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(54) **NEPENTHESIN-1 DERIVED RESISTANCE TO FUNGAL PATHOGENS IN MAJOR CROP PLANTS**

NEPENTHESIN-ABGELEITETE RESISTENZ GEGEN PATHOGENE PILZE IN GETREIDE

RÉSISTANCE DÉRIVÉE PAR NEPENTHESINE À DES AGENTS PATHOGÈNES FONGIQUES DANS DES CÉRÉALES

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Description

Field of the invention

[0001] The invention provides a genetically modified cereal plant having a recombinant DNA construct comprising a gene encoding a polypeptide having aspartyl protease activity (EC 3.4.23.12) whose enhanced expression, particularly in grain, confers enhanced fungal disease resistance as compared to a parent cereal plant from which said genetically modified cereal plant was derived. The invention further provides a method for producing a genetically modified cereal plant of the invention comprising transforming one or more cells of a parent plant with a recombinant DNA construct. Further provided is a method for manufacturing the genetically modified grain production of a crop of genetically modified cereal plants which exhibit increased resistance to a fungal disease due to expression of the recombinant DNA construct. Furthermore, use of grain produced by a genetically modified cereal plant of the invention includes it use in the manufacture of a composition, comprising a milled grain composition, an animal fodder, or steam-pelleted animal fodder.

Background of the Invention

[0002] Fungal pathogens cause considerable yield and quality losses of economically important crops. *Fusarium* head blight (FHB) or scab is one of the major fungal diseases of the *Triticeae* family in temperate, and warm humid regions of the world. The disease is linked to several Fusarium species, where *F. graminearum* and *F. culmorum* are economically the most relevant. FHB infection causes a significant reduction in crop yield and quality due to shrivelled grains and their contamination with mycotoxins. In the 1990s, FHB epidemics caused an estimated economic loss of 2.7 billion USD in the US alone. *Fusarium* species, causing FHB, produce toxins that belong to the trichothecenes such as Deoxynivalenol (DON), nivalenol (NIV) and their derivatives including 3-acetyldeoxynivalenol (3-ADON), 15-ADON and 4-acetylnivalenol. They also produce mycotoxins such as zearalenone (ZEA), moniliformin, fumonisins and butenolide. Most of these mycotoxins are associated with fungal virulence and cause toxicosis in humans and animals. FHB management based on the use of resistant cultivars with good agronomic traits would potentially provide a simple and effective control strategy. However, to date, few wheat and barley accessions, or other major crop plants with moderate resistance to FHB have been reported. Resistance to FHB is a quantitative trait, governed by the combined effects of several quantitative trait loci (QTL), epistasis and the environment. A major QTL (Fhb1) on chromosome 3BS and other minor QTL derived from the Chinese cultivar Sumai are the main sources of genetic resistance to FHB in wheat. In contrast, sources of FHB resistance in barley are limited and only provide a modest level of resistance. Due to the polygenic nature of FHB resistance, development of resistant cultivars with suitable agronomic traits is still a challenge. The discovery of antifungal or antitoxin genes provides a potential strategy for the development of FHB resistant cultivars; which may additionally confer resistance to other fungal diseases. Accordingly, the present invention addresses the problem of providing antifungal genes of plant origin that are capable of conferring resistance to FHB caused by Fusarium; and other fungal diseases (e.g. Aspergillus) when expressed in cereal cultivars, as well as in other crop plants such as legumes and cotton.

Summary of the invention

[0003] According to a first embodiment, the invention provides a genetically modified cereal plant having a recombinant DNA construct stably-integrated into the genome of the cereal plant; said construct comprising a gene operably linked to a promoter of heterologous or homologous origin, wherein

- said promoter directs specific expression of said operably linked gene in grain of said plant, and
- said gene comprises a coding sequence encoding a signal peptide N-terminally fused to a polypeptide having aspartic endoprotease activity (EC 3.4.23.12), and wherein the amino acid sequence of said polypeptide has at least 88% identity to a sequence selected from the group consisting of: SEQ ID No.: 4; amino acid residues 30-451 of SEQ ID No: 6; amino acid residues 30-451 of SEQ ID No: 8; amino acid residues 30-451 of SEQ ID No: 10; and amino acid residues 28-446 of SEQ ID No: 12, and

wherein expression of said gene confers enhanced resistance to a fungal disease caused by a species of *Fusarium* and/or *Aspergillus* as compared to a parent cereal plant from which said genetically modified cereal plant was derived.

[0004] Preferably the nucleotide sequence of said heterologous promoter is selected from the group consisting of: SEQ ID No: 25; SEQ ID No: 26; SEQ ID No: 27; SEQ ID No: 28, and SEQ ID No: 29; and

the amino acid sequence of said polypeptide having aspartic endoprotease activity (EC 3.4.23.12) has at least 88% sequence identity to a sequence selected from the group consisting of: SEQ ID No.: 4; amino acid residues 30-451 of SEQ ID No: 6; amino acid residues 30-451 of SEQ ID No: 8; amino acid residues 30-451 of SEQ ID No: 10; and amino acid residues 28-446 of SEQ ID No: 12; and
said promoter directs endosperm-specific expression of said gene.
Furthermore, preferably the amino acid sequence of

said signal peptide is selected from the group consisting of: SEQ ID No: 14, 16, 18, 20, 22, and 24.

[0005] When the genetically modified crop plant is a cereal, preferably said crop plant is a species of *Triticum* or *Hordeum or Zea.*

[0006] The invention further provides genetically modified grain of the genetically modified cereal plant of the invention, wherein the genome of said grain comprises the recombinant DNA construct integrated into the genome of said genetically modified cereal plant.

[0007] In a second embodiment, the invention provides a method for producing a genetically modified cereal plant of the invention comprising:

a) transforming one or more cells of a parent cereal plant with a recombinant DNA construct comprising a gene operably linked to a promoter of heterologous origin, wherein:

- said promoter directs specific expression of said operably linked gene in grain of said plant, and,
- said gene comprises a coding sequence encoding a signal peptide N-terminally fused to a polypeptide having aspartyl protease activity (EC

3.4.23.12), and wherein the amino acid sequence of said polypeptide has at least 88% identity to a sequence selected from the group consisting of: SEQ ID No.: 4; amino acid residues 30-451 of SEQ ID No: 6; amino acid residues 30-451 of SEQ ID No: 8; amino acid residues 30-451 of SEQ ID No: 10; and amino acid residues 28-446 of SEQ ID No: 12, , and

b) selecting transformed cereal plant cells, wherein the genome of said cells comprises a copy of said recombinant DNA construct; and

c) regenerating a genetically modified cereal plant from cells obtained in step (b).

[0008] In a third embodiment, the invention provides a method for detecting a cereal plant exhibiting increased resistance to a fungal disease caused by a species of *Fusarium* and/or *Aspergillus,* said method comprising:

a) obtaining a sample of genomic DNA from a genetically modified cereal plant according to the invention, or portion thereof;

b) detecting in said sample the presence of said recombinant DNA construct;

wherein said recombinant DNA construct comprises a gene operably linked to a promoter of heterologous origin, wherein

- said promoter directs specific expression of said operably linked gene in grain of said plant, and

- said gene comprises a coding sequence encoding a signal peptide N-terminally fused to a polypeptide having aspartyl protease activity (EC 3.4.23.12), and wherein the amino acid sequence of said polypeptide has at least 88% identity to a sequence selected from the group consisting of: SEQ ID No.: 4; amino acid residues 30-451 of SEQ ID No: 6; amino acid residues 30-451 of SEQ ID No: 8; amino acid residues 30-451 of SEQ ID No: 10; amino acid residues 28-446 of SEQ ID No: 12.

[0009] Preferably, said recombinant DNA construct is detected by amplification of a region of the nucleic acid sequence of said construct, wherein said region has a 5' end within the promoter and a 3' end within the gene.

[0010] Preferably, the genetically modified cereal plant of the second, third and fourth embodiment is a species of *Triticum* or *Hordeum* or *Zea.*

[0011] In a fourth embodiment, the invention provides for a use of genetically modified grain of the genetically modified cereal plant of the invention, for the manufacture of a composition, wherein said composition is any one of:

a. a milled grain or seed composition,
b. animal fodder, and
c. steam-pelleted animal fodder

wherein the genome of said grain comprises the recombinant DNA construct integrated into the genome of said genetically modified cereal plant.

Description of the invention

FIGURES

[0012]

Figure 1. Cartoon showing (a) the primary sequence annotation and (b) the predicted 3D structure of HvNEP-1 protein, identifying the signal peptide (SP) residues 1 to 29, prodomain (PD), nepenthesin specific insert sequence comprising amino acid residues 151 to 172 (NAP-I), and D116 and D322, the two catalytic aspartic residues within the catalytic pocket (DAS and DPG) and tyrosine flap (Y186) that holds the substrate within the pocket.

Figure 2. Multiple sequence alignment of the HvNEP-1 protein and related plant aspartic endoprotease proteins. The sequences in Figure 2A are: *Hordeum vulgare* nepenthesin 1 (HvNEP-1) (M0W9B2: SEQ ID No.: 2); *Aegilops tauschii* (XP-020183092.1); *Triticum aestivum* (W5EU17); *Triticum urartu* (T1NBT2); *Hordeum vulgare* phytep-

sin (P42210: SEQ ID No.: 36); *Nepenthes mirabilis Nep1* (UNIPROT: K4MIM1: SEQ ID No.:37) and *Hordeum vulgare* UNIPROT: CND41 (BAK02683: SEQ ID No.:38). The sequences in Figure 2B are: *Hordeum vulgare* nepenthesin 1 (HvNEP-1) (M0W9B2); *Aegilops tauschii* (XP-020183092.1); *Triticum aestivum* (W5EU17); *Triticum aestivum* (A0A1D6RYR6); and *Triticum urartu* (T1NBT2). Residues are shaded light gray or dark gray depending on the level of conservation among the sequences

Figure 3 Graphical presentation of HvNEP-1 inhibitory activity, shown as percent inhibition of phytase activity, over (i) a pH range and (ii) a temperature range. The assay comprised 5 μg of HvNEP-1, 2.5 U/ml of *A. ficuum* phytase and 2 mM of sodium phytate substrate, which was incubated for 1h using the following buffers: pH 2.0 to 2.5, 100 mM formate; pH 3.0 to 5.5, 100 mM acetate; pH 6.0 to 7.0, 100 mM sodium phosphate; pH 8.0, 100mM Tris-HCl at 37 °C. The assay in (ii) was performed using 100 mM acetate buffer pH 5.0, incubated for 1h. The activity of HvNEP-1 was calculated as percent phytase inhibition, compared to the corresponding sample without HvNEP-1, as controls. Values are mean of 3 independent technical replicates, and error bars represent means ±sd of replicates.

Figure 4 Graphical presentation (histogram) of the residual inhibitory activity of HvNEP-1 following incubation for 1h at 37°C in the presence of the protease inhibitors: E-64 (50 μM), pepstatin A (100μM), phenylmethylsulfonyl fluoride (PMSF, 1 mM), EDTA (5 mM) and DMSO (3%). Residual inhibitory activity was measured as described in Figure 3, and percent residual activity was calculated relative to the corresponding sample without protease inhibitor, as control. Values are mean of 3 independent technical replicates, and error bars represent means ±sd of replicates.

Figure 5 Graphical presentation of residual phytase activity of *A. ficuum* (A) and TaPAPhy (B) phytases after treatment with the proteases HvNEP-1 or pepsin at different concentration ratios of phytase to protease (w:w). Values are mean of 3 independent technical replicates, and error bars represent means+ ±sd of replicates.

Figure 6 Graphical presentation of residual phytase activity detected in crude phytase extracts (100 μg) from *F. graminearum* 7775 and *F. culmorum* 8984 measured in the presence of with increasing concentration ratios of HvNEP-1 protease (w:w), using sodium phytate as substrate. Values are mean of 3 independent technical replicates, and error bars represent means+ ±sd of replicates.

Figure 7 Graphical presentation of biomass of *F. graminearum* strain JCM 9873 during growth over an 8 day period in the presence or absence of HvNEP-1 protease; values are mean of 3 independent technical replicates, and error bars represent means ±sd of replicates.

Figure 8 Graphical presentation of 15-ADON production by *F. graminearum* JCM9873 strain during growth over an 8 day period in the presence or absence of HvNEP-1 protease. In the presence of HvNEP1, 15-ADON production by *F. graminearum* was not detectable. Values are mean of 3 independent technical replicates, and error bars represent means ±sd of replicates.

Figure 9 Graphical presentation of the relative expression levels of TRI genes in *F. graminearum* JCM9873 strain following culture with and without and then detected by qPCR analysis. Gene expression of TRI4, TRI5, TRI6 and TRI12 were normalized using GADPH gene expression levels. The asterisks on the bars represent: significant (*), highly significant (**) and very highly significant (***) differences in TRI gene expression with and without HvNEP-1 protease.

Figure 10 Graphical presentation of the relative HvNEP-1 protease expression levels, in selected HvNEP-1 transgenic barley lines determined by RT-PCR analysis. The selected lines are transformed with a gene construct comprising a D-hordein promoter operably linked to a gene encoding a D-hordein signal peptide fused to ΔHvNEP-1 having an C-terminal KDEL sequence, operably linked to a NOS terminator. Values are mean of three independent technical replicates, and error bars represent means ±sd.

Figure 11 Graphical presentation of the percent infection of selected HvNEP-1 transgenic barley lines scored 3 weeks after inoculation with either spores of *F. graminearum* (FG) or *F. culmorum* (FC) spores, or inoculated with water control (MQ). Values are mean of three independent technical replicates, and error bars represent means ±sd.

Figure 12 Graphical presentation of the AUDPC (area under disease progress curve) analysis of selected HvNEP-1 transgenic barley lines scored 3 weeks after inoculation with either spores of selected HvNEP-1 transgenic barley lines scored 3 weeks after inoculation with either spores of *F. graminearum* (FG) or *F. culmorum* (FC) spores, or inoculated with water control (MQ). (FG) or *F. culmorum* (FC) spores, or inoculated with water control (MQ). The minimum and maximum AUDPC per treatment are indicated with error bars.

Figure 13 tabulates the levels of deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZON) mycotoxins detected in selected HvNEP-1 transgenic barley lines scored 3 weeks after inoculation with either spores of*F. graminearum* (FG) or *F. culmorum* (FC) spores, or inoculated with water control (MQ). FC+ and FG+ denotes grains showing FHB symptoms, whereas FC- and FG- denotes grains without FHB symptoms with *F. culmorum* (FC) and *F. graminearum* (FG). Detection limits for DON, NIV and ZEA are >50 μg, >50 μg and >5 μg per kg of DW, respectively.

Figure 14. Multiple sequence alignment of the *H. vulgare* nepenthesin-1 protein (HvNEP-1) from *Hordeum vulgare* and NEP-1 proteins encoded by NEP-1 orthologues from *Zea mays, Glycine max* and *Gossypium hirsutum.* The aligned sequences are: HvNEP-1 (UNIPROT: M0W9B2; SEQ ID No.: 2); ZmNEP-1 (protein ID: XP_008668084.1; SEQ ID No.:45); GmNEP-1 (protein ID: XP_003523200.1; SEQ ID No.:47); and GhNEP-1 (protein ID: XP_016704203.1; SEQ ID No.:49). Residues of the catalytic triads (D[A/T][S/G]) and (D[P/S]G) are boxed, the tyrosine flap (Y) is boxed; the position of the NEP-I "insert" sequence, ([V/L].....[A/M/V/I) characterised by 4 cysteine residues in the orthologue-encoded NEP-1s, is indicated by a solid line.

Abbreviations and terms:

[0013] gi number: (genInfo identifier) is a unique integer which identifies a particular sequence, independent of the database source, which is assigned by NCBI to all sequences processed into Entrez, including nucleotide sequences from DDBJ/EMBL/GenBank, protein sequences from SWISS-PROT, PIR and many others.

[0014] Amino acid sequence identity: The term "sequence identity" as used herein, indicates a quantitative measure of the degree of homology between two amino acid sequences of substantially equal length. The two sequences to be compared must be aligned to give a best possible fit, by means of the insertion of gaps or alternatively, truncation at the ends of the protein sequences. The sequence identity can be calculated as ((Nref-Ndif) 100)/(Nref), wherein Ndif is the total number of non-identical residues in the two sequences when aligned and wherein Nref is the number of residues in one of the sequences. Sequence identity can alternatively be calculated by the BLAST program e.g. the BLASTP program (Pearson W.R and D.J. Lipman (1988)) (www.ncbi.nlm.nih.gov/cgi-bin/BLAST). In one embodiment of the invention, alignment is performed with the sequence alignment method ClustalW with default parameters as described by Thompson J., et al 1994, available at http://www2.ebi.ac.uk/clustalw/.

[0015] Preferably, the numbers of substitutions, insertions, additions or deletions of one or more amino acid residues in the polypeptide as compared to its comparator polypeptide is limited, i.e. no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 substitutions, no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 insertions, no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 additions, and no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 deletions. Preferably the substitutions are conservative amino acid substitutions: limited to exchanges within members of group 1: Glycine, Alanine, Valine, Leucine, Isoleucine; group 2: Serine, Cysteine, Selenocysteine, Threonine, Methionine; group 3: proline; group 4: Phenylalanine, Tyrosine, Tryptophan; Group 5: Aspartate, Glutamate, Asparagine, Glutamine.

[0016] Cereal plant: is a member of the Family *Poaceae;* this family encompassing the tribe *Triticeae,* as well as other members include the genus *Oryza* (e.g. *Oryza sativa), Zea* (e.g. *Zea mays)* and *Sorghum* (e.g. *Sorghum bicolor).* The tribe *Triticeae* encompasses the genus *Triticum* (e.g. *Triticum aestivum)* and *Hordeum* (e.g. *Hordeum vulgare).*

[0017] Heterologous promoter: a promoter is a region of DNA that initiates transcription of an operatively-linked gene. A heterologous promoter is a promoter of heterologous origin with respect to the gene to which it is operatively-linked, which is a promoter having a nucleic acid sequence and function that is different (heterologous in origin) from the promoter that is operatively-linked to the respective gene in nature.

[0018] A heterologous promoter and the gene to which it is operably-linked may originate from the genome of a common plant of origin. In this case, when an individual member of the plant of origin is transformed with a DNA fragment comprising said heterologous promoter operably-linked to said gene, the resulting transformed plant is defined as an intragenic plant.

[0019] Homologous promoter: is a promoter that is homologous in origin to the gene to which it is operatively-linked; such that a contiguous nucleic acid sequence comprising said promoter and its operatively-linked gene is present at a locus within the genome of a plant of origin. When an individual member of the plant of origin is transformed with a DNA fragment comprising said promoter operably-linked to said gene, the resulting transformed plant is defined as a cisgenic plant.

[0020] Native gene: is an endogenous gene present in the genome of a plant found in nature.

[0021] Recombinant DNA construct: is a non-natural polynucleotide comprising nucleic acid fragments derived from polynucleotides of different origin that are combined by the use of recombinant DNA technology and whose nucleic acid sequence is not present in the genomes of plants found in nature. The recombinant DNA construct is suitable for insertion into the genome of an organism (e.g. cereal plant genome) by means of transformation. Genes that are stably-integrated into the genome of a host plant are inherited in the progeny produced in subsequent plant generations of the transformed plant. **Spike:** is the grain-bearing organ of a cereal plant, which develops on one or more shoots

(tillers) that grow after the initial parent shoot grows from a germinating cereal seed.

Detailed description of the invention

[0022] Fungal pathogens of the major crop plants, such as cereals, legumes (e.g. soybean) and cotton, require a source of phosphorous. A key source of phosphorous for such pathogens is phosphorous stored as phytate in the grain or seeds of these crop plants. In cereal grains, phosphorous is also found in a bound form, predominantly (~70%) as phytate stored in the aleurone layer of the grain. In order to access phytate-bound phosphorous in such seeds and cereal grains and successfully establish an infection, a pathogen needs phytase activity. Phytases are often among the palette of secreted enzymes produced by fungal pathogens of the major crop plants, including cereals, legumes and cotton.

[0023] Plants have evolved inhibitors of pathogenic microbial enzymes as defence components. The present invention addresses the problem of developing genetically improved cereal plants having enhanced resistance to fungal pathogens, in particular species of *Fusarium* and *Aspergillus,* which is the cause of the major fungal diseases, including *Fusarium* head blight (FHB) or scab in cereals.

I A genetically modified crop plant of the invention

[0024] The invention provides a genetically modified cereal plant. Genetically modified cerealplants are cereals belonging to the family *Poaceae,* in particular a member of the tribe *Triticeae* or the tribe *Andropogoneae*.

[0025] The genome of the cereal plant is genetically modified by introduction of a gene encoding a polypeptide having nepenthesin-1-type aspartic proteinase activity. This polypeptide belongs to a new family of nepenthesin-1-type aspartic endoproteases identified herein that are native to cereal plants (*Triticeae* and *Andropogoneae).* Identification is based on structural homology between the polypeptide and the nepenthesin-1 and nepenthesin-2 found in the pitcher fluid of carnivorous plants, in particular the presence of catalytic pocket formed by the catalytic triads (DAS and DPG) and possession of a nepenthesin-specific insert sequence (NAP-I), as detailed in Example 1.3 (figure 2, 14). Those members of this new family found in *Triticeae* share a high degree of structural homology, distinguishing them from other aspartic proteases found in cereals. The polypeptide members of this new family further exhibit some functional properties in common with nepenthesins (EC 3.4.23.12), based on the properties exhibited by one polypeptide member (obtained by recombinant expression in yeast), as detailed in Example 2.3. Accordingly, the catalytic activity of the polypeptide may be classified as belonging to EC 3.4.23.12.

[0026] One native member of the nepenthesin-1-type aspartic endoproteases found in the cereal plant, *Hordeum vulgare,* is HvNEP-1. The native *H. vulgare* gene encoding HvNEP-1 (having nucleic acid sequence SEQ ID No: 1), encodes a polypeptide having 453 amino acids (SEQ ID No: 2). The primary amino acid sequence encoded by the native HvNEP-1 gene includes a putative N-terminal signal peptide (amino acid residues 1-29) and a predicted prodomain (amino acid residues 30-80) and a mature protein domain. The primary amino acid sequence of additional members of the new family of nepenthesin-1-type aspartic endoprotease that are native to cereal plants (in particular *Triticeae*), as well as the crop plants *Glycine max* and *Gossypium hirsutum,* are aligned with the sequence of HvNEP-1 in Figure 2B and 14, respectively.

[0027] The primary amino acid sequence of a polypeptide having nepenthesin-1-type aspartic endoprotease activity expressed in a genetically modified cereal plant comprises an N-terminal signal peptide that co-translationally targets the expressed polypeptide for transport into the endoplasmic reticulum. The signal peptide is fused to the transported polypeptide comprising a prodomain and mature domain. The amino acid sequence of the transported polypeptide, having nepenthesin-1-type aspartic proteinase activity, has at least 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 % amino acid sequence identity to amino acid residues 30-451 of SEQ ID No: 2 [HvNEP-1; UNIPROT: M0W9B2] or residues 1-425 of SEQ ID No.: 4. Alternatively, the amino acid sequence of the transported polypeptide, having nepenthesin-1-type aspartic proteinase activity, has at least 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 % amino acid sequence identity to: amino acid residues 30-451 of SEQ ID No: 6 [Ae.tNEP-1; NCBI: XP_020183092.1]; amino acid residues 30-451 of SEQ ID No: 8 [TaNEP-1; UNIPROT: W5EU17_WHEAT]; amino acid residues 30-451 of SEQ ID No: 10 [TaNEP-1; UNIPROT: A0A1D6RYR6_WHEAT); and amino acid residues 28-446 of SEQ ID No: 12 [TuNEP-1; UNIPROT: T1NBT2_TRIUA].

[0028] In one embodiment, the N-terminal signal peptide fused to the transported polypeptide is a signal peptide derived from a native cereal grain storage protein. Suitable signal peptides include a D hordein signal peptide having SEQ ID No: 14 (derived from UNIPROT:I6TRS8); C hordein signal peptide having SEQ ID No: 16 (derived from UNIPROT: Q41210); a B hordein signal peptide having SEQ ID No: 18 (derived from UNIPROT: Q0PIV6), a glutenin signal peptide having SEQ ID No: 20 (derived from UNIPROT: P08488), and a gliadin signal peptide having SEQ ID No:22 (derived from UNIPROT: Q41529). Additionally, a suitable signal peptide include the native signal peptide corresponding to the selected NEP-1 polypeptide; for example the HvNEP-1 signal peptide having SEQ ID No:24. Herein disclosed are amino acid residues 1-29 of SEQ ID No: 6 from [Ae.t-NEP-1]; amino acid residues 1-29 of SEQ ID No: 8 from [TaNEP-1]; amino acid residues 1-29 of SEQ ID No: 10 from [TaNEP-1); and amino acid residues 1-27 of SEQ ID

No: 12 [TuNEP-1].

[0029] In a further embodiment, the primary amino acid sequence of a polypeptide having nepenthesin-1-type aspartic proteinase activity expressed in a genetically modified cereal plant may include an endoplasmic reticulum (ER)-retention signal fused to the C-terminal of the encoded and expressed polypeptide. Suitable ER-retention signals maybe selected from among a KDEL, SEKDEL and HDEL tag.

[0030] In wild-type cereal plants, nepenthesin-1-type aspartic proteinase activity was initially detected in the cereal grain (Example 1). Transformation of wild-type cereal plants with a gene encoding a polypeptide of the invention serves to enhance the level of expression of this gene in the plant and correspondingly to enhance the level of nepenthesin-1-type aspartic proteinase activity. The gene encoding the polypeptide having nepenthesin-1-type aspartic proteinase activity in a genetically modified cereal plant, may be tissue-specifically expressed in a tissue of the cereal grain during grain development or it may be expressed constitutively in both tissues of the cereal grain and other plant parts. In order to obtain grain-specific gene expression, a cereal grain-specific promoter of heterologous origin is cognately fused to the gene encoding the polypeptide. For example, the heterologous promoter may be used to direct tissue-specific expression of the cognate gene of the invention in either the endosperm storage tissue, lemma or aleurone of the grain. Heterologous promoters suitable for directing endosperm-specific expression during development of a cereal grain include a promoter that in nature directs expression of a D hordein gene having SEQ ID No: 25; a C hordein gene having SEQ ID No: 26, B hordein gene having SEQ ID No: 27; a glutenin gene having SEQ ID No: 28, and an α-gliadin gene having SEQ ID No: 29. Heterologous promoters disclosed herein that are suitable for directing aleurone-specific expression during development of a cereal grain include a promoter that in nature directs expression of a LTP1 gene having SEQ ID No: 41. Constitutive promoters include the CaMV35S and ubiquitin promoters [NCBI accession no.: AR287190].

[0031] The genetically modified cereal plant of the invention belongs to the family Poaceae; and may for example be selected from among the genus of *Triticum, Hordeum, Secale, Triticale, Sorghum, Zea* and *Oryza.* In particular cereal plant may be a species selected from among *Triticum aestivum, Hordeum vulgare, Secale cereale, Oryza sativa, Zea mays* and a *Triticale* hybrid. More particularly, the genetically modified cereal plant of the invention is a species of *Triticum* or *Hordeum.*

[0032] An intragenic genetically modified cereal plant comprising a recombinant DNA construct integrated into the genome of the cereal plant, is one where the construct comprises a heterologous promoter operably-linked to a gene encoding a polypeptide having aspartic endoprotease activity (EC 3.4.23.12), and where the heterologous promoter and its operably-linked gene are both derived from the genome of the parent of the genetically modified cereal plant.

[0033] A cisgenic genetically modified cereal plant comprising a recombinant DNA construct integrated into the genome of the cereal plant is one where the construct comprises a homologous promoter operably-linked to a gene encoding a polypeptide having aspartic endoprotease activity (EC 3.4.23.12), where the homologous promoter is the native promoter for its operably-linked gene and both are derived from the genome of the parent of the genetically modified cereal plant.

[0034] A preferred embodiment of the invention provides a genetically modified species of *Hordeum,* comprising a recombinant DNA construct, said construct comprising a gene encoding a signal peptide fused to a HvNEP-1 having SEQ ID No: 4; wherein the gene is operably linked to a heterologous promoter having a sequence selected from among SEQ ID No: 25, 26 or 27. Preferably the signal peptide has an amino acid sequence selected from among SEQ ID No: 14, 16 and 18.

[0035] A preferred embodiment of the invention provides a genetically modified species of *Triticum,* comprising a recombinant DNA construct, said construct comprising a gene encoding a signal peptide fused to NEP-1 protein having a sequence selected from among the group: amino acid residues 30-451 of SEQ ID No: 6 [Ae.tNEP-1; NCBI: XP_020183092.1]; amino acid residues 30-451 of SEQ ID No: 8 [TaNEP-1; UNIPROT: W5EU17_WHEAT]; amino acid residues 30-451 of SEQ ID No: 10 [TaNEP-1; UNIPROT: A0A1D6RYR6_WHEAT); amino acid residues 28-446 of SEQ ID No: 12 [TuNEP-1; UNIPROT: T1NBT2_TRIUA]; wherein the gene is operably linked to a heterologous promoter having a sequence of SEQ ID No: 28 or 29. Preferably the signal peptide has an amino acid sequence selected from SEQ ID No: 20 and 22. Herein disclosed are amino acid residues 1-29 of SEQ ID No: 6 are from [Ae.tNEP-1]; amino acid residues 1-29 of SEQ ID No: 8 from [TaNEP-1]; amino acid residues 1-29 of SEQ ID No: 10 [TaNEP-1); amino acid residues 1-27 of SEQ ID No: 12 from [TuNEP-1].

[0036] Another preferred embodiment of the invention provides a genetically modified *Zea mays,* comprising a recombinant DNA construct, said construct comprising a gene encoding a signal peptide fused to a HvNEP-1 having SEQ ID No: 4; wherein the gene is operably linked to a heterologous promoter. Preferably the signal peptide has an amino acid sequence selected from among SEQ ID No: 14, 16 and 18.

II Fungal resistance of a genetically modified cereal plant of the invention

[0037] A genetically modified cereal plant comprising a gene that directs enhanced expression of a polypeptide having nepenthesin-1-type aspartic proteinase activity in developing grain of the plant is more resistant to fungal

disease than the parent plant from which it was derived by genetic modification.

[0038] In particular, the genetically modified cereal plant of the invention, exhibits enhanced resistance to infection by *Fusarium* and preferably both *Fusarium* and *Aspergillus* pathogens. Enhanced resistance to pathogen attack by isolates of *F. graminearum* and *F. culmorum* is illustrated in respect of genetically modified cereal plants according to the invention in Example 5. In this example, mean percent of infection of developing spikes ranged from 3.41 to 23.08 % in genetically modified *Hordeum vulgare* plants, whereas mean percent infection in spikes of control parent plants ranged from 31.88 to 50 % for both *F. graminearum* and *F. culmorum* strains. The progression of FHB in the infected spikes over a period of weeks was also reduced in the genetically modified *Hordeum vulgare* plants as compared to the control plants.

[0039] Indications as to the underlying mechanism whereby expression of the nepenthesin-1-type aspartic proteinase in a genetically modified cereal plant of the invention enhances fungal resistance are seen from the effect of recombinantly-expressed HvNEP-1 on the growth and toxin production by *Fusarium* cultivated on controlled growth media. Growth of *Fusarium* cultures was significantly inhibited when cultured in the presence of HvNEP-1, which mirrors the inhibitory effect on infection by *Fusarium* and progression of the fungal disease on genetically modified cereal plants expressing HvNEP-1. Importantly, both toxin production and the expression of genes (TRI4, TRI5 and TRI6) required for fungal trichothecene synthesis was inhibited in *Fusarium* cultures by the presence of HvNEP-1 (as show in Example 3.3). More specifically, the phytase enzymes produced by *Fusarium* cultures, that play an essential role in releasing phosphate required for *Fusarium* growth on cereal grains, are strongly inhibited by HvNEP-1 (a shown in Example 3.1). Surprisingly, fungal phytases are more sensitive to inhibition by nepenthesin-1-type aspartic endoprotease of the invention as compared to phytases native to cereal grains (see Example 2). Furthermore, the ability of nepenthesin-1-type aspartic proteinases of the invention to inhibit fungal phytases is not shared by other known aspartic proteases (pepsin) indicating that the nepenthesin-1-type aspartic endoprotease form a distinct and unique class of enzymes, whose substrate selective properties confer resistance to fungal attack.

III Methods for producing and detecting a genetically modified cereal plant of the invention

[0040] A nucleic acid molecule having a nucleic acid sequence encoding a polypeptide having nepenthesin-1-type aspartic proteinase activity, to be expressed in crop plant of the invention (see section I), may be derived by sequence specific amplification of the corresponding sequence of the native NEP-1 gene from genomic DNA extracted from the respective plant. The nucleic acid molecule can also be produced synthetically, to comprise a coding sequence for the respective polypeptide; and whose nucleotide sequence is preferably optimised for expression in the respective plant. Examples of suitable nucleic acid molecules encoding polypeptides having nepenthesin-1-type aspartic proteinase activity for expression in a cereal plant according to the invention is provided in the sequence listing. The nucleic acid molecule, encoding a polypeptide for use in the invention, is operably linked (fused) to cis-regulatory regions comprising a promoter nucleic acid molecule of heterologous origin. The promoter is a tissue-specific promoter that directs tissue-specific expression in developing grain of the cereal plant. Preferably the promoter is an endosperm-specific promoter, for example a promoter that drives expression of a storage protein gene native to the cereal plant to be genetically modified. A terminator nucleic acid molecule may be derived from a terminator that terminates expression of a storage protein gene native to the cereal plant to be genetically modified; or the terminator can be a CaMV 35S terminator (SEQ ID No.: 30) or a terminator derived from the nopaline synthase gene (SEQ ID No.: 31), isolated from *Agrobacterium tumefaciens.*

[0041] A nucleic acid molecule, encoding a polypeptide for use in the invention, operably linked to cis-regulatory regions, is introduced into a nucleic acid construct (pWBVec8 vector; Gynheung et al., 1988) to ensure efficient cloning in *F. coli* and subsequently *Agrobacterium* strains, and which make it possible to stably transform the cereal plants of the invention. Such vectors include various binary and co-integrated vector systems, which are suitable for the T-DNA-mediated transformation. The vector systems are generally characterized by having at least the *vir* genes, which are required for *Agrobacterium*-mediated transformation, and T-DNA border sequences.

[0042] *Agrobacterium* transformation typically involves the transfer of the binary vector carrying the foreign DNA of interest (e.g., pWBVec8 vector) to an appropriate *Agrobacterium* strain, and may be performed as described by Gynheung et al., (1988). For example, transformation of a parent cereal plant species by recombinant *Agrobacterium* may be performed by co-cultivation of a suspension of transformed Agrobacterium cells with isolated immature cereal grain embryos on a solid selective growth medium following the procedure described by Bartlett et al., (2008) and Holme, et al. (2012). Transformed tissue is regenerated on selectable medium carrying an antibiotic or herbicide resistance marker present between the T-DNA borders of the binary vector.

[0043] Positive transformants can be identified by PCR using a 5' primer with binding a site located in the promoter region upstream of the NEP-1 coding sequence and a 3' primer located inside the coding sequence for the nepenthesin-1-type aspartic proteinase; such as to dis-

tinguish the inserted gene from a native gene encoding an aspartic proteinase.

[0044] Cisgenes in cisgenic plants can be identified using standard southern blot analysis or by means of iPCR (Triglia et al., 1988), where one or more copies of a gene and their respective flanking regions in the genome are amplified, and then compared. In this manner iPCR can be used to distinguish and identify a gene inserted into the genome of a cisgenic genetically modified cereal plant of the invention by transformation and a native copy of the gene in the genome.

III Use of genetically modified cereal plants of the invention

[0045] Genetically modified grain and seeds of the genetically modified cereal plants of the invention have a lower risk of contamination with toxins and mycotoxins due to their enhanced resistance to infection by fungal diseases, in particular *Fusarium* infections. Infection by these fungal diseases is accompanied by the production of toxins belonging to the trichothecenes (e.g. Deoxynivalenol (DON), nivalenol (NIV) and their derivatives including 3-acetyldeoxynivalenol (3-ADON), 15-ADON and 4-acetylnivalenol) and mycotoxins (e.g. zearalenone, moniliformin, fumonisins and butenolide) Since both toxins and mycotoxins carry a health risk when used as feed for animals or for human consumption, there is an advantage in using grain derived from genetically modified cereal plants of the invention. Accordingly, grain produced by genetically modified cereal plants of the invention can be used in the production of animal fodder; processed for human consumption or used for fibre/thread manufacture.

[0046] Traditional processing steps performed when using genetically modified cereal grain of the invention include one or more of the following steps:

i. Cleaning/conditioning cereal grain: First the genetically modified grain is cleaned. For example the grain may be passed through magnets and/or metal detectors to remove any metal contamination. Machines can be used to separate any other seeds, stones or dust that may have got mixed with the wheat.

ii. Gristing grain: The cleaned and conditioned grain is blended with other types of grain in different proportions to make different kinds of flour.
The gristed grain passes through special rollers called break rolls. They break each grain into its three parts: cereal grain germ, bran and endosperm. Sieves sift the three separated parts into different streams.

iii. Mixing: The bran, germ and endosperm fractions, having been separated out, can optionally be blended, and can be milled to make different types of milled cereal grain composition, such as Wholemeal flour using all parts of the grain; Brown flour contains about 85% of the original grain, but with some bran and germ removed; and White flour is made from the endosperm only.

iv. Steam pelleting: Milled cereal grain composition may be combined with other fodder ingredients in a steam-pelleting machine, where the components are exposed to steam at a temperature of about 80°C - 90°C for a period of time sufficient to reduce the microbial population to levels safe for animal consumption, and the product is converted to dried pellets.

Examples

Example 1: Detection, isolation and identification of a fungal phytase inhibitor from *Hordeum vulgare*

[0047] Crude protein extract (CPE) was extracted from the grains of barley cv. Invictus, fractionated and analyzed for the ability to inhibit *A. ficuum* phytase, as follows:

1.1 Phytase extraction: The grains (5 g) were ground to a fine powder using a rotary mill (IKA Tube mill control), and grain proteins were extracted in 1:10 (w/v) 25mM sodium acetate buffer (pH 5.5) containing 0.1mM $CaCl_2$, by constant shaking (300 rpm) at 25°C for 1h. The extract supernatant was collected by centrifugation (3392×g, for 30 minutes at 4°C), to which ammonium sulfate was added to 60% saturation, and the precipitated proteins were collected by centrifugation (7000×g, 15 min, 259 4°C). The protein pellet was re-suspended in 50 ml of 25 mM acetate buffer (pH 4.5) and dialyzed against 50 mM Tris-HCl buffer (pH 7.5) overnight. The supernatant was collected by centrifugation (7000×g, 30 min, 4°C), and concentrated (Vivaspin Turbo 30 kDa cut off). Proteins (>30 kDa) were loaded onto an ÄKTA Fast Protein Liquid Chromatography (FPLC) device equipped with a Superdex G200 column, and the collected FPLC fractions were assessed for *Aspergillus ficuum* phytase inhibition employing the phytase assay described below. Fractions having phytase inhibitory activity were analyzed by Mass Spectrometry (MS) according to Dionisio, G. et al. (2011), to identify the phytase inhibitor amongst the detected proteins.

1.2 Phytase assay: Phytase activity and its inhibition was measured according to an ammonium-molybdate method (Engelen AJ, et al., 1994). In brief, 100 μl of FPLC fraction (0 ± 1 mg ml^{-1}) was incubated with 10 μl (2.5 U ml^{-1}) of A. *ficuum* phytase, 1mM sodium phytate and 400 μl of 25 mM sodium acetate buffer (pH 5.5) containing 0.1mM $CaCl_2$, at 37°C for 1 hour. The reaction was terminated by adding 800 μl of stop

solution (20mM ammonium heptamolybdate, 5mM ammonium vanadate and 6% nitric acid to the final concentration) to the reaction mixture. After centrifugation (4226×g, 5 min), the absorbance of the supernatant was measured at 415 nm using 96 well plate reader (Epoch, Bio-Tek, USA). The residual phytase activity was determined relative to a blank sample.

1.3 Identity of the candidate phytase inhibitor: MS analysis of the most inhibitory fraction identified peptides from 30 different proteins; of which 4 peptides corresponded to an uncharacterized protein annotated to have aspartyl protease activity (Uniprot: M0W9B2). This candidate inhibitor was estimated by MS to have a molecular weight 48.915 kDa.

[0048] A candidate barley gene was predicted from the identified Uniprot accession number (M0W9B2) and tblastN against the barley genomic sequence in the NCBI database and the IPK Barley BLAST server. The candidate gene had an open reading frame (ORF) of 1362 bp encoding a protein of 453 amino acids with a predicted molecular weight of 48.9 kDa. The deduced protein encoded a preproenzyme with a putative signal peptide, a prodomain and a long polypeptide interrupted by the nepenthesin-specific insert sequence (NAP-I) (Fig. 1a). The NAP-I sequence is predicted based on NAP-I sequences described for nepenthesins and homologues (Athauda et al., 2004). Based on the characteristic Nepenthesin aspartic endoprotease (NPAP)-type primary structure organization of the deduced protein it was identified as an HvNEP-1 (i.e. a barley nepenthesin-1-type aspartic endoprotease). The predicted 3D structure of the mature protein displays a catalytic pocket formed by the two catalytic triads (DAS and DPG) supported by Tyr residue (Y186) as a flap (Fig. 1b). Multiple sequence alignment of HvNEP-1 and related aspartic proteases revealed that catalytic Asp residues are conserved but not the flap Tyr. Residues forming the catalytic triads with Asp differ from the characteristic aspartic proteases (DTG/DSG and DTG). Besides, the NAP-I sequence contains two Cys residues rather than four described for most of NPAPs proteins (Fig. 2). The protein showed <20% homology to the nepenthesins from *Nepenthes* species.

Example 2: Cloning, expression and properties of the HvNEP-1

[0049] *2.1 Cloning HvNEP-1 gene:* A candidate gene was predicted from the sequence of Uniprot: M0W9B2, and tblastN against the barley genomic sequence in the NCBI database and the IPK Barley BLAST server. Genomic DNA (gDNA) was extracted from the leaves of 6-day old barley cv. Invictus seedlings as described by Doyle et al., 1991. The HvNEP-1 coding sequence, corresponding to encoded amino acid residues 30-453 (minus signal peptide coding sequence; ΔHvNEP-1) was PCR amplified using gDNA as template and gene-specific primers, and Herculase II DNA polymerase, according to the manufacturer's instructions (Invitrogen). The amplified 1.5 kbp DNA fragment was gel purified and cloned into pCRII-TOPO Blunt vector according to the manufacturer's instructions (Invitrogen). Selected clones were evaluated for the insert by restriction digestion, and sequencing (Eurofins Genomics).

[0050] *2.2 HvNEP-1 gene expression:* The ΔHvNEP-1 sequence, further comprising 3' sequence encoding a C-terminal His6 tag, was cloned into the pGAPZoA vector downstream of an alpha mating factor secretion signal coding sequence, using In-fusion (Zhu et al., 2007), under control of the glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter (Fig. 3); and transformed into *Pichia pastoris* strain KM71H. HvNEP-1 protein expression in Pichia was confirmed by matrix-assisted laser-desorption ionization time of flight (MALDI-TOF)-mass spectrometry (MS), SDS-PAGE and Western blotting. The levels of HvNEP-1 in the growth media was 1.2 mg/ml. Western blot analysis, using anti His6 mouse monoclonal antibodies (Roche) and and goat anti-mouse IgG alkaline phosphatase conjugate (BioRad, Hercules, CA), identified a protein with an approximate size of 92 kDa. The predicted theoretical mass of the truncated HvNEP-1 is 47 kDa, indicating that Pichia expressed HvNEP-1 forms a homodimer.

[0051] *2.3 Properties of HvNEP-1:* The enzymatic activity of HvNEP-1 (expressed in Pichia), was measured indirectly, by incubating the enzyme in the presence of *Aspergillus ficuum* phytase, as substrate, and then detecting percent inhibition of the phytase activity measured according to Engelen (1994). HvNEP-1 exhibited peak activity for inhibiting *A. ficuum* phytase at pH 5.0 and at temperature 40 °C (Fig. 3). The sensitivity of HvNEP-1 to protease inhibitors was characteristic of a nepenthesin-1 type aspartic endoprotease. HvNEP-1 was strongly inhibited the protease inhibitor, Pepstatin A (98.2 % loss of activity), while PMSF, E-64, EDTA and DMSO inhibited the enzyme activity by 13.5%, 6.4%, 9.7% and 2.7% respectively (Fig. 4).

[0052] The substrate selectivity of HvNEP-1 was compared with pepsin (aspartic acid protease on the activity of *A. ficuum* (EC 3.1.3.8) and wheat TaPAPhy phytase (EC 3.1.3.26). Although both fungal and wheat phytases were highly sensitive to HvNEP-1 inhibition (Fig. 5); the sensitivity of fungal phytase was clearly stronger, since residual phytase activity of *A. ficuum* was reduced at phytase: protease ratios of 1:500 (Fig. 5i), while residual TaPAPhy phytase activity was first reduced at phytase: protease ratios of 1:100 (Fig. 5ii). In contrast, both phytases were resistant to pepsin, as phytase activity was unaffected after exposure to pepsin even at phytase: protease ratio of 1:20.

Example 3: HvNEP-1 is an inhibitor of *Fusarium* phytases and the growth and toxin production of *Fusarium* species.

[0053] *3.1 HvNEP-1 inhibits Fusarium phytase:* HvNEP-1 strongly inhibited phytases in crude extracts derived from *F. graminearum* 7775 and *F. culmorum* 8984. Incubation with HvNEP-1 in a ratio of only 1: 500 phytase: HvNEP-1 protease (w/w), at room temperature for 1 h was sufficient to cause inhibition (Fig. 6).

[0054] *3.3 HvNEP-1 inhibits Fusarium growth and toxins production:* Antifungal activity of recombinantly-expressed HvNEP-1 against *Fusarium* was analyzed using fungal cultures prepared according to Etzerodt, T. et al. (2015). A composition comprising either HvNEP-1 (3.47 mg) or Ronozyme ProAct serine protease (L) EC 3.4.21.- (supplied by Novozymes) as a control, in 100 μl of 100 mM acetate buffer pH 5.5 were added to 1ml fungal culture (10^7spores/ml) on day 1 and again on 2 day of incubation with shaking (22°C, 130 rpm) for 2, 3, 6 and 8 days. On the respective days, mycelial mass was collected by centrifugation (max speed for 20 min), freeze dried and weighed. Toxin profiles were analyzed according to Etzerodt, T. et al. (2015). Expression of genes involved in fungal trichothecene synthesis were analysed by extracting total RNA from mycelial mass, harvested after 10 days culture (Chomczynski et al. 2006). RNA samples were treated with DNase (Roche) and reverse transcribed using Superscript III-RT (Invitrogen) and oligo (dT) 21T-anchor containing primer. Reverse transcripts of the coding sequences TRI4 [XM_011323872.1; SEQ ID No.:32], TRI5 [XM_011323870.1; SEQ ID No.: 33], TRI6 [encoding GenBank: CEF78358.1] and TRI12 [encoding GenBank: ANO39668.1] were quantified by qPCR (6 μl Power SYBR Green master mix (Applied Biosystems), 1 μl diluted cDNA, 2.4 μl of μM primer mix and 2.6 μl sterile Milli Q water), in a final volume of 12 μL; and products detected in an AB7900HT sequence detection system (Applied Biosystems).

[0055] HvNEP-1 strongly inhibited both growth and toxin production, as seen by the reduction in biomass accumulation in the fungal cultures over a period of 8 days incubation (Fig. 7 and 8). The expression of TRI4, TRI5 and TRI6 genes were suppressed by HvNEP-1, (Fig. 9), in particular TRI6, whose suppression was highly significant.

Example 4: HvNEP-1 overexpressing *Hordeum vulgare* lines

[0056] Transgenic *Hordeum vulgare* lines expressing an HvNEP-1 gene were obtained by Agrobacterium-mediated transformation, as follows:

4.1 HvNEP-1 gene transformation vector construction: The HvNEP-1 coding sequence [SEQ ID No.:3] encoding ΔHvNEP-1 (lacking the native HvNEP-1 signal peptide) was PCR amplified from *Hordeum vulgare* gDNA. PCR amplification was used engineer a nucleic acid sequence encoding a fusion protein comprising an N-terminal HordD signal peptide [SEQ ID No.: 14] and a C-terminal SEKDEL [SEQ ID No.: 39] serving as an endoplasmic reticulum (ER) sorting sequence. The nucleic acid sequence encoding this HvNEP-1 fusion protein was fused downstream of a HordD promoter [SEQ ID No.:25] and inserted upstream of the *Agrobacterium tumefaciens-derived* NOS terminator [SEQ ID No.: 31] in the transformation vector pWBVec8 (Gynheung et al., 1988).

4.2 Generation of HvNEP-1 transgenic Hordeum vulgare lines: The HvNEP-1 transformation vector construct was introduced into competent *Agrobacterium strain* AGL0, as described Gynheung et al., (1988). Transformants were selected by growth on LB plates containing 100 μg/ml spectinomycin and 25 μg /ml Rifampicin for 72 h at 28°C; and positive colonies were identified by PCR. Positive clones were cultured in MG/L medium ((5 g/l Mannitol, 1 g/l L-glutamic acid, 0.25 g/ l KH_2PO_4, 0.1 g/l NaCl, 0.1 g/ l $MgSO_4$*$7H_2O$, 1ng/ l Biotin, 5 g/l Tryptone, 2.5 g/l Yeast extract) containing 100 μg/ml spectinomycin and 25 μg/ml Rifampicin and then used for immature barley embryo transformation following the procedure described by Bartlett et al., (2008) and Holme, et al. (2012).

[0057] Following transformation, selection and regeneration of T0 plants, gDNA was isolated from young leaves (according to Doyle et al., 1991); and selection of positive transformants was confirmed by PCR using forward and reverse primers [SEQ ID No.: 34 and 35] with binding sites inside the HordD promoter and the HvNEP-1 gene yielding a PCR fragment of 759 bp.

[0058] Twenty HvNEP-1 transgenic lines (T0 generation) showed detectable HvNEP-1 expression, the highest expression was seen in line NEP20 (0.4166), the lowest in line NEP20-02(0.0114) (Fig. 10) relative to un-transformed lines (GP).

Example 5: Transgenic HvNEP-1 *Hordeum vulgare* lines exhibit *Fusarium* resistance

Fusarium-infected HvNEP-1 transgenic lines were assessed for Fusarium Head Blight (FHB) resistance and mycotoxin accumulation at the 85-87 growth stage (according to Zadoks scale (Zadoks, et al., 1974)).

[0059] *5.1 Fusarium infection:* Spore suspensions of *F. graminearum* 7775 and *F. culmorum* 8984 isolates, having a DON chemotype, were prepared according to Etzerodt, T. et al. (2015). Each spore suspension (1×10^5 spores per ml in water, containing 0.04% tween 20) was used to spray-inoculate spikes of T0 HvNEP-1 transgenic

lines 8 weeks of germination (Zadoks stages 60). Control spikes were sprayed with MQ water. Untransformed golden promise (GP) plants at the same stage of development were treated similarly with the *Fusarium* spore suspensions and MQ water. The inoculated and mock-inoculated plants were covered with plastic bags and cultivated in a controlled environment (18-21°C and relative humidity 70-75%). FHB disease severity of 10 T0 transgenic lines was compared to untransformed *Hordeum vulgur*e cv Golden Promise (GP) plants, and scored as percentage of infected seeds in the first 3 matured spikes in each plant at 1, 2 and 3 weeks after inoculation.

[0060] *5.2 Disease severity:* Disease scoring showed a substantial reduction in FHB severity in HvNEP-1 transgenic lines (Fig. 11) whose mean percent of infection ranged from 3.41 to 23.08 %, whereas mean percent infection in the control GP plants were ranging from 31.88 to 50 % for both *F. graminearum* and *F. culmorum* strains. The progression of FHB in the spikes of transgenic lines and control GP plants was assessed for the first three weeks after inoculation, and AUDPC (area under disease progress curve) calculated (Fig. 12). The mean AUDPC of FHB progress was higher in the control GP barley plants than in the HvNEP-1 transgenic lines.

[0061] 5.3 *Mycotoxin production*: Mycotoxin levels detected following inoculation with spores of *F. graminearum* or *F. culmorum* strains showed a general reduction in mycotoxin production in HvNEP-1 transgenic lines as compared to control GP barley plants (Fig. 13).

Example 6 Cloning and transgenic expression of HvNEP-1 and its orthologue genes in maize *(Zea mays)*

[0062] *6.1 Cloning Zea mays, ZmNEP-1 cDNA:* mRNA is extracted from leaves of *Zea mays* seedlings and used to generate cDNA as described by Yockteng et al (2013). The ZmNEP-1 cDNA has NCBI Ref sequence number: XM_008669862.2, and comprises a coding sequence for the ZmNEP-1 protein having protein ID: XP_008668084.1. A DNA sequence comprising the coding sequence for ZmNEP-1 having amino acid residues 1- 453 [SEQ ID No.: 45]; and the mature protein having amino residues 27- 453 [SEQ ID No.:45], are PCR amplified using cDNA as template and gene-specific primers, and Herculase II DNA polymerase, according to the manufacturer's instructions (Invitrogen). The amplified DNA fragment is gel purified and cloned into pCRII-TOPO Blunt vector according to the manufacturer's instructions (Invitrogen). Selected clones are evaluated for the insert by restriction digestion, and sequencing (Eurofins Genomics).

6.2 *Transgenic constructs and their transformation and expression* in *Zea mays*

[0063] The nucleic acid sequence encoding the protein: ZmNEP-1 is fused downstream of a seed-specific promoter and inserted upstream of the *Agrobacterium tumefaciens-derived* NOS terminator [SEQ ID No.: 31] in the transformation vector pWBVec8 (Gynheung et al., 1988). The seed-specific promoter used is as follows: α-zein gene promoter [SEQ ID No.: 50] for expression in Z. *mays.*

[0064] For Z. *mays* transformation, the vector, comprising the respective ZmNEP-1 expression construct, is transformed into competent *Agrobacterium strain* AH101, which is introduced into Z. *mays* embryos as described Ishida Y et al., (2007).

[0065] Positive transformants are detected by PCR using gene specific primers; and selected transformants are cultured to regenerate plants.

6.3 *Screening transformants for fungal disease resistance*

[0066] For Z. *mays,* ears are selected and inoculated at early silking stage with an inoculum of *Fusarium graminearum* and *Aspergillus niger* spores (5×10^5 spores/ml), and disease severity is evaluated after 3-4 weeks of humid growth conditions using a 7-class rating scale as described by Reid LM et al., (2002).

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[0067]

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Claims

1. A genetically modified cereal plant having a recombinant DNA construct integrated into the genome of the cereal plant; said construct comprising a gene operably linked to a heterologous promoter, wherein:

   i. said heterologous promoter directs grain-specific expression of said operably linked gene, and

   ii. said gene comprises a coding sequence encoding a signal peptide N-terminally fused to a polypeptide having aspartic endoprotease activity (EC 3.4.23.12), and wherein the amino acid sequence of said polypeptide has at least 88% sequence identity to a sequence selected from the group consisting of: SEQ ID No.: 4; amino acid residues 30-451 of SEQ ID No: 6; amino acid residues 30-451 of SEQ ID No: 8; amino acid residues 30-451 of SEQ ID No: 10; and amino acid residues 28-446 of SEQ ID No: 12; and

   wherein expression of said gene confers enhanced resistance to a fungal disease caused by a species of *Fusarium* and/or *Aspergillus* as compared to a parent cereal plant from which said genetically modified cereal plant was derived.

2. The genetically modified cereal plant of claim 1, wherein the nucleotide sequence of said heterologous promoter is selected from the group consisting of: SEQ ID No: 25; SEQ ID No: 26; SEQ ID No: 27; SEQ ID No: 28, and SEQ ID No: 29; and
   wherein said promoter directs endosperm-specific expression of said gene.

3. The genetically modified cereal plant of claims 1 or 2, where the amino acid sequence of said signal peptide is selected from the group consisting of: SEQ ID No: 14, 16, 18, 20, 22, and 24.

4. The genetically modified cereal plant of any one of claims 1 to 3,
   wherein the gene further encodes an endoplasmic reticulum retention peptide fused to the C-terminal of the polypeptide having aspartic endoprotease activity (EC 3.4.23.12), wherein the amino acid sequence of said peptide is selected from the group consisting of: KDEL, SEKDEL, and HDEL.

5. The genetically modified cereal plant of any one of claims 1 to 4, wherein said cereal plant is a species of *Triticum* or *Hordeum or Zea.*

6. Genetically modified grain of the genetically modified cereal plant of any one of claims 1 to 5, wherein the genome of said grain comprises the recombinant

DNA construct integrated into the genome of said genetically modified cereal plant.

7. A method for producing a genetically modified cereal plant of any one of claims 1 to 6 comprising:

a. transforming one or more cells of a parent cereal plant with a recombinant DNA construct comprising a gene operably linked to a heterologous promoter, wherein

i. said promoter directs grain-specific expression of said operably linked gene, and
ii. said gene comprises a coding sequence encoding a signal peptide N-terminally fused to a polypeptide having aspartate endoprotease activity (EC 3.4.23.12), and wherein the amino acid sequence of said polypeptide has at least 88% identity to a sequence selected from the group consisting of: SEQ ID No.: 4; amino acid residues 30-451 of SEQ ID No: 6; amino acid residues 30-451 of SEQ ID No: 8; amino acid residues 30-451 of SEQ ID No: 10; and amino acid residues 28-446 of SEQ ID No: 12; and

b. selecting transformed cells of said cereal plant, wherein the genome of said cells comprises a copy of said recombinant DNA construct; and
c. regenerating a genetically modified cereal plant from cells obtained in step (b).

8. A method for detecting a cereal plant exhibiting increased resistance to a fungal disease caused by a species of *Fusarium* and/or *Aspergillus,* said method comprising:

a. obtaining a sample of genomic DNA from a cereal plant according to claim 1 or portion thereof;
b. detecting in said sample the presence of said recombinant DNA construct;
wherein said recombinant DNA construct comprises a gene operably linked to a heterologous promoter, wherein

i. said promoter directs grain-specific expression of said operably linked gene, and
ii. said gene comprises a coding sequence encoding a signal peptide N-terminally fused to a polypeptide having aspartyl protease activity (EC 3.4.23.12), and wherein the amino acid sequence of said polypeptide has at least 88% identity to a sequence selected from the group consisting of: SEQ ID No.: 4; amino acid residues 30-451 of SEQ ID No: 6; amino acid residues 30-451 of SEQ ID No: 8; amino acid residues 30-451 of SEQ ID No: 10; and amino acid residues 28-446 of SEQ ID No: 12.

9. The method of claim 8, where said recombinant DNA construct is detected by amplification of a region of nucleic acid sequence of said construct, wherein said region has a 5' end within the promoter and a 3' end within the gene.

10. The method of any one of claims 7 to 9, wherein said cereal plant is a species of *Triticum* or *Hordeum* or *Zea.*

11. Use of the genetically modified grain of claim 6 for the manufacture of a composition, wherein said composition is any one of:

a. a milled grain or seed composition,
b. animal fodder, and
c. steam-pelleted animal fodder,

wherein the genome of said grain comprises the recombinant DNA construct integrated into the genome of said genetically modified cereal plant.

Patentansprüche

1. Genetisch veränderte Getreidepflanze, welche ein in das Genom der Getreidepflanze integriertes rekombinantes DNA-Konstrukt aufweist; wobei das Konstrukt ein Gen umfasst, welches funktionell mit einem heterologen Promotor verknüpft ist, wobei:

i. der heterologe Promotor die getreidespezifische Expression des funktionell verknüpften Gens steuert, und
ii. das Gen eine codierende Sequenz umfasst, welche ein Signalpeptid codiert, welches N-terminal an ein Polypeptid mit Aspartat-Endoprotease-Aktivität (EC 3.4.23.12) fusioniert ist, und wobei die Aminosäuresequenz des Polypeptids zumindest 88 % Sequenzidentität mit einer Sequenz aufweist, welche ausgewählt ist aus der Gruppe bestehend aus: SEQ ID Nr.: 4; Aminosäureresten 30-451 von SEQ ID Nr.: 6; Aminosäureresten 30-451 von SEQ ID Nr.: 8; Aminosäureresten 30-451 von SEQ ID Nr.: 10; und Aminosäureresten 28-446 von SEQ ID Nr.: 12; und

wobei die Expression dieses Gens eine erhöhte Resistenz gegen eine Pilzerkrankung, welche durch eine Art von *Fusarium* und/oder *Aspergillus* verursacht wird, im Vergleich zu einer Mutter-Getreidepflanze verleiht, von welcher die genetisch verän-

derte Getreidepflanze abstammt.

2. Genetisch veränderte Getreidepflanze nach Anspruch 1, wobei die Nukleotidsequenz des heterologen Promotors ausgewählt ist aus der Gruppe bestehend aus: SEQ ID Nr.: 25; SEQ ID Nr.: 26; SEQ ID Nr.: 27; SEQ ID Nr.: 28 und SEQ ID Nr.: 29; und
wobei der Promotor die endospermspezifische Expression des Gens steuert.

3. Genetisch veränderte Getreidepflanze nach Anspruch 1 oder 2, wobei die Aminosäuresequenz des Signalpeptids ausgewählt ist aus der Gruppe bestehend aus SEQ ID Nr.: 14, 16, 18, 20, 22 und 24.

4. Genetisch veränderte Getreidepflanze nach einem der Ansprüche 1 bis 3, wobei das Gen weiter ein endoplasmatisches Retikulum-Retentionspeptid codiert, welches an dem C-Terminus des Polypeptids mit Aspartat-Endoprotease-Aktivität (EC 3.4.23.12) fusioniert ist, wobei die Aminosäuresequenz des Peptids ausgewählt ist aus der Gruppe bestehend aus: KDEL, SEKDEL und HDEL.

5. Genetisch veränderte Getreidepflanze nach einem der Ansprüche 1 bis 4, wobei die Getreidepflanze eine Art von *Triticum* oder *Hordeum* oder Zea ist.

6. Genetisch verändertes Korn der genetisch veränderten Getreidepflanze nach einem der Ansprüche 1 bis 5, wobei das Genom des Korns das rekombinante DNA-Konstrukt umfasst, welches in das Genom der genetisch veränderten Getreidepflanze integriert ist.

7. Erzeugungsverfahren einer genetisch veränderten Getreidepflanze nach einem der Ansprüche 1 bis 6, umfassend:

a) Transformieren einer oder mehrerer Zellen einer Getreidepflanze mit einem rekombinanten DNA-Konstrukt, welches ein Gen umfasst, welches funktionell mit einem heterologen Promotor verknüpft ist, wobei

i. der Promotor die getreidespezifische Expression des funktionell verknüpften Gens steuert, und
ii. das Gen eine codierende Sequenz umfasst, welche ein Signalpeptid codiert, welches N-terminal an ein Polypeptid mit Aspartat-Endoprotease-Aktivität (EC 3.4.23.12) fusioniert ist, und wobei die Aminosäuresequenz des Polypeptids zumindest 88 % Identität mit einer Sequenz aufweist, welche ausgewählt ist aus der Gruppe bestehend aus: SEQ ID Nr.: 4; Aminosäureresten 30-451 von SEQ ID Nr.: 6; Aminosäureresten 30-451 von SEQ ID Nr.: 8; Aminosäureresten 30-451 von SEQ ID Nr.: 10; und Aminosäureresten 28-446 von SEQ ID Nr.: 12; und

b) Auswählen transformierter Zellen der Getreidepflanze, wobei das Genom der Zellen eine Kopie des rekombinanten DNA-Konstrukts umfasst; und
c) Regenerieren einer genetisch veränderten Getreidepflanze aus den in Schritt b) gewonnenen Zellen.

8. Verfahren zum Nachweisen einer Getreidepflanze, welche eine erhöhte Resistenz gegen eine Pilzerkrankung aufweist, welche durch eine Art von *Fusarium* und/oder *Aspergillus* verursacht wird, wobei das Verfahren umfasst:

a) Gewinnen einer Probe genomischer DNA aus einer Getreidepflanze nach Anspruch 1 oder einem Abschnitt davon;
b) Nachweisen des Vorhandenseins des rekombinanten DNA-Konstrukts in der Probe;
wobei das rekombinante DNA-Konstrukt ein Gen umfasst, welches funktionell mit einem heterologen Promotor verknüpft ist, wobei

i. der Promotor die getreidespezifische Expression des funktionell verknüpften Gens steuert, und
ii. das Gen eine codierende Sequenz umfasst, welche ein Signalpeptid codiert, welches N-terminal an ein Polypeptid mit Aspartyl-Protease-Aktivität (EC 3.4.23.12) fusioniert ist, und wobei die Aminosäuresequenz des Polypeptids zumindest 88 % Identität mit einer Sequenz aufweist, welche ausgewählt ist aus der Gruppe bestehend aus: SEQ ID Nr.: 4; Aminosäureresten 30-451 von SEQ ID Nr.: 6; Aminosäureresten 30-451 von SEQ ID Nr.: 8; Aminosäureresten 30-451 von SEQ ID Nr.: 10; und Aminosäureresten 28-446 von SEQ ID Nr.: 12.

9. Verfahren nach Anspruch 8, wobei das rekombinante DNA-Konstrukt durch Amplifikation eines Bereichs der Nukleinsäuresequenz des Konstrukts nachgewiesen wird, wobei der Bereich ein 5'-Ende innerhalb des Promotors und ein 3'-Ende innerhalb des Gens aufweist.

10. Verfahren nach einem der Ansprüche 7 bis 9, wobei die Getreidepflanze eine Art von *Triticum* oder *Hordeum* oder *Zea* ist.

11. Verwendung des genetisch veränderten Korns nach Anspruch 6 zur Herstellung einer Zusammensetzung, wobei die Zusammensetzung eine der Folgenden ist:

a) eine gemahlene Korn- oder Saatzusammensetzung,
b) Tierfutter, und
c) durch Dampfpelletierung hergestelltes Tierfutter,

wobei das Genom des Korns das rekombinante DNA-Konstrukt umfasst, welches in das Genom der genetisch veränderten Getreidepflanze integriert ist.

Revendications

1. Plante céréalière génétiquement modifiée présentant une construction d'ADN recombinant intégrée dans le génome de la plante céréalière ; ladite construction comprenant un gène lié fonctionnellement à un promoteur hétérologue, dans laquelle :

i. ledit promoteur hétérologue dirige une expression spécifique au grain dudit gène lié fonctionnellement et
ii. ledit gène comprend une séquence de codification codant pour un peptide signal fusionné en N-terminal à un polypeptide présentant une activité d'endoprotéase aspartique (EC 3.4.23.12), et dans laquelle la séquence d'acides aminés dudit polypeptide présente au moins 88 % d'identité de séquence avec une séquence choisie dans le groupe constitué par : SEQ ID No.: 4 ; des résidus d'acides aminés 30-451 de SEQ ID No.: 6 ; des résidus d'acides aminés 30-451 de SEQ ID No.: 8 ; rdes ésidus d'acides aminés 30-451 de SEQ ID No.: 10 ; et des résidus d'acides aminés 28-446 de SEQ ID No.: 12 ; et

dans laquelle l'expression dudit gène confère une résistance accrue à une maladie fongique causée par une espèce de *Fusarium* et/ou *Aspergillus* par comparaison à une plante céréalière mère à partir de laquelle ladite plante céréalière génétiquement modifiée est dérivée.

2. Plante céréalière génétiquement modifiée selon la revendication 1, dans laquelle la séquence nucléotidique dudit promoteur hétérologue est choisie dans le groupe constitué par : SEQ ID No.: 25 ; SEQ ID No.: 26 ; SEQ ID No.: 27 ; SEQ ID No.: 28 et SEQ ID No.: 29 ; et
dans laquelle ledit promoteur dirige une expression spécifique à l'endosperme dudit gène.

3. Plante céréalière génétiquement modifiée selon la revendication 1 ou 2, dans laquelle la séquence d'acides aminés dudit peptide signal est choisie dans le groupe constitué par : SEQ ID No.: 14, 16, 18, 20, 22 et 24.

4. Plante céréalière génétiquement modifiée selon l'une quelconque des revendications 1 à 3, dans laquelle le gène code en outre un peptide de rétention du réticulum endoplasmique fusionné à l'extrémité C-terminale du polypeptide présentant une activité d'endoprotéase aspartique (EC 3.4.23.12), dans laquelle la séquence d'acides aminés dudit peptide est choisie dans le groupe constitué pat : KDEL, SEKDEL et HDEL.

5. Plante céréalière génétiquement modifiée selon l'une quelconque des revendications 1 à 4, dans laquelle ladite plante céréalière est une espèce de *Triticum* ou *Hordeum* ou Zea.

6. Grain génétiquement modifié de la plante céréalière génétiquement modifiée selon l'une quelconque des revendications 1 à 5, dans lequel le génome dudit grain comprend la construction d'ADN recombinant intégrée dans le génome de ladite plante céréalière génétiquement modifiée.

7. Procédé de production d'une plante céréalière génétiquement modifiée selon l'une quelconque des revendications 1 à 6, comprenant :

a) la transformation d'une ou de plusieurs cellules d'une plante céréalière mère avec une construction d'ADN recombinant comprenant un gène lié fonctionnellement à un promoteur hétérologue, dans lequel

i. ledit promoteur dirige l'expression spécifique au grain dudit gène lié fonctionnellement, et
ii. ledit gène comprend une séquence de codification codant pour un peptide signal fusionné en N-terminal à un polypeptide présentant une activité d'endoprotéase d'aspartate (EC 3.4.23.12) et dans lequel la séquence d'acides aminés dudit polypeptide présente au moins 88 % d'identité avec une séquence choisie dans le groupe constitué par : SEQ ID No. : 4 ; des résidus d'acides aminés 30-451 de SEQ ID No.: 6 ; des résidus d'acides aminés 30-451 de SEQ ID No.: 8 ; des résidus d'acides aminés 30-451 de SEQ ID No.: 10 ; et des résidus d'acides aminés 28-446 de SEQ ID No.: 12 ; et

b) la sélection de cellules transformées de ladite

plante céréalière, dans lequel le génome desdites cellules comprend une copie de ladite construction d'ADN recombinant ; et
c) la régénération d'une plante céréalière génétiquement modifiée à partir de cellules obtenues à l'étape (b).

8. Procédé de détection d'une plante céréalière présentant une résistance accrue à une maladie fongique causée par une espèce de *Fusarium* et/ou *Aspergillus,* ledit procédé comprenant :

a) l'obtention d'un échantillon d'ADN génomique à partir d'une plante céréalière selon la revendication 1 ou d'une partie de celle-ci ;
b) la détection, dans ledit échantillon, de la présence de ladite construction d'ADN recombinant ;
dans lequel ladite construction d'ADN recombinant comprend un gène lié fonctionnellement à un promoteur hétérologue, dans lequel

i. ledit promoteur dirige l'expression spécifique au grain dudit gène lié fonctionnellement, et
ii. ledit gène comprend une séquence de codification codant pour un peptide signal fusionné en N-terminal à un polypeptide présentant une activité de protéase d'aspartyl (EC 3.4.23.12), et dans lequel la séquence d'acides aminés dudit polypeptide présente au moins 88 % d'identité avec une séquence choisie dans le groupe constitué par : SEQ ID No.: 4 ; des résidus d'acides aminés 30-451 de SEQ ID No.: 6 ; des résidus d'acides aminés 30-451 de SEQ ID No.: 8 ; des résidus d'acides aminés 30-451 de SEQ ID No.: 10 ; et des résidus d'acides aminés 28-446 de SEQ ID No.: 12.

9. Procédé selon la revendication 8, dans lequel ladite construction d'ADN recombinant est détectée par amplification d'une région de séquence d'acides nucléiques de ladite construction, dans lequel ladite région présente une extrémité 5' dans le promoteur et une extrémité 3' dans le gène.

10. Procédé selon l'une quelconque des revendications 7 à 9, dans lequel ladite plante céréalière est une espèce de *Triticum* ou *Hordeum* ou *Zea.*

11. Utilisation du grain génétiquement modifié selon la revendication 6 pour la fabrication d'une composition, dans laquelle ladite composition est l'un quelconque :

a) d'une composition de grains moulus ou de graines moulues,
b) d'un fourrage pour animaux, et
c) d'un fourrage pour animaux granulé à la vapeur,

dans laquelle le génome dudit grain comprend la construction d'ADN recombinant intégrée dans le génome de ladite plante céréalière génétiquement modifiée.

Figure 1

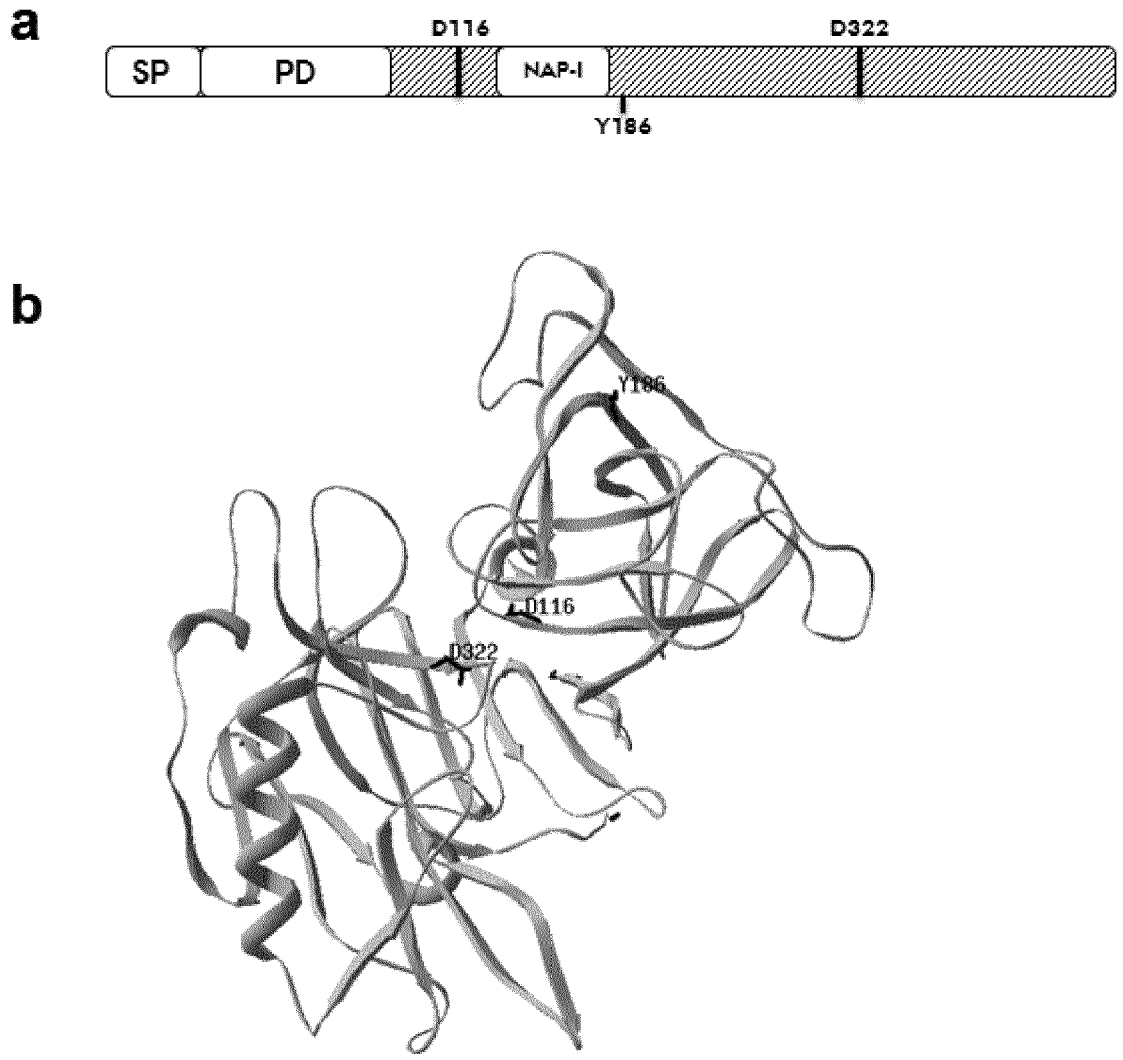

Figure 2A

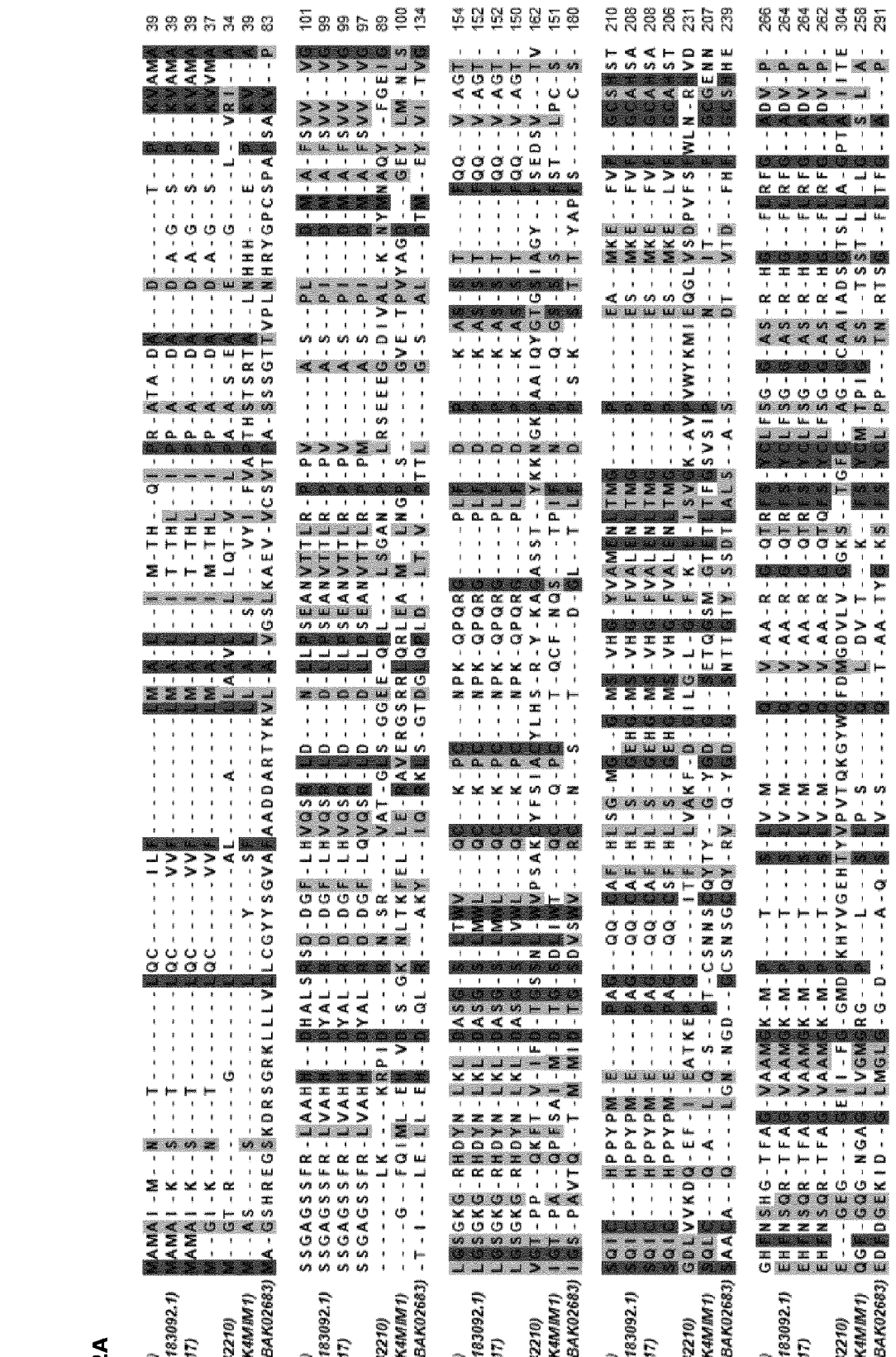
HvNEP-1 (M0W9B2)
A.tauschii (XP_020183092.1)
T. aestivum (W5EU17)
T. Urartu (T1NBT2)
H. vulgare phyt (P42210)
N. mirabilis Nep1 (K4MIM1)
H. vulgare CND41 (BAK02683)

Figure 2A continued

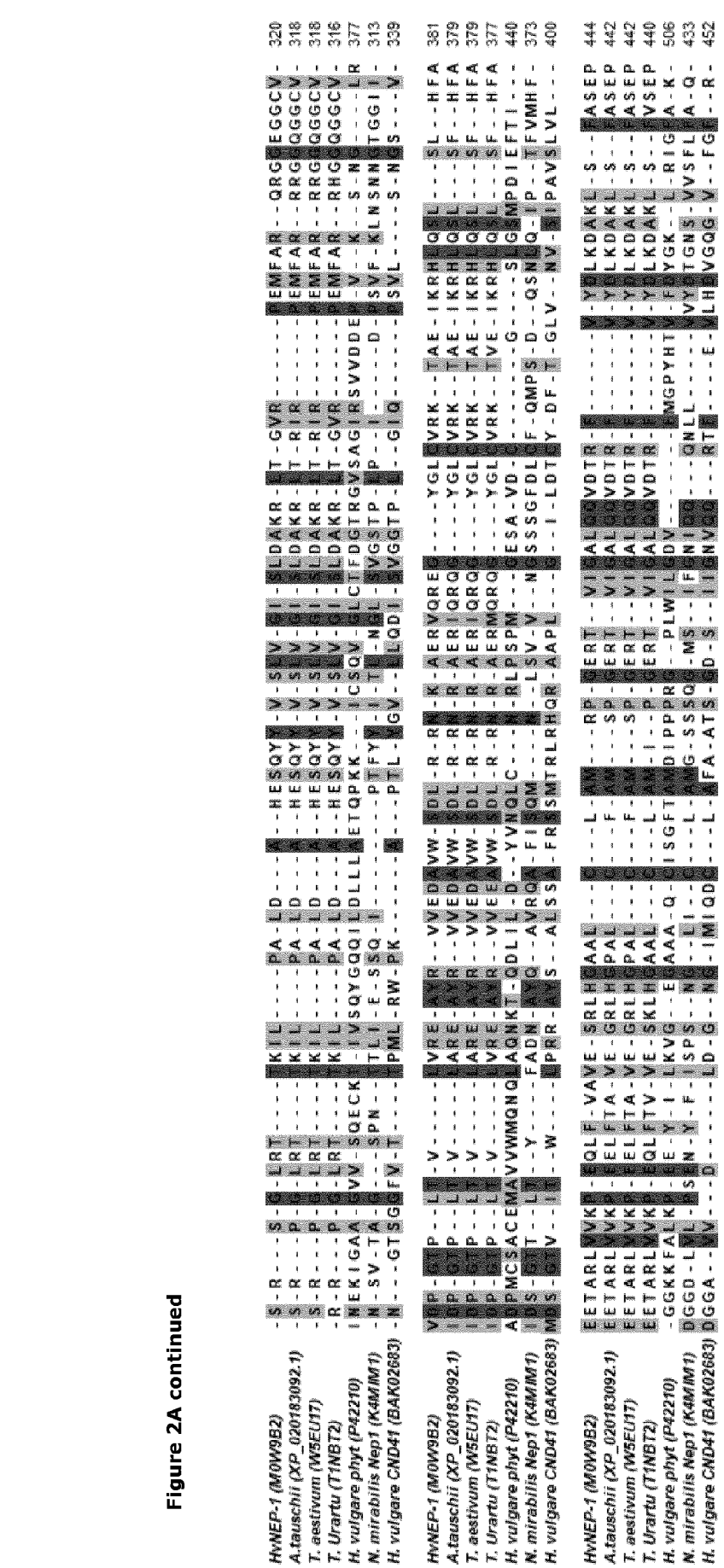

Figure 2B

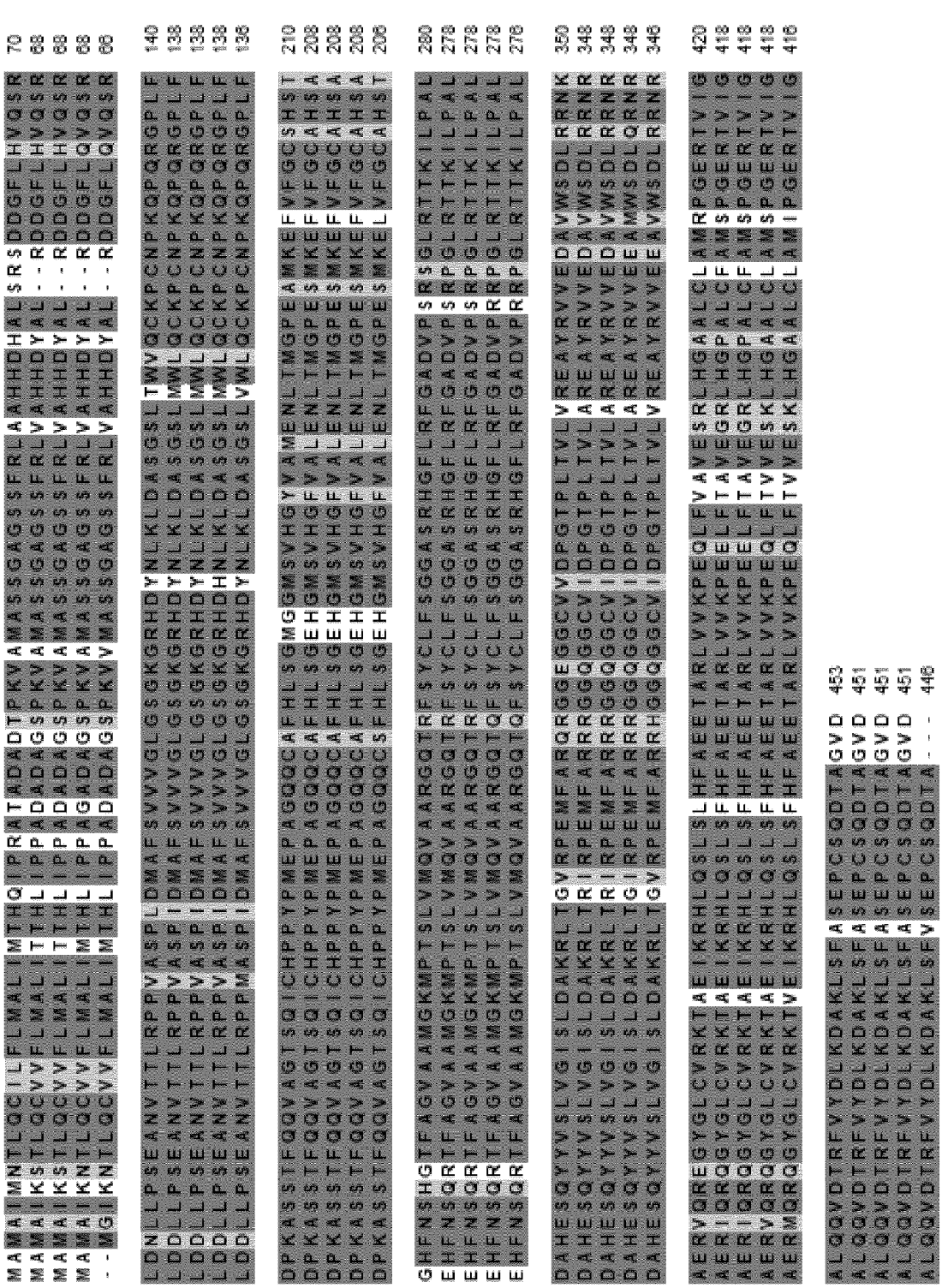
HvNEP-1 (M0W9B2)
Aegilops tauschii(XP_020183092
T. aestivum(WSEU17)
T. aestivum(A0A1D6RYR6)
T. Urartu (T1NBT2)

Figure 3

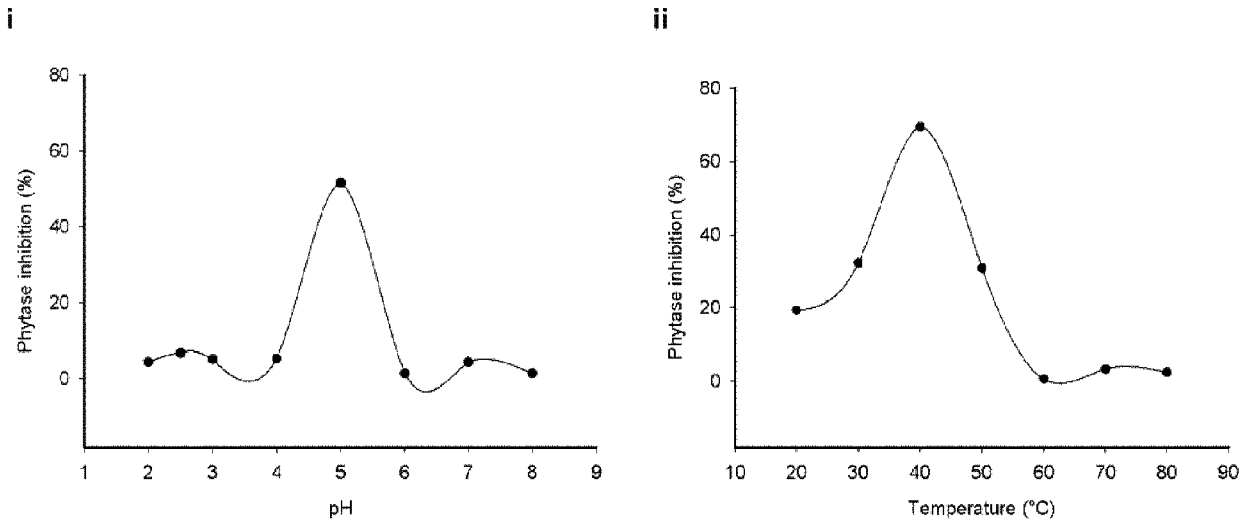

Figure 4

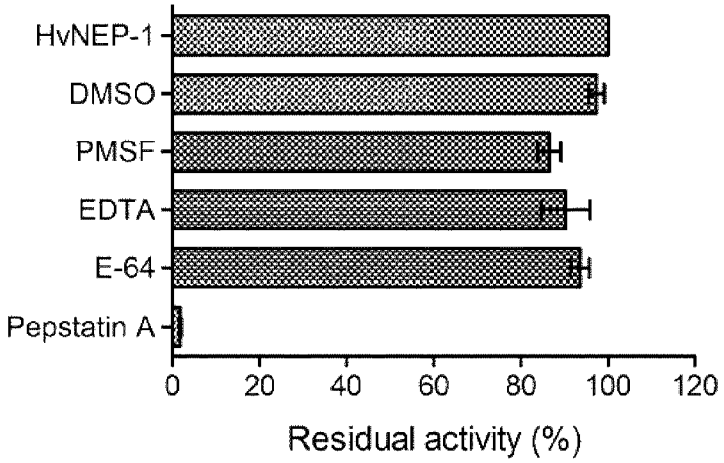

Figure 5

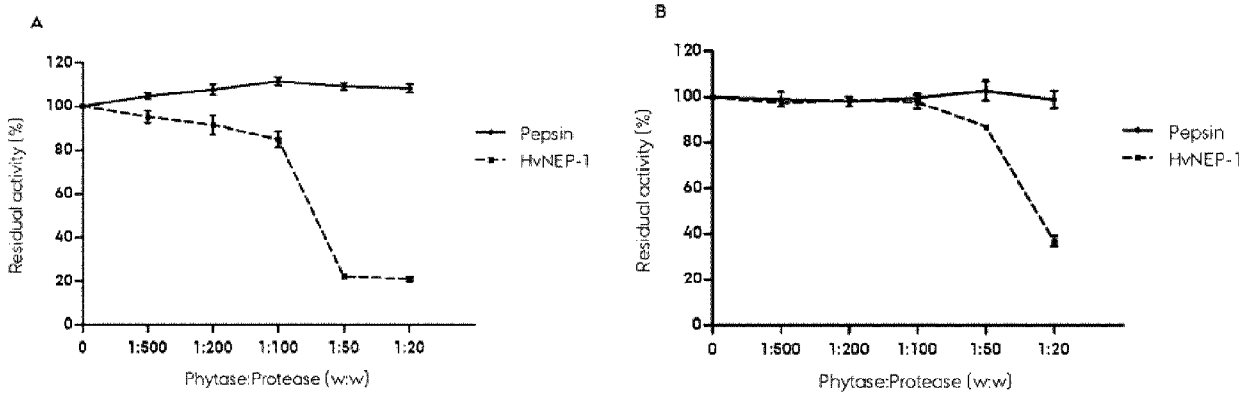

Figure 6

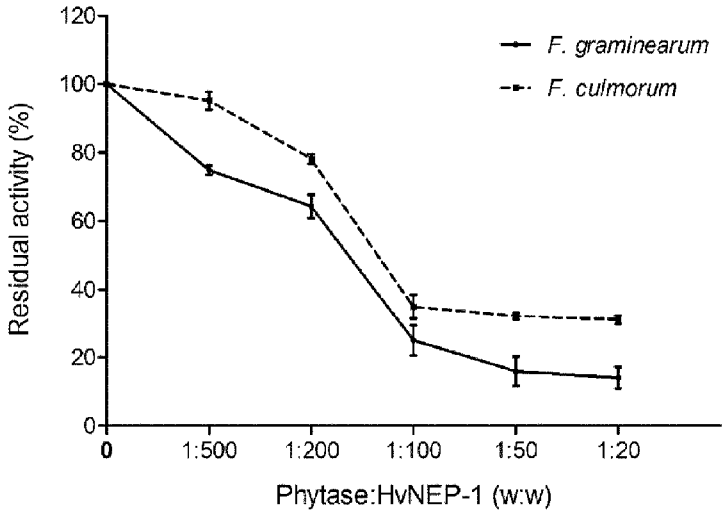

Figure 7

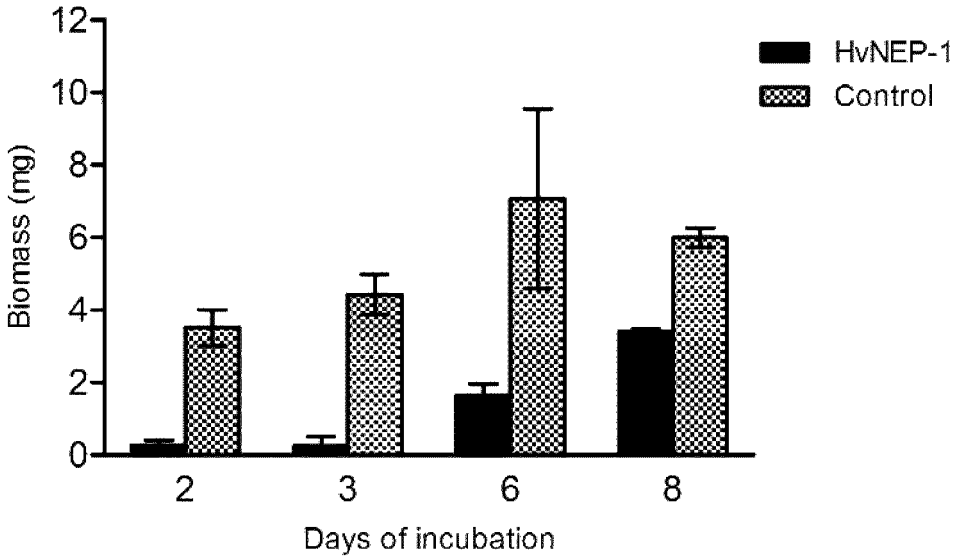

Figure 8

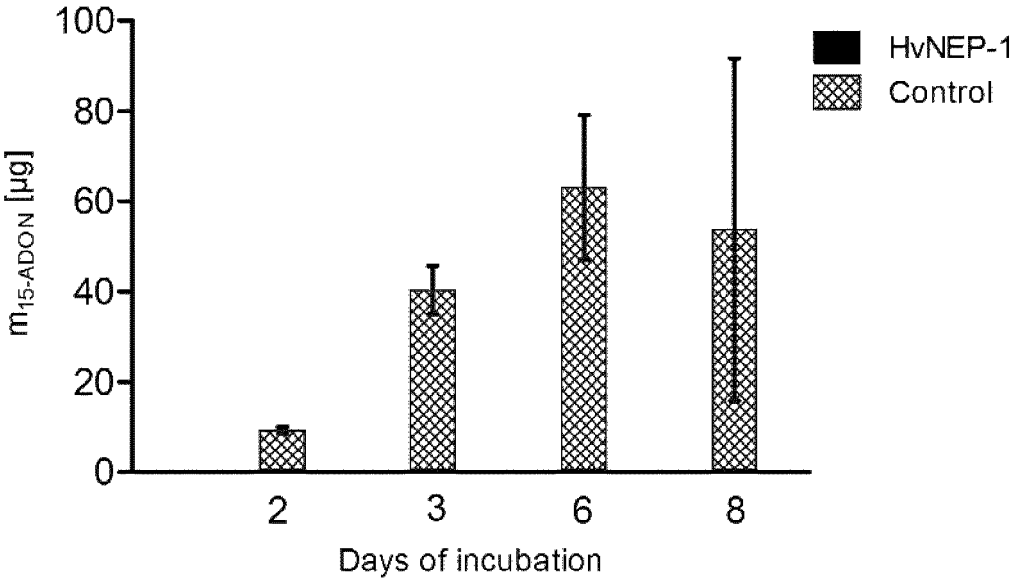

Figure 9

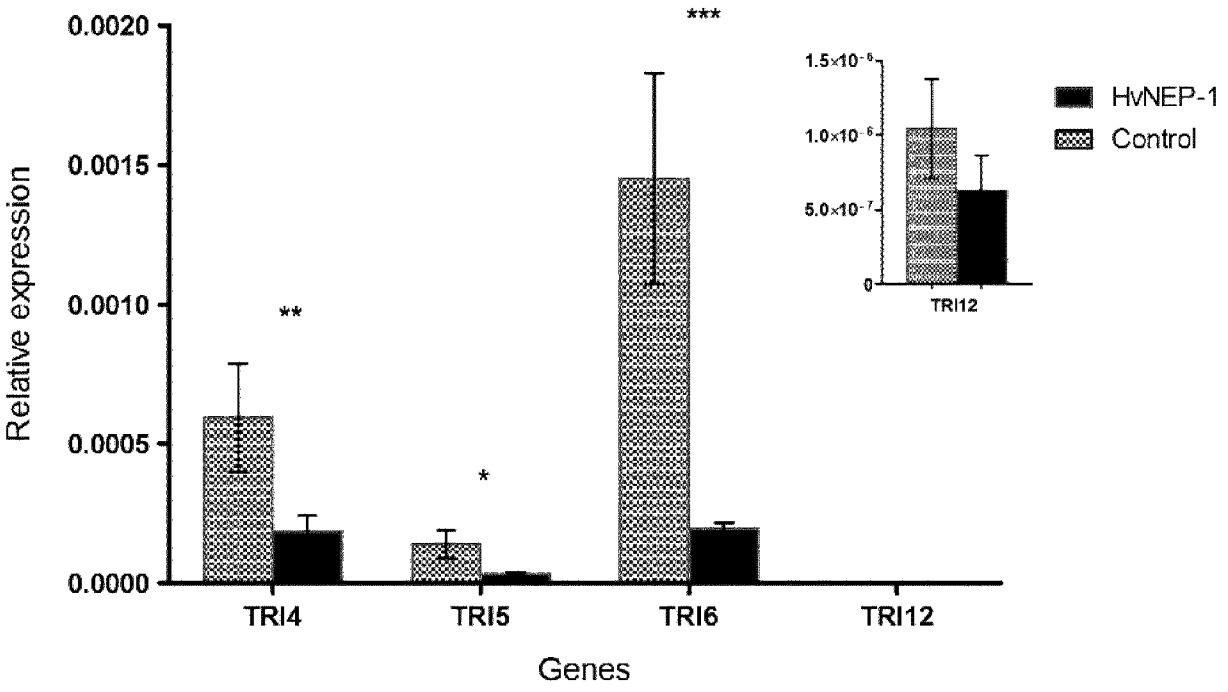

Figure 10

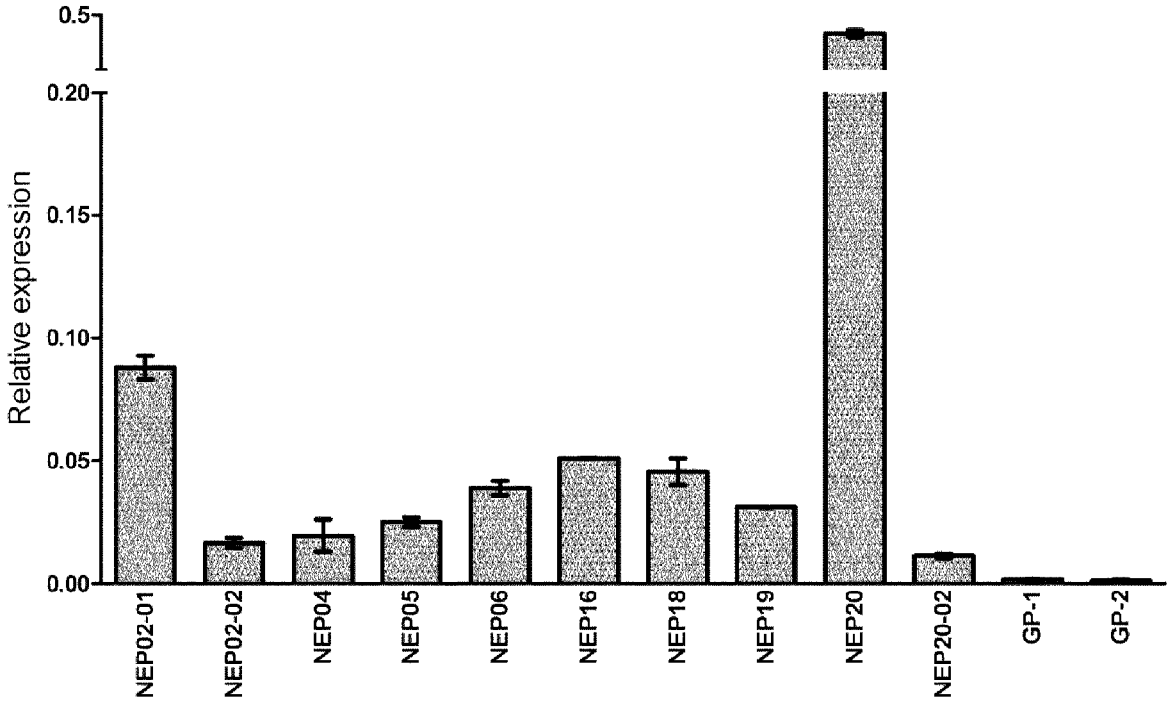

Figure 11

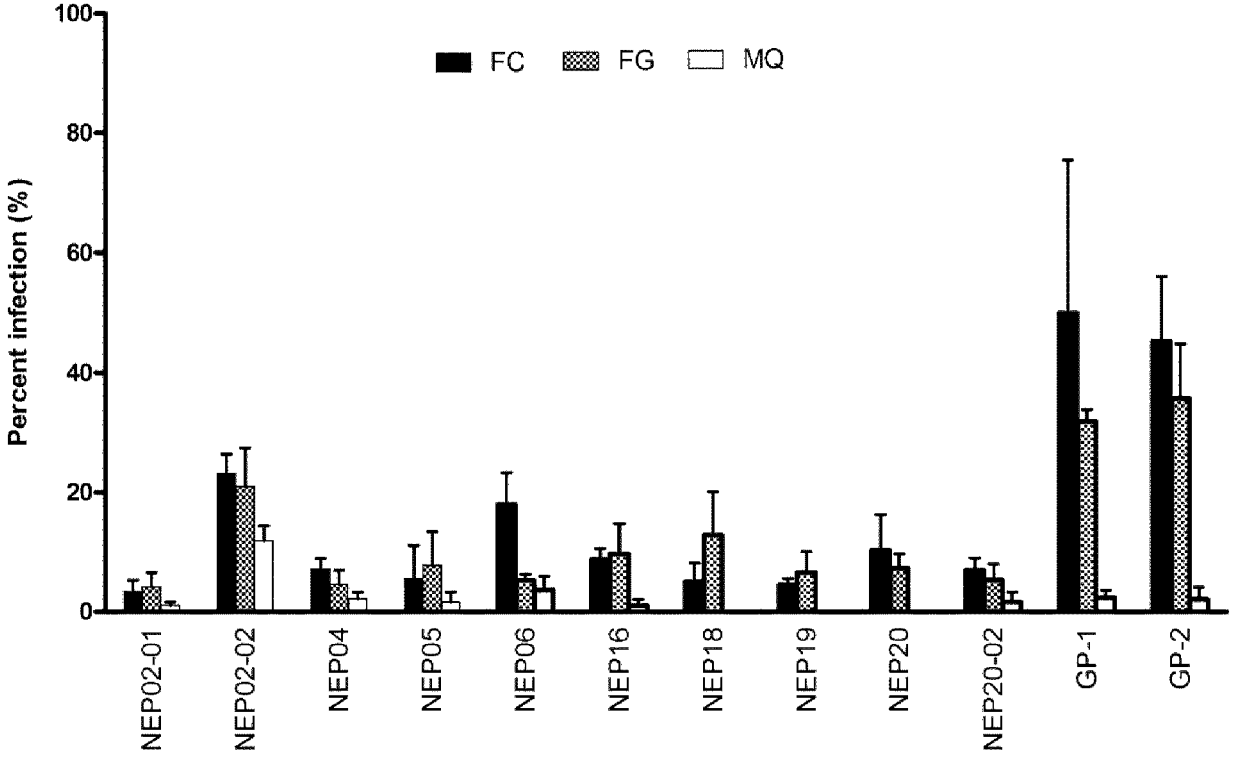

Figure 12

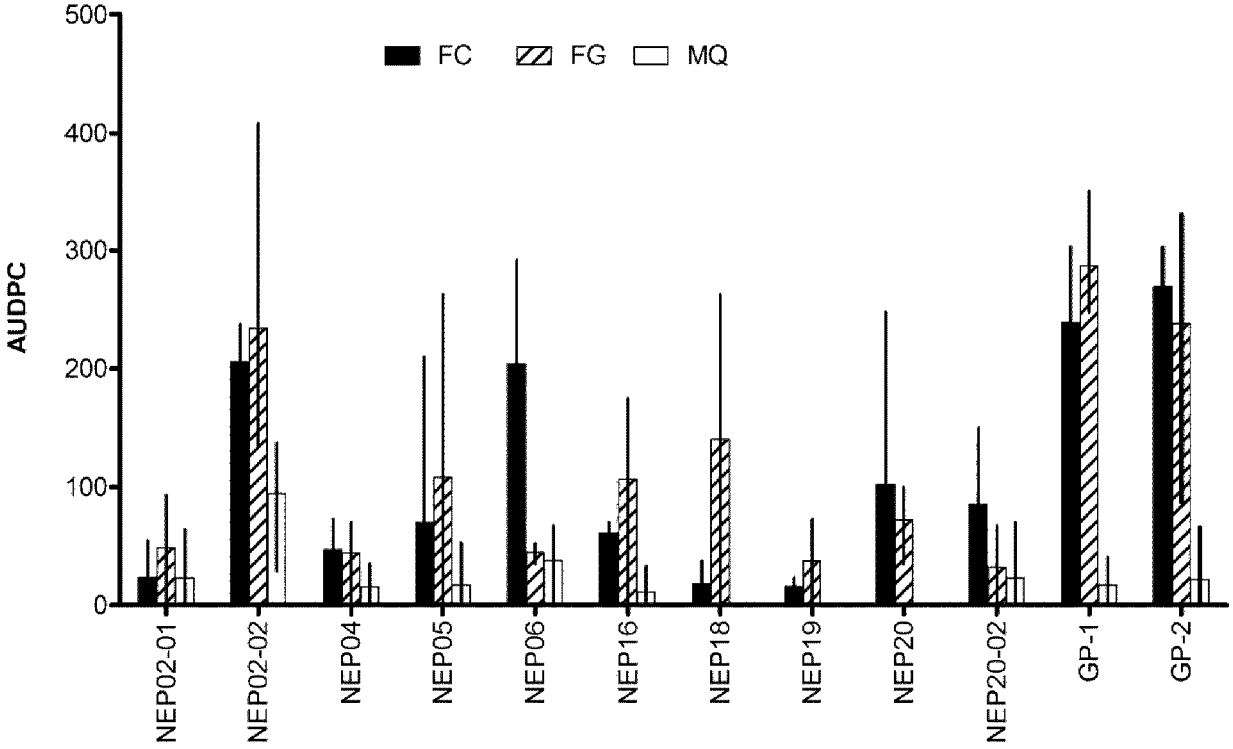

Figure 13

		HvNEP-1 transgenic lines and GP controls													
		NEP02-01	**NEP02-02**	**NEP04**	**NEP05**	**NEP06**	**NEP16**	**NEP18**	**NEP19**	**NEP20**	**NEP20-02**	**GP-1**	**GP-2**		
Samples used for analysis	**FC+**	15938	794	1627	1231	9198	925	1257	<50	25043	462	7264	77	**NIV**	**Mycotoxins (µg/kg DW)**
		<50	<50	<50	<50	52	<50	<50	<50	152	<50	37091	2324	**DON**	
		<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	186	55	**ZEA**	
	FC-	77	<50	<50	55	<50	<50	<50	<50	<50	<50	<50	<50	**NIV**	
		<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	142	855	**DON**	
		<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	15	**ZEA**	
	FG+	35510	6243	1543	16377	29727	597	97	<50	53	2399	13050	<50	**NIV**	
		5295	<50	56	<50	<50	<50	<50	8170	40073	<50	6022	1664	**DON**	
		<5	<5	<5	<5	<5	<5	<5	<5	256	<5	<5	38	**ZEA**	
	FG-	124	<50	<50	<50	171	<50	<50	<50	<50	<50	<50	<50	**NIV**	
		<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	569	240	**DON**	
		<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	**ZEA**	
	MQ	<50	52	<50	<50	<50	163	<50	<50	<50	<50	<50	<50	**NIV**	
		<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	**DON**	
		<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	**ZEA**	

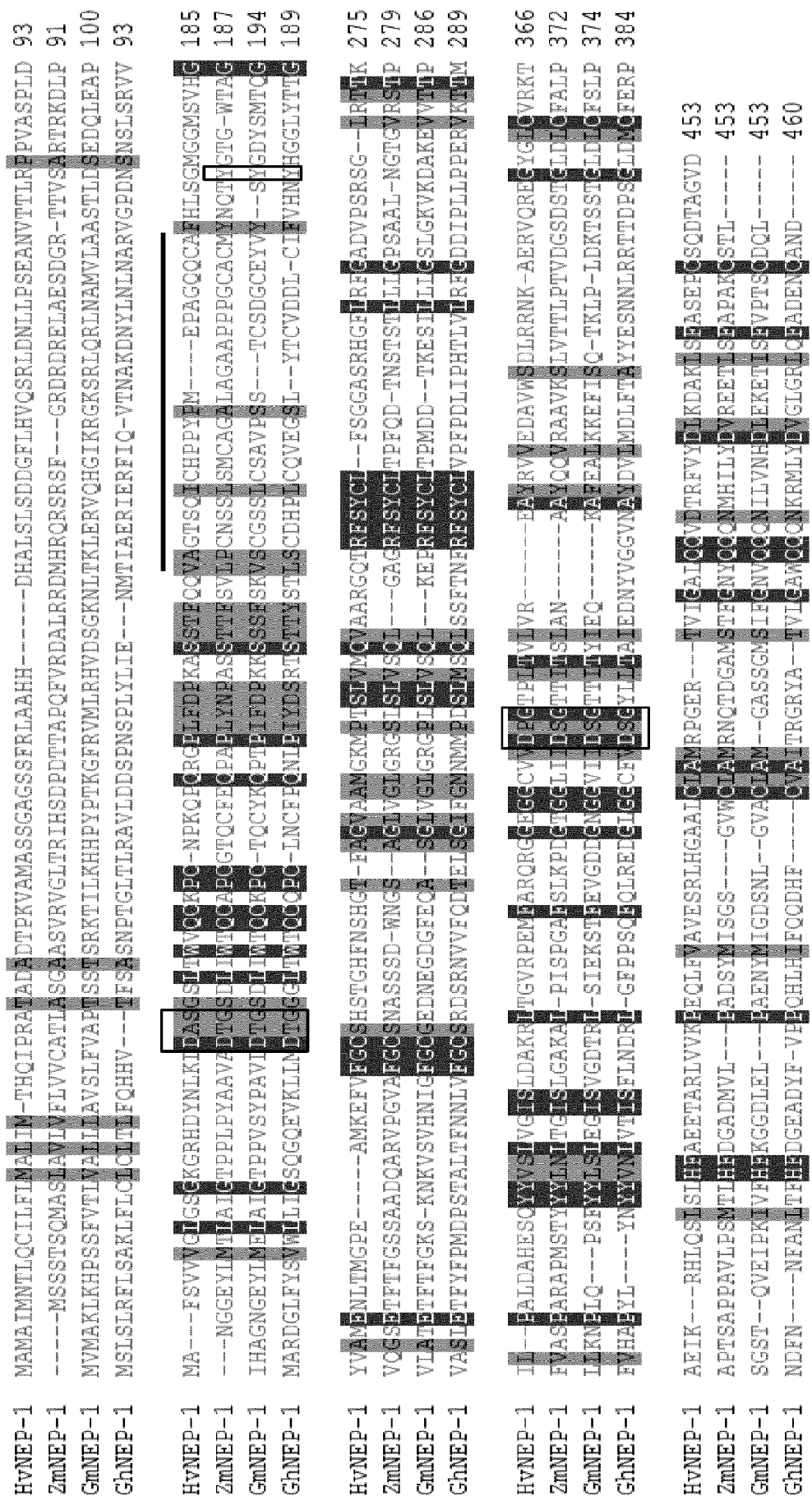

Figure 14

REFERENCES CITED IN THE DESCRIPTION

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