L17_Mt60D2	0	0	0	0	0	++
297	423	Mt14D1_Bn47L2_Mt14D2	0	2.5	1	0	0	band not visible
298	424	Mt14D1_MALdo11L12_Mt14D2	0	1.5	1	0	0	band not visible
299	425	Mt14D1_Bn74L3_Mt14D2	0	4	1.5	1.5	0	band not visible
300	426	Mt14D1_Bn75L13_Mt14D2	0	2	0	0	0	band not visible
301	427	Mt14D1_Mt60L4_Mt14D2	0	3	1	1	0	band not visible
302	428	Mt14D1_Mt77L5_Mt14D2	0	1	0	0	0	band not visible

NUC SEQ ID NO	PRT SEQ ID NO	template_id	Colletotrichum graminicola	Fusarium graminearum	Fusarium verticillioides	Stenocarpella maydis	Phakopsora pachyrhizi	protein expression
303	429	Mt14D1_CITcl3L14_Mt14D 2	0	2.5	1.5	0	0	++
304	430	Mt14D1_Mt78L9_Mt14D2	0	3.5	1.5	0	0	band not visible
305	431	Mt14D1_CITcl2L17_Mt14D 2	0	0	0	0	0	band not visible
306	432	Bn79D1_Bn35L11_Bn79D2	0	0	0	0	0	band not visible
307	433	Bn79D1_Bn47L2_Bn79D2	0	0	0	0	0	+
308	434	Bn79D1_Bn74L3_Bn79D2	0	0	0	0	0	+
309	435	Bn79D1_Bn75L13_Bn79D2	0	0	0	0	0	band not visible
310	436	Bn79D1_Mt60L4_Bn79D2	0	0	0	0	0	band not visible
311	437	Bn79D1_Mt77L5_Bn79D2	0	0	0	0	0	+
312	438	Bn79D1_Mt14L6_Bn79D2	0	0	0	0	0	+
313	439	Bn79D1_Mt66L15_Bn79D2	0	0	0	0	0	++
314	440	Bn79D1_Mt65L8_Bn79D2	0	0	0	0	0	+
315	441	Bn79D1_Mt78L9_Bn79D2	0	0	0	0	0	+
316	442	Bn79D1_CITcl2L17_Bn79D 2	0	0	0	2.5	0	+
317	443	Bn79D1_ARAly26L10_Bn79 D2	0	0	0	0	0	failed transformation
318	444	Mt65D1_Bn35L11_Mt65D2	0	2	0	0	0	+
319	445	Mt65D1_Bn36L1_Mt65D2	0	0	0	0	0	band not visible
320	446	Mt65D1_MALdo11L12_Mt6 5D2	0	2	1	0	0	band not visible

NUC SEQ ID NO	PRT SEQ ID NO	template_id	Colletotrichum graminicola	Fusarium graminearum	Fusarium verticillioides	Stenocarpella maydis	Phakopsora pachyrhizi	protein expression
321	447	Mt65D1_Bn75L13_Mt65D2	0	0	0	0	0	band not visible
322	448	Mt65D1_Mt77L5_Mt65D2	0	0	0	0	0	band not visible
323	449	Mt65D1_CITcl3L14_Mt65D 2	0	0.5	0	0	0	band not visible
324	450	Mt65D1_Bn79L7_Mt65D2	0	0	0		0	band not visible
325	451	Mt65D1_Mt66L15_Mt65D2	0	0	0	0	0	+
326	452	Mt65D1_Mt78L9_Mt65D2	0	0	0	0	0	band not visible
327	453	Mt65D1_CITcl2L17_Mt65D 2	0	0	0	0	0	band not visible
328	454	Mt65D1_ARAly26L10_Mt65 D2	0	0	0	0	0	band not visible
		MEDsa.AFPm1	3	3	2.25	2.6	2	N/A
		Empty Vector	0	0	0	0.5	0	N/A
		20mM Tris-HCl pH8.0	0	0	0	0	0	N/A

[079] This data demonstrates that linkers have a broad capacity for linking two defensin regions together to create synthetic 2D defensin that are active against one or more fungal pathogens. Eight of the seventeen linkers tested (Bn35L11, Bn47L2, Bn74L3, Bn75L13, CITcl2L17, CITcl3L14, MALdo11L12, and Mt65L8) showed promise in working across different defensin backgrounds. Many of the synthetic multi-domain or 2D defensins had higher levels of protein accumulation in plant protoplasts with the potential for new or altered spectrums of activity against different fungal pathogens.

**Example 10. Disease Reduction by Synthetic Heterodimeric 2D Defensins
in Transgenic Soybean Plants**

[080] Transgenic soybean plants expressing heterodimeric 2D defensin Cl.AFP22/linker Mt.AFP65/AMArU.AFP10 (nucleotide SEQ ID NO: 1155 and polypeptide SEQ ID NO: 1156) were established and grown in soil in growth chambers and inoculated with a pathogen suspension containing *Phakopsora pachyrhizi* (pathogen for Asian soy rust, ASR), at the V2 stage. The plants were scored 12-14 days after inoculation for percent leaf infection. The score was compared to that of plants transformed with an empty vector. The transgenic soybean plants expressing the heterodimeric 2D defensin showed significant disease reduction as compared to the control plants.

Table 15. Transgenic soybean plants expressing heterodimeric 2D defensin.

Sample ID	NUC SEQ ID NO/ PRT SEQ ID NO	Numbers of Sample tested	Mean of percent leaf infection *	Std Error
Empty Vector Control	/	33	41.8182	1.7029
Cl.AFP22/linker Mt.AFP65/AMArU.AFP10	1155/1156	30	31.3333	1.7860

* Means are statistically different at $p < 0.05$

Example 11. 1D Defensin capable of inhibiting fungal growth

[081] Several native 1D defensins were also identified that may be useful either alone or as defensin regions/domains when designing synthetic 2D or other MD defensins. Table 16 provides a list of native 1D defensins with nucleotide and protein sequence identifiers and annotated to identify the boundaries between different sequence regions or domains. The ninety-nine (99) defensins listed in Table 16 were selected based on their activity in the fungal plate assay described above in Example 6. Of the 413 1D defensin proteins tested, the 99 1D defensins listed in Table 16 had an average fungal growth inhibition score of 2 or greater over at least two repeated experiments against at least one of the five fungi listed in the Tables above.

[082] As similarly described above, the polynucleotide sequence positions defining the boundaries of the polynucleotide sequences encoding the N-terminal TS sequence (TS_Start, TS_End), the defensin sequence portion (Def_Start, Def End), and the C-terminal extension sequence (CT_Start, Ct_End; if present) are identified for each of the 1D defensins listed. Based on the information provided in Table 16, one can discern the corresponding protein sequence boundaries of the N-terminal TS sequence, defensin sequence portion, and C-terminal extension sequence (if present) of a 1D defensin encoded by the polynucleotide sequences in Table 15 by dividing the nucleotide positions by three (an amino acid corresponding to one codon of three nucleotides). As an exemplary illustration, the 1D defensin protein “ARAl_y_AFP15” has an amino acid length of 83 amino acids with its TS sequence corresponding to amino acids 1-19, its defensin sequence portion corresponding to amino acids 20-75, and its C-terminal extension sequence corresponding to amino acids 76-83 of SEQ ID NO: 1054.

Table 16. Identification of 1D defensins.

Locus_ID	DNA SEQ ID NO	PRT SEQ ID NO	Targeting Signal (TS) Sequence				Def_Start	Def_End	CT_Start	CT_End
			DNA SEQ ID NO	PRT SEQ ID NO	TS_Start	TS_End				
ARAlly_AFP15	955	1054	713	859	1	57	58	225	226	249
Zm_AFP82	956	1055	714	860	1	81	82	237	238	321
IPOtr_AFP2	957	1056	715	861	1	96	97	237	No CT	No CT
ABUth_AFP1	958	1057	716	862	1	81	82	222	No CT	No CT
ABUth_AFP6	959	1058	717	863	1	24	25	180	No CT	No CT
AMBar_AFP1	960	1059	718	864	1	93	94	234	No CT	No CT
AMBar_AFP7	961	1060	719	865	1	117	118	249	No CT	No CT
AMArU_AFP16	962	1061	720	866	1	90	91	240	No CT	No CT
AMArU_AFP10	963	1062	721	867	1	81	82	240	No CT	No CT
AMIBtr_AFP6	964	1063	722	868	1	213	214	360	No CT	No CT
AMIBtr_AFP7	965	1064	723	869	1	93	94	234	No CT	No CT
BRAdi_AFP13	966	1065	724	870	1	96	97	240	No CT	No CT
Bn_AFP43	967	1066	725	871	1	78	79	240	241	255
Bn_AFP1	968	1067	726	872	1	75	76	231	232	240
Bn_AFP51	969	1068	727	873	1	90	91	267	268	282
Bn_AFP98	970	1069	728	874	1	87	88	252	253	264
Bn_AFP52	971	1070	729	875	1	231	232	402	403	417
Bn_AFP103	972	1071	730	876	1	81	82	228	229	273
Sb_AFP1	973	1072	731	877	1	78	79	231	232	384
CANro_AFP2	974	1073	732	878	1	78	79	219	No CT	No CT

Locus_ID	DNA SEQ ID NO	PRT SEQ ID NO	Targeting Signal (TS) Sequence				Def_Start	Def_End	CT_Start	CT_End
			DNA SEQ ID NO	PRT SEQ ID NO	TS_Start	TS_End				
CANro_AFP1	975	1074	733	879	1	93	94	234	No CT	No CT
CANro_AFP13	976	1075	734	880	1	84	85	213	214	273
CHEal_AFP7	977	1076	735	881	1	108	109	249	No CT	No CT
CHEal_AFP1	978	1077	736	882	1	78	79	222	No CT	No CT
CONca_AFP1	979	1078	737	883	1	96	97	237	No CT	No CT
STEme_AFP1	980	1079	738	884	1	96	97	231	No CT	No CT
ERAre_AFP11	981	1080	739	885	1	87	88	234	No CT	No CT
Mt_AFP52	982	1081	740	886	1	9	10	171	No CT	No CT
ERAre_AFP14	983	1082	741	887	1	90	91	276	No CT	No CT
ERAre_AFP23	984	1083	742	888	1	90	91	255	256	303
ERAre_AFP26	985	1084	743	889	1	87	88	258	No CT	No CT
ERAre_AFP29	986	1085	744	890	1	87	88	249	250	291
ERAre_AFP30	987	1086	745	891	1	66	67	207	208	303
ERAre_AFP36	988	1087	746	892	1	72	73	216	217	324
ERAre_AFP37	989	1088	747	893	1	99	100	258	259	303
Cl_AFP22	990	1089	748	894	1	93	94	240	No CT	No CT
Cl_AFP24	991	1090	749	895	1	69	70	210	211	312
ERAre_AFP56	992	1091	750	896	1	69	70	213	214	333
ERAre_AFP57	993	1092	751	897	1	87	88	249	No CT	No CT
Cl_AFP39	994	1093	752	898	1	12	13	186	187	228
ERAre_AFP58	995	1094	753	899	1	60	61	219	220	354
Cl_AFP44	996	1095	754	900	1	96	97	240	No CT	No CT
ERAre_AFP65	997	1096	755	901	1	81	82	249	No CT	No CT

Locus_ID	DNA SEQ ID NO	PRT SEQ ID NO	Targeting Signal (TS) Sequence				Def_Start	Def_End	CT_Start	CT_End
			DNA SEQ ID NO	PRT SEQ ID NO	TS_Start	TS_End				
STEme_AFP2	998	1097	756	902	1	30	31	162	No CT	No CT
STEme_AFP6	999	1098	757	903	1	138	139	282	No CT	No CT
ERAt_e_AFP87	1000	1099	758	904	1	87	88	234	No CT	No CT
ERAt_e_AFP92	1001	1100	759	905	1	144	145	351	No CT	No CT
STEme_AFP4	1002	1101	760	906	1	96	97	246	No CT	No CT
ERAt_e_AFP94	1003	1102	761	907	1	90	91	276	No CT	No CT
ERAt_e_AFP95	1004	1103	762	908	1	81	82	246	247	312
ARAl_y_AFP1	1005	1104	763	909	1	87	88	231	232	246
At_AFP1	1006	1105	764	910	1	90	91	231	No CT	No CT
Ps_AFP1	1007	1106	---	---	---	---	1	138	No CT	No CT
PINsy_AFP1	1008	1107	765	911	1	99	100	246	247	252
Ta_AFP1	1009	1108	---	---	---	---	1	141	No CT	No CT
Ph_AFP2	1010	1109	766	912	1	75	76	222	223	306
DIGsa_AFP1	1011	1110	767	913	1	192	193	348	349	399
SACra_AFP18	1012	1111	768	914	1	75	76	195	196	282
SACra_AFP20	1013	1112	769	915	1	15	16	171	172	207
SACra_AFP23	1014	1113	770	916	1	81	82	228	229	324
SACra_AFP27	1015	1114	771	917	1	78	79	231	232	330
EUPma_AFP6	1016	1115	772	918	1	87	88	237	No CT	No CT
EUPma_AFP7	1017	1116	773	919	1	87	88	237	No CT	No CT
EUPma_AFP2	1018	1117	774	920	1	81	82	222	No CT	No CT
FOEvu_AFP4	1019	1118	775	921	1	102	103	273	No CT	No CT
BASsc_AFP18	1020	1119	776	922	1	54	55	207	208	261

Locus_ID	DNA SEQ ID NO	PRT SEQ ID NO	Targeting Signal (TS) Sequence				Def_Start	Def_End	CT_Start	CT_End
			DNA SEQ ID NO	PRT SEQ ID NO	TS_Start	TS_End				
BASsc_AFP20	1021	1120	777	923	1	90	91	246	No CT	No CT
BASsc_AFP3	1022	1121	778	924	1	81	82	225	No CT	No CT
BASsc_AFP25	1023	1122	779	925	1	36	37	351	No CT	No CT
BASsc_AFP11	1024	1123	780	926	1	24	25	174	No CT	No CT
Os_AFP12	1025	1124	781	927	1	69	70	213	214	321
Os_AFP9	1026	1125	782	928	1	72	73	213	214	258
Mt_AFP36	1027	1126	783	929	1	87	88	213	214	222
Ta_AFP4	1028	1127	---	---	---	---	1	375	No CT	No CT
Mt_Def4	1029	1128	784	930	1	87	88	228	No CT	No CT
PANha_AFP7	1030	1129	785	931	1	96	97	237	No CT	No CT
PANvi_AFP55	1031	1130	786	932	1	75	76	303	No CT	No CT
PANvi_AFP17	1032	1131	787	933	1	69	70	216	217	369
PLAma_AFP7	1033	1132	788	934	1	15	16	156	157	330
PORol_AFP12	1034	1133	789	935	1	81	82	225	No CT	No CT
PORol_AFP15	1035	1134	790	936	1	87	88	231	No CT	No CT
PORol_AFP4	1036	1135	791	937	1	90	91	231	No CT	No CT
PORol_AFP6	1037	1136	792	938	1	24	25	165	No CT	No CT
RAPra_AFP6	1038	1137	793	939	1	81	82	219	No CT	No CT
RAPra_AFP8	1039	1138	794	940	1	87	88	240	No CT	No CT
RAPra_AFP9	1040	1139	795	941	1	87	88	240	No CT	No CT
RAPra_AFP11	1041	1140	796	942	1	87	88	237	No CT	No CT
RAPra_AFP20	1042	1141	797	943	1	87	88	237	No CT	No CT
RAPra_AFP12	1043	1142	798	944	1	87	88	240	No CT	No CT

Locus_ID	DNA SEQ ID NO	PRT SEQ ID NO	Targeting Signal (TS) Sequence				Def_Start	Def_End	CT_Start	CT_End
			DNA SEQ ID NO	PRT SEQ ID NO	TS_Start	TS_End				
ROSbl_AFP13	1044	1143	799	945	1	30	31	198	No CT	No CT
ROSbl_AFP7	1045	1144	800	946	1	81	82	225	No CT	No CT
ROSbl_AFP8	1046	1145	801	947	1	81	82	222	No CT	No CT
Sb_AFP6	1047	1146	802	948	1	96	97	237	No CT	No CT
SPIol_AFP13	1048	1147	803	949	1	93	94	249	No CT	No CT
SPIol_AFP15	1049	1148	804	950	1	84	85	231	No CT	No CT
TARof_AFP4	1050	1149	805	951	1	84	85	231	232	414
TARof_AFP6	1051	1150	806	952	1	39	40	189	190	345
Zm_AFP73	1052	1151	807	953	1	90	91	246	247	314
Zm_AFP10	1053	1152	808	954	1	99	100	246	247	318

**Example 12. Transgenic Soybean Plants Expressing 1D Defensin Have
Enhanced Disease Resistance to Phakopsora Pachyrhizi**

[083] Transgenic soybean plants expressing 1D defensins listed in Table 17 were established and grown in soil in growth chambers and inoculated at the V2 stage with a pathogen suspension containing soybean rust pathogen, *Phakopsora pachyrhizi*. The plants were scored 12-14 days after inoculation for percent leaf infection. Disease reduction is reported as percent disease relative to the negative control (plants transformed with an empty vector) in Table 17. These results show that the transgenic soybean plants expressing 1D defensin, Cl.AFP44, ERAte_AFP29, or ERAte_AFP30 have enhanced resistance to *Phakopsora pachyrhizi*.

Table. 17 Disease testing for transgenic soybean plants expressing 1D defensins

Tests	NUC SEQ ID NO/PRT SEQ ID NO	Locus ID	% disease relative to negative control	Statistically Significant Activity?
1	991/1090	Cl.AFP44	31	yes
2	991/1090	Cl.AFP44	38	yes
3	986/1085	ERAte_AFP29	71	yes
4	987/1086	ERAte_AFP30	65	yes

**Example 13. Transgenic Soybean Plants Expressing PINSY.AFP1
Have Enhanced Disease Resistance to Phakopsora Pachyrhizi**

[084] Transgenic soybean plants expressing a codon optimized PINSY.AFP1 gene (SEQ ID NO: 1157) under the control of a synthetic promoter as set forth in SEQ ID NO: 1158 were generated. Field trials of transgenic plants expressing PINSY.AFP1 (polypeptide SEQ ID NO: 1107) were conducted to assess plant resistance to *Phakopsora pachyrhizi* utilizing multiple planting dates. The field trials were conducted using a randomized complete block design for each of two planting dates. Efficacy parameters were scored throughout the field trial season and at the end of the season yield was determined. Trait efficacy was measured by scoring disease prevalence 3 times at 14 day intervals once ASR was observed in the wildtype plots. Disease Ratings were taken as a 1-9 rating of overall plot infection (RST) and as a percent disease infection in the plots (DPPF). Efficacy data was utilized to calculate area under the disease

progress curve (AUDPC) to determine overall disease resistance of each event. Data are presented in Table 18.

Table 18. Field Trial Efficacy Results as Rated disease infection (RST) and Percent disease infection reduction (DPPF)

Event	Area under disease progress curve RST	Area under disease progress curve DPPF	RST LSD(.10)	DPPF LSD(.10)
Non-transgenic control	31.9	319.4	7.4 total	99.7 total
Transgenic Event 1	26.3	117.7**	7.4 total	99.7 total
Transgenic Event 2	21.0**	95.4**	7.4 total	99.7 total
Transgenic Event 3	26.3	207.8*	7.4 total	99.7 total
Transgenic Event 4	22.8**	163.6**	7.4 total	99.7 total

* Significant reduction in disease at $p \leq 0.1$

** Significant reduction in disease at $p \leq 0.05$

[085] The average rated disease for the control plants was 31.9. The average rated crop injury by disease for transgenic PINSy.AFLP1 soybean Event 1, Event 2, Event 3, and Event 4 was 26.3, 21.0, 26.3, and 22.8 respectively. The rated disease reduction least significant difference (LSD) at 0.10 was 7.4 for all events tested. The average percent disease for the non-transgenic control utilizing Area Under the Disease Progress Curve (AUDPC) 319.4. The average AUDPC for percent crop injury by disease for transgenic PINSy.AFP1 soybean Event 1, Event 2, Event 3, and Event 4 was 117.7, 95.4, 207.8 and 163.6 respectively. The AUDPC percent disease reduction least significant difference (LSD) at 0.10 was 99.7 for all transgenic events tested. These results indicate that all transgenic soybean events expressing PINSY.AFP1 have enhanced disease resistance to *Phakopsora pachyrhizi*.

Example 14. Transgenic Corn Plants Expressing 1D Defensins have Enhanced Disease Resistance

[086] Transgenic corn plants expressing 1D defensin were generated and grown in the greenhouse. At the VT growth stage, two nodes were infected by wounding the stalk and injecting a suspension of *Colletotrichum graminicola* inoculum into the wound. Corn plants were evaluated ~2-3 weeks after inoculation. Stalks were harvested, leaves were removed and the stalks were split longitudinally. Disease severity was reported as the percent necrosis of the cut surface and is compared to the percent necrosis of non-transformed plants as shown in Table 19. Transgenic corn plants expressing defensin AMBtr_AFP7, Cl_AFP22, Cl_AFP44,

ERAt_e_AFP95, Ph_AFP2, or PINsy_AFP1 as shown in Table 19 have enhanced disease resistance to *Colletotrichum graminicola*. Transgenic corn plants expressing defensin Ph_AFP2 were further tested for resistance to other pathogens and were shown to have enhanced broad-spectrum disease resistance to *S. maydis*, *F. verticillioides*, *F. graminearum*.

Table 19. Transgenic corn plants expressing 1D defensins have enhanced disease resistance

Defensin	NUC SEQ ID NO	PRT SEQ ID NO	Number of events with significant disease reduction/number of events tested (at p-value<0.2)
AMBtr_AFP7	961	1060	5 of 7
Cl_AFP22	990	1089	3 of 7
Cl_AFP44	996	1095	3 of 20
ERAt _e _AFP95	1004	1103	6 of 9
Ph_AFP2	1010	1109	9 of 17
PINsy_AFP1	1108	1107	4 of 9

* * *

[087] As various modifications could be made in the constructions and methods herein described and illustrated without departing from the scope of the invention, it is intended that all matter contained in the foregoing description or shown in the accompanying drawings shall be interpreted as illustrative rather than limiting. The breadth and scope of the invention should not be limited by any of the above-described exemplary embodiments, but should be defined only in accordance with the following claims appended hereto and their equivalents. All patent and non-patent documents cited in this specification are incorporated herein by reference in their entireties.

CLAIMS

What is claimed is:

Claim 1. A recombinant DNA construct comprising a nucleic acid sequence encoding a multi-domain defensin polypeptide comprising a first defensin region connected to a second defensin region by a linker region, the first defensin region and the second defensin region each comprising a gamma-thionin domain, wherein the nucleic acid sequence encoding the multi-domain defensin polypeptide is operably linked to a promoter functional in a plant cell.

Claim 2. The recombinant DNA construct of claim 1, wherein the first defensin region is heterologous with respect to the second defensin region or the linker region.

Claim 3. The recombinant DNA construct of claim 1, wherein the first defensin region is identical to the second defensin region.

Claim 4. The recombinant DNA construct of claim 1, wherein the first defensin region is different from the second defensin region.

Claim 5. The recombinant DNA construct of claim 1, wherein the first defensin region or the second defensin region comprises a polypeptide having at least 80% identity to an amino acid sequence selected from the group consisting of SEQ ID NOs: 559-662.

Claim 6. The recombinant DNA construct of claim 5, wherein the first defensin region or the second defensin region comprises a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NOs: 559-662.

Claim 7. The recombinant DNA construct of claim 1, wherein the linker region comprises a polypeptide having at least 80% identity to an amino acid sequence selected from the group consisting of SEQ ID NOs: 153-202.

Claim 8. The recombinant DNA construct of claim 7, wherein the linker region comprises a polypeptide having an amino acid sequence selected from the group consisting of: SEQ ID NOs: 153-202.

Claim 9. The recombinant DNA construct of claim 1, wherein the multi-domain defensin polypeptide comprises an amino acid sequence having at least 80% identity to an amino acid sequence selected from the group consisting of SEQ ID NOs: 52-102, 329-454 and 1156.

Claim 10. The recombinant DNA construct of claim 9, wherein the multi-domain defensin polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 52-102, 329-454 and 1156.

Claim 11. The recombinant DNA construct of claim 1, wherein the first defensin region or the second defensin region comprises an amino acid sequence having at least 80% identity to a sequence selected from the group consisting of SEQ ID NOs: 1054-1152 and 1154.

Claim 12. The recombinant DNA construct of claim 11, wherein the first defensin region or the second defensin region comprises a sequence selected from the group consisting of SEQ ID NOs: 1054-1152 and 1154.

Claim 13. The recombinant DNA construct of claim 1, wherein the multi-domain defensin polypeptide encoded by nucleic acid sequence further comprises an N-terminal transit signal sequence having at least 80% identity to an amino acid sequence selected from the group consisting of SEQ ID NOs: 809-954.

Claim 14. A plant, seed, plant tissue, plant part, or cell comprising the recombinant DNA construct of claim 1.

Claim 15. The plant, seed, plant tissue, plant part, or cell of claim 14, comprising the multi-domain defensin polypeptide encoded by the recombinant DNA construct of claim 1.

Claim 16. The plant, seed, plant tissue, plant part, or cell of claim 15, wherein the plant, seed, plant tissue, plant part, or cell has tolerance or activity against at least one plant fungal pathogen selected from the group consisting of *Fusarium*, *Collectotrichum*, *Stenocarpella*, and *Phakopsora*.

Claim 17. The plant, seed, plant tissue, plant part, or cell of claim 16, wherein the plant, seed, plant tissue, plant part, or cell has tolerance or activity against at least one fungal species

selected from the group consisting of *Fusarium graminearum*, *Fusarium verticilloides*, *Collectotrichum graminicola*, *Stenocarpella maydis*, and *Phakopsora pachyrhizi*.

Claim 18. A microorganism comprising the recombinant DNA construct of claim 1.

Claim 19. A DNA molecule or vector comprising the recombinant DNA construct of claim 1.

Claim 20. The DNA molecule or vector of claim 19, wherein the nucleic acid sequence comprises a polynucleotide sequence having at least 70% identity to a nucleotide sequence selected from the group consisting of SEQ ID NOs: 1-51, 203-328 and 1155.

Claim 21. The DNA molecule or vector of claim 19, wherein the nucleic acid sequence comprises a polynucleotide having a sequence selected from the group consisting of SEQ ID NOs: 1-51, 203-328 and 1155.

Claim 22. A method for conferring fungal pathogen tolerance or resistance to a plant, seed, cell, or plant part comprising expressing in said plant, seed, cell, or plant part the multi-domain defensin polypeptide encoded by the recombinant DNA construct of claim 1.

Claim 23. A method for producing a transgenic plant with tolerance to a fungal pathogen comprising transforming a plant cell or tissue with the recombinant DNA molecule or vector of claim 18, and regenerating a transgenic plant.

Claim 24. A recombinant DNA construct comprising a nucleic acid sequence encoding a defensin polypeptide having at least 80% identity to an amino acid sequence selected from the group consisting of SEQ ID NOs: 1054-1152, wherein the nucleic acid sequence encoding the defensin polypeptide is operably linked to a promoter functional in a plant cell.

Claim 25. The recombinant DNA construct of claim 24, wherein the defensin polypeptide has an amino acid sequence selected from the group consisting of SEQ ID NOs: 1054-1152.

Claim 26. The recombinant DNA construct of claim 24, wherein the promoter comprises a nucleotide sequence as set for in SEQ ID NO: 1158.

Claim 27. A plant, seed, plant tissue, plant part, or cell comprising the recombinant DNA construct of claim 24.

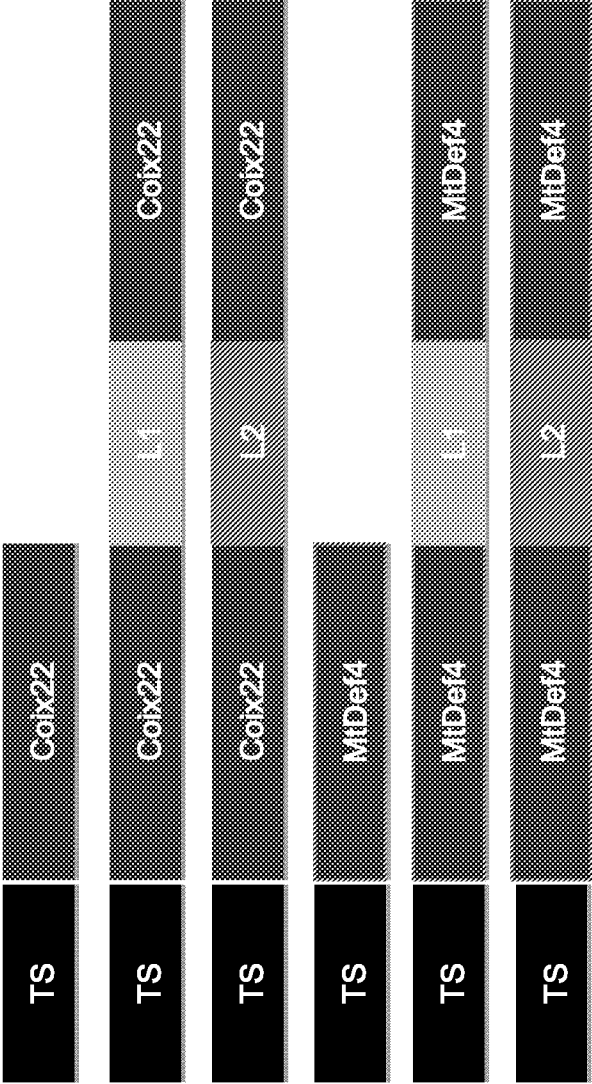
Claim 28. The plant, seed, plant tissue, plant part, or cell of claim 27, wherein the plant, seed, plant tissue, plant part, or cell has tolerance or activity against at least one plant fungal pathogen selected from the group consisting of *Fusarium*, *Collectotrichum*, *Stenocarpella*, and *Phakopsora*.

Claim 29. The plant, seed, plant tissue, plant part, or cell of claim 28, wherein the plant, seed, plant tissue, plant part, or cell has tolerance or activity against at least one fungal species selected from the group consisting of *Fusarium graminearum*, *Fusarium verticilloides*, *Collectotrichum graminicola*, *Stenocarpella maydis*, and *Phakopsora pachyrhizi*.

Claim 30. A method for producing a transgenic plant with tolerance to a fungal pathogen comprising transforming a plant cell or tissue with the recombinant DNA molecule or vector of claim 24, and regenerating a transgenic plant.

FIG. 1

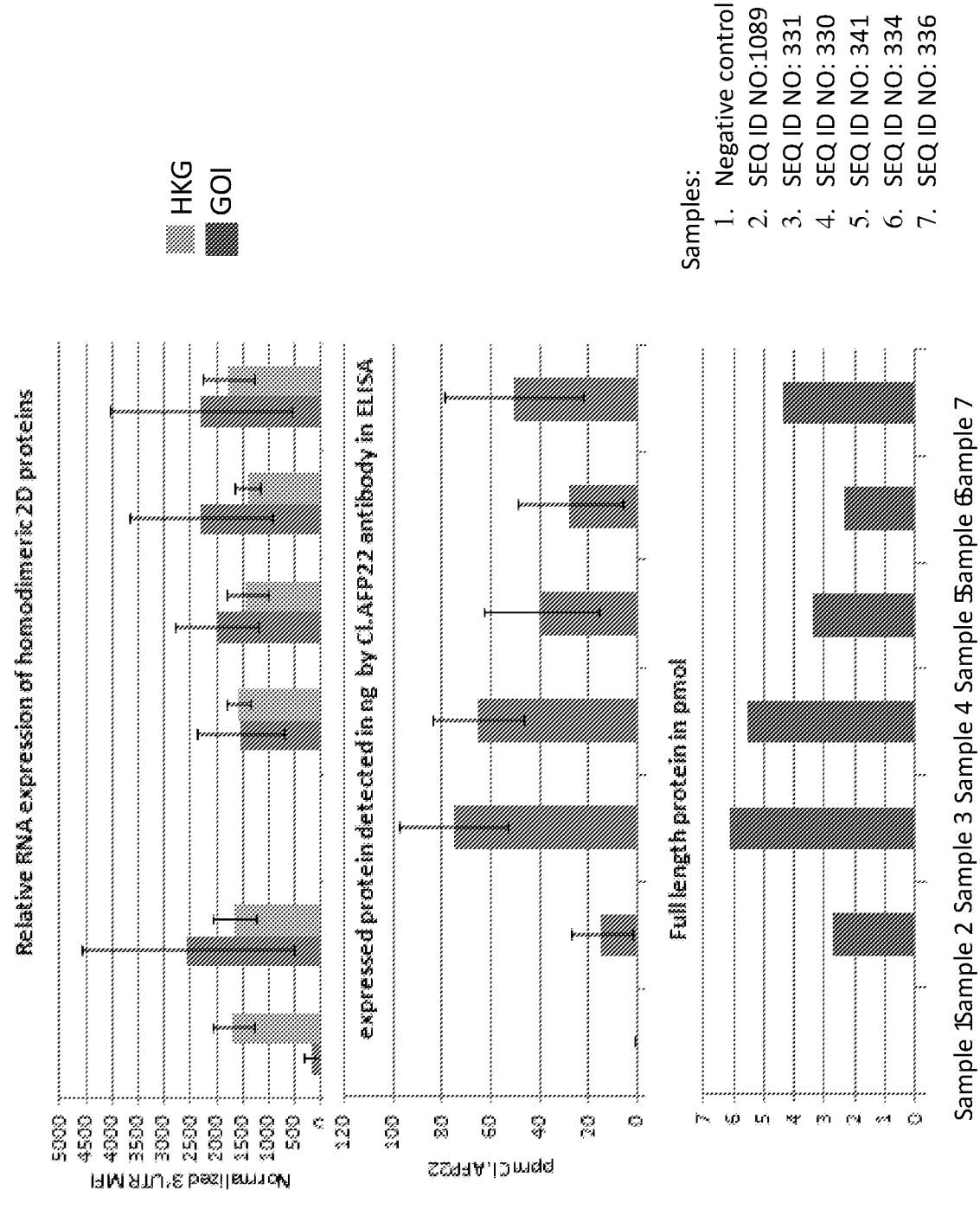
Homodimeric synthetic 2Ds



Heterodimeric synthetic 2D



FIG. 2



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/14153

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 15/82, A01P 3/00 (2017.01)

CPC - C07K 14/415, C07K 2319/02, C12N 15/8282

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 2015/0283204 A1 (HEXIMA LIMITED) 8 October 2015 (08.10.2015) para [0007], [0014], [0033], [0046], [0093], [0094], [0097], [0098], [0142], [0144]	1-4, 14-19, 22-23 ----- 5-6, 13 ----- 7-8
Y	~ NCBI Reference Sequence XP_002863804 (17 November 2009) [retrieved on 07 May 2017 from https://www.ncbi.nlm.nih.gov/protein/297791839] entire doc.	13
Y	~ UniProtKB D7MTG8 (10 August 2010) [retrieved on 07 May 2017 from http://www.uniprot.org/uniprot/D7MTG8] entire doc.	5-6
A	~ NCBI Reference Sequence XP_002864616.1 (17 November 2009) [retrieved on 07 May 2017 from https://www.ncbi.nlm.nih.gov/protein/297793463] entire doc. [XP_002864616.1 displays 77% identity to the applicant's SEQ ID NO: 560]	5-6
A	US 8,999,291 B2 to (GOODMAN et al.) 7 April 2015 (07.04.2015) SEQ ID NO: 229	7-8
A	~ THOMMA et al. Plant defensins. Planta (8 October 2002) vol 216, pp 193-202, pg 193, col 2 para 2	1-8, 13-19, 22-23

☐ 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

7 May 2017

Date of mailing of the international search report

26 MAY 2017

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/14153

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:

----- see extra sheet -----

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.:
1-8, 13-19, 22-23, limited to SEQ ID NOs: 559, 560, 153 and 809

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 17/14153

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

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.

Group I+: 1-30, directed to a DNA construct encoding a multi-domain defensin polypeptide comprising a first defensin region connected to a second defensin region by a linker region, a method of production and a method of use, thereof. The multi-domain defensin polypeptide will be searched to the extent that the first defensin region and the second defensin region encompasses SEQ ID NOs: 559 and 560, respectively; the linker region encompasses SEQ ID NO: 153, and the N-terminal transit signal encompasses SEQ ID NO: 809. It is believed that claims 1-8, 13-19, 22-23 encompass this first named invention, and thus these claims will be searched without fee to the extent that they encompass SEQ ID NOs: 559, 560, 153 and 809. Additional multi-domain defensin polypeptide(s) will be searched upon the payment of additional fees. Applicants must specify the claims that encompass additionally elected multi-domain defensin polypeptide(s). 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 a multi-domain defensin polypeptide encompasses SEQ ID NO: 52 and its corresponding nucleic acid SEQ ID NO: 1 (claims 1-4, 9-10, 14-23). Additional exemplary election would be a multi-domain defensin polypeptide encompasses SEQ ID NO: 1054 and promoter nucleic acid SEQ ID NO: 1158 (claims 1-4, 11-12, 14-19, 22-30).

The inventions listed as Group I+ do not relate to a single general inventive concept 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

The technical feature of each of the inventions listed as Group I+ is the DNA construct encoding a multi-domain defensin polypeptide, recited therein. Each invention of Group I+ requires a DNA construct comprising a specific multi-domain defensin polypeptide, not required by any of the other inventions.

Common Technical Features

The inventions of Group I+ share the technical feature of a nucleic acid sequence encoding a defensin polypeptide, wherein the nucleic acid sequence encoding the defensin polypeptide is operably linked to a promoter functional in a plant cell. However, these shared technical features do not represent a contribution over prior art in view of US 2006/0150276 A1 to Anderson et al. (hereinafter 'Anderson'). Anderson teaches said nucleic acid sequence (para [0021] "a genetic construct comprising a promoter or functional equivalent thereof operably linked to a nucleotide sequence encoding a floral-derived, defensin-like molecule", para [0008] "The defensins have previously been known as gamma-thionins ... Most defensins isolated and studied to date have been derived from seeds ... Seed defensins are small (5 kDa) basic, cysteine-rich proteins and many have anti-fungal activity.").

The inventions of Group I+ further share the technical feature of a nucleic acid sequence encoding a multi-domain defensin polypeptide comprising a first defensin region connected to a second defensin region by a linker region, the first defensin region and the second defensin region each comprising a gamma-thionin domain. Anderson teaches multidomain defensin polypeptides (para [0327] "a genetic construct comprising a promoter or functional equivalent thereof operably linked to a nucleotide sequence encoding a floral-derived, defensin-like molecule having a mature domain", para [0313] "the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a polypeptide comprising two or more mature domains having activity against one or more plant pests and optionally an N-terminal signal domain and optionally an acidic C-terminal domain. The polypeptide according to the latter embodiment would be regarded as a fusion polypeptide.", para [0310] "the nucleic acid molecule may encode for the N-terminal signal domain operably linked to the mature domain. ...The nucleic acid molecule may also encode all three domains or comprise heterologous domains from other defensin or defensin-like molecules"). Anderson does not expressly teach a linker, however, an artisan of ordinary skill in molecular biology art would have readily appreciated that linkers are commonly inserted between domains of recombinant proteins, such as to provide flexibility or separation between domains.

The inventions of Group I+ further share the technical feature of a plant, seed, plant tissue, plant part, or cell comprising the recombinant DNA construct wherein the plant or plant part has tolerance or activity against a fungal species. Anderson teaches said plant or plant part property (abstract "The present invention provides nucleic acid molecules ... which encode defensin-like molecules. The present invention contemplates the use of such nucleic acid molecules in the generation of transgenic plants having resistance or at least reduced sensitivity to plant pests including ... fungi").

As the technical features were known in the art at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the inventions.

Group I+ therefore lack unity under PCT Rule 13 because they do not share the same or corresponding special technical feature.