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(54) Title: NOVEL NECROSIS AND ETHYLENE INDUCING PROTEINS FROM BOTRYTIS

(57) Abstract: The present invention relates to necrosis and ethylene inducing proteins from Botrytis species and their use. Provided are also methods for determining the susceptibility of a plant's tissue to a necrosis and ethylene inducing protein and methods for selecting plants being resistant to one or more of these proteins and to Botrytis infection.



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Novel necrosis and ethylene inducing proteins from *Botrytis*

Field of the invention

5 The present invention relates to novel necrosis and ethylene inducing proteins (referred to as BxNEP1 and BxNEP2) from *Botrytis*, and to nucleic acids encoding *Botrytis* NEP proteins, as well as methods for producing NEP proteins. The invention further relates to the use of these proteins and/or nucleic acids in plant breeding, in enforcing plant sanitation and in methods for screening compounds that are active
10 against *Botrytis*. In addition, new uses for non-*Botrytis* NEP proteins are provided. In another embodiment, the use of broad range and host-specific BxNEP1 and BxNEP2 proteins as bioherbicides is provided.

Background of the invention

15 The plant pathogenic fungus *Botrytis cinerea* (teleomorph: *Botryotinia fuckeliana*) causes severe pre-and post harvest diseases (e.g. "grey mould") in more than 235 plant species (Jarvis, 1977, *Botryotinia* and *Botrytis* species – Taxonomy, physiology and pathogenicity. A guide to the literature, Monograph no.14, Ottawa, Research Branch, Canada Department of Agriculture), including commercially
20 important species such as grapevine, tomato, strawberry, cucumber, bulb flowers and ornamental plants. The fungus is considered a necrotroph; it kills the host tissue prior to colonization. Little information about the mechanism of cell killing is available but the role of diffusible toxins, oxalic acid, production of active oxygen species and cell wall degrading enzymes were proposed. For a review on the infection strategies of *B.*
25 *cinerea* we refer to Prins *et al.* (2000, in: Fungal pathology. Ed. Kronstad, J., Kluwer Academic Publishers, Dordrecht, The Netherlands. Pp 32-64).

For many phytopathogenic microorganisms necrosis and ethylene inducing proteins (NEP) have been described to play a role in pathogenesis. Currently, some
30 homologues of genes encoding NEPs have been described in various bacteria, oomycetes and fungi. The proteins encoded by these genes have been referred to as 'necrosis and ethylene inducing protein' (NEP), 'necrosis-inducing-like peptide' (NIP), '25 kDa elicitor-like protein', 'necrosis-inducing *Phytophthora* protein' (NPP) or 'necrosis-inducing elicitor' (NIE). More in particular, the species for which such

NEP homologues have been described are: *Fusarium oxysporum* f.sp. *erythroxyli* (e.g. Genbank accession number AAC97382), *Fusarium graminearum* (teleomorph: *Giberella zae*), *Verticillium dahliae* (e.g. AAS45247), *Magnaporthe grisea* (e.g. EAA46838; EAA49539; EAA48743), *Neurospora crassa* (XP_331114), *Aspergillus*
5 *nidulans* (EAA62936), *Pythium aphanidermatum* (AAD53944), *Pythium middletoni* (AAQ89594), *Pythium monospermum* (AAQ89593), *Phytophthora sojae* (AAM48171; AAK01636); AAM48172, *Phytophthora infestans* (AAK25828), *Phytophthora parasitica* (AAK19753), *Erwinia carotovora* (YP_051177), *Bacillus halodurans* (NP_241261), *Streptomyces coelicolor* (NP_631398), *Bacillus licheniformis* and *Vibrio pommerensis*.
10 These proteins can be characterized by both their activity (in particular necrosis induction) and by structural similarity, such as the presence of the conserved GHRHDWE domain (which may comprise one amino acid change in some NEP proteins). They have an overall amino acid sequence identity of above around 30% using the Blast algorithm and above around 20% using the Needleman and Wunsch
15 algorithm for global pairwise alignments. The size of the proteins ranges from about 200 to about 300 amino acids, i.e. having a molecular weight of around about 20 to 30kDa. The activity of NEP proteins and their role as elicitors have been reviewed by Pemberton and Salmond (2004, Molec. Plant Pathol. 5, 353-359). In hemibiotrophic plant pathogens it was shown that the genes encoding these proteins are expressed
20 when the pathogens switch from the biotrophic phase to the necrotrophic phase. All NEPs analysed so far were found to be toxic for dicotyledonous plants and Bailey and co-workers have suggested using the *Fusarium oxysporum* NEP as a bioherbicide (Jennings *et al.* 2000, Weed Science 48, 7-14).

However, the art thus far has not described NEP homologues in the genus
25 *Botrytis*. Thus, it is an object of the invention to provide NEP homologues in the commercially important phytopathogens of the genus *Botrytis*. It is a further object of the invention to provide for nucleic acids encoding *Botrytis* NEPs, as well as methods for using these proteins and/or nucleic acids in plant breeding, in enforcing plant sanitation and in methods for screening compounds that are active against *Botrytis*. In
30 addition, BcNEP proteins may be used as ingredients in the manufacture of herbicide compositions.

Description of the invention

Definitions

The term “necrosis” refers herein to the development of necrotic lesions or necrotic areas (or completely necrotized organs, such as leaves) on plant cells or tissue
5 contacted with a NEP protein according to the invention. Visible necrosis is due to sufficient numbers of dead plant cells so that the necrotic areas become visible to the eye (macro-lesions, in contrast to micro-lesions, which are only visible following magnification). “Absolute necrosis” refers to necrosis of substantially all cells or tissues brought into contact with a suitable amount of a NEP protein according to the
10 invention.

“Phytotoxin” refers to a protein which causes plant cell death when brought into contact with plant cell(s).

“*Botrytis*” refers to all species of the genus *Botrytis*, in particular to *Botrytis cinerea*, but also other species, such as *B. tulipae*, *B. elliptica*, *B. fabae*, *B. aclada*, *B.*
15 *allii*, *B. byssoideae*, *B. convoluta*, *B. croci*, *B. galanthina*, *B. gladiolorum*, *B. globosa*, *B. hyacinthi*, *B. porri*, *B. polyblastis*, *B. sphaerosperma*, *B. squamosa*, *B. pelargonii*, *B. calthae*, *B. ficariarum*, *B. paeoniae*, *B. ranunculi*, *B. narcissicola*.

The term “gene” means a DNA fragment comprising a region (transcribed region), which is transcribed into an RNA molecule (e.g. an mRNA) in a cell, operably
20 linked to suitable regulatory regions (e.g. a promoter). A gene may thus comprise several operably linked fragments, such as a promoter, a 5' leader sequence, a coding region and a 3' nontranslated sequence (3' end) comprising a polyadenylation site. “Expression of a gene” refers to the process wherein a DNA region which is operably linked to appropriate regulatory regions, particularly a promoter, is transcribed into an
25 RNA, which is biologically active, i.e. which is capable of being translated into a biologically active protein or peptide or which is active itself (e.g. in posttranscriptional gene silencing or RNAi). In one embodiment the 5'-end of the coding sequence preferably encodes a (homologous or heterologous) secretion signal, so that the encoded protein or peptide is secreted out of the cell. The coding sequence is preferably
30 in sense-orientation and encodes a desired, biologically active protein or protein fragment.

A “chimeric” (or recombinant) gene refers to any gene, which is not normally found in nature in a species, in particular a gene in which different parts of the nucleic

acid region are not associated in nature with each other. For example the promoter is not associated in nature with part or all of the transcribed region or with another regulatory region. The term "chimeric gene" is understood to include expression constructs in which a promoter or transcription regulatory sequence is operably linked to one or more coding sequences or to an antisense (reverse complement of the sense strand) or inverted repeat sequence (sense and antisense, whereby the RNA transcript forms double stranded RNA upon transcription).

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

A "nucleic acid construct" or "nucleic acid vector" is herein understood to mean a man-made nucleic acid molecule resulting from the use of recombinant DNA technology. The term "nucleic acid construct" therefore does not include naturally occurring nucleic acid molecules although a nucleic acid construct may comprise (parts of) naturally occurring nucleic acid molecules.

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

A "truncated protein" refers herein to a protein which is reduced in amino acid length compared to the wild type protein. Especially, certain domains may be absent, such as the necrosis-inducing domain. In a preferred embodiment a truncated protein lacks the necrosis-inducing domain but retains the receptor binding domain.

A "chimeric protein" or "hybrid protein" is a protein composed of various protein "domains" (or motifs) which is not found as such in nature but which are joined to form a functional protein, which displays the functionality of the joined domains (for example receptor binding). A chimeric protein may also be a fusion protein of two or more proteins occurring in nature. The term "domain" as used herein means any part(s)

or domain(s) of the protein with a specific structure or function that can be transferred to another protein for providing a new hybrid protein with at least the functional characteristic of the domain.

The term “expression vector” refers to nucleotide sequences that are capable of affecting expression of a gene in host cells or host organisms compatible with such sequences. These expression vectors typically include at least suitable transcription regulatory sequences and optionally, 3’ transcription termination signals. Additional factors necessary or helpful in effecting expression may also be present, such as expression enhancer elements. DNA encoding the polypeptides of the present invention will typically be incorporated into the expression vector. The expression vector will be introduced into a suitable host cell and be able to effect expression of the coding sequence in an *in vitro* cell culture of the host cell. The expression vector will be suitable for replication in a eukaryotic host cell or organism, such as a cultured mammalian, plant, insect, yeast, fungi or other eukaryotic cell line, or in a prokaryotic host, such as a bacterial host.

As used herein, the term “promoter” or “transcription regulatory sequence” refers to a nucleic acid fragment that functions to control the transcription of one or more coding sequences, and is located upstream with respect to the direction of transcription of the transcription initiation site of the coding sequence, and is structurally identified by the presence of a binding site for DNA-dependent RNA polymerase, transcription initiation sites and any other DNA sequences, including, but not limited to transcription factor binding sites, repressor and activator protein binding sites, and any other sequences of nucleotides known to one of skill in the art to act directly or indirectly to regulate the amount of transcription from the promoter. A “constitutive” promoter is a promoter that is active in most tissues under most physiological and developmental conditions. An “inducible” promoter is a promoter that is physiologically or developmentally regulated, e.g. by the application of a chemical inducer. A “tissue specific” promoter is only active in specific types of tissues or cells.

The term “selectable marker” is a term familiar to one of ordinary skill in the art and is used herein to describe any genetic entity which, when expressed, can be used to select for a cell or cells containing the selectable marker. Selectable marker gene products confer for example antibiotic resistance. Genes conferring resistance to antibiotics such as kanamycin, rifampicin, erythromycin, actinomycin,

chloramphenicol, tetracyclines, nisin and lactacin F are generally known in the art. The term "reporter" may be used interchangeably with marker, although it is mainly used to refer to visible markers, such as green fluorescent protein (GFP). Selectable markers may be dominant or recessive or bidirectional.

5 As used herein, the term "operably linked" refers to a linkage of polynucleotide elements in a functional relationship. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a transcription regulatory sequence is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the DNA
10 sequences being linked are typically contiguous and, where necessary to join two protein encoding regions, contiguous and in reading frame.

"Gene delivery" or "gene transfer" refers to methods for reliable introduction of recombinant or foreign DNA into host cells. The transferred DNA can remain non-integrated or preferably integrates into the genome of the host cell. Gene delivery can
15 take place for example by transduction, using viral vectors, or by transformation of host cells, using known methods, such as electroporation, cell bombardment, *Agrobacterium* mediated transformation and the like.

A "host cell" or a "recombinant host cell" or "transformed cell" are terms referring to a new individual cell (or organism) arising as a result of at least one nucleic acid molecule, especially comprising a chimeric gene encoding a desired protein or a
20 nucleic acid sequence which upon transcription yields an antisense RNA or an inverted repeat RNA (or hairpin RNA) for silencing of a target gene/gene family, having been introduced into said cell. The host cell may be any eukaryotic or prokaryotic cell e.g. a plant cell, microbial, insect or mammal (including human) cell. The host cell may
25 contain the nucleic acid construct as an extra-chromosomally (episomal) replicating molecule, or more preferably, comprises the chimeric gene integrated in the nuclear or plastid genome of the host cell. Included are any derivatives of the host cell, such as tissues, whole organism, cell cultures, explants, protoplasts, further generations, etc. derived from the cell, which retain the introduced gene or nucleic acid. It is understood
30 that when referring to a pathogen's "host" reference is made to the plant species which the pathogen is able to attack during its disease-cycle. In this context, "host-specific" means that only a limited number of specific host species are affected, while "broad range" means that a large number of plant species are affected. A broad range

phytotoxin (or broad range herbicide composition) thus affects many plant species, while a host-specific phytotoxin (or host specific herbicide composition) affects a defined, limited number of species.

A "recombinant micro-organism" refers to a micro-organism comprising a (man made) nucleic acid construct within its cell(s), in particular one or more chimeric genes. The recombinant micro-organism preferably contains the nucleic acid construct or vector as an episomally replicating molecule, or alternatively and more preferably, integrated into its genome. The latter has the advantage of greater genetic stability of the introduced DNA. It is immaterial by what method the nucleic acid construct is introduced into the micro-organism. Suitable transformation methods for introducing nucleic acid constructs into cells of micro-organisms, such as e.g. electroporation, are available to a skilled person. "Micro-organism" refers to bacteria, fungi (including yeasts), oomycetes, archaea and viruses.

A "transgene" is herein defined as a gene that has been newly introduced into a cell, i.e. a gene that does not normally occur in the cell. The transgene may comprise sequences that are native to the cell, sequences that naturally do not occur in the cell, and it may comprise combinations of both. A transgene may contain sequences coding for one or more proteins that may be operably linked to appropriate regulatory sequences for expression of the coding sequences in the cell. The transgene may be integrated into the host cell's genome.

The terms "target peptide" refers to amino acid sequences which target a protein to intracellular organelles such as vacuoles, plastids, preferably chloroplasts, mitochondria, leucoplasts or chromoplasts, the endoplasmic reticulum, or to the extracellular space (secretion signal peptide). A nucleic acid sequence encoding a target peptide may be fused (in frame) to the nucleic acid sequence encoding the amino terminal end (N-terminal end) of the protein or may replace part of the amino terminal end of the protein.

The term "ortholog" of a gene or protein refers herein to the homologous gene or protein found in another species, which has the same function as the gene or protein, but is (usually) diverged in sequence from the time point on when the species harbouring the genes diverged (i.e. the genes evolved from a common ancestor by speciation).

The term “homologous” when used to indicate the relation between a given (recombinant) nucleic acid or polypeptide molecule and a given host organism or host cell, is understood to mean that in nature the nucleic acid or polypeptide molecule is produced by a host cell or organisms of the same species, preferably of the same variety or strain. If homologous to a host cell, a nucleic acid sequence encoding a polypeptide will typically (but not necessarily) be operably linked to another (heterologous) promoter sequence and, if applicable, another (heterologous) secretory signal sequence and/or terminator sequence than in its natural environment. It is understood that the regulatory sequences, signal sequences, terminator sequences, etc. may also be homologous to the host cell. In this context, the use of only “homologous” sequence elements allows the construction of “self-cloned” genetically modified organisms (GMO’s) (self-cloning is defined herein as in European Directive 98/81/EC Annex II). When used to indicate the relatedness of two nucleic acid sequences the term “homologous” means that one single-stranded nucleic acid sequence may hybridize to a complementary single-stranded nucleic acid sequence. The degree of hybridization may depend on a number of factors including the amount of identity between the sequences and the hybridization conditions such as temperature and salt concentration as discussed later.

The term “substantially identical”, “substantial identity” or “essentially similar” or “essential similarity” means that two peptide or two nucleotide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default parameters, share at least a certain percentage of sequence identity as defined elsewhere herein. GAP uses the Needleman and Wunsch global alignment algorithm to align two sequences over their entire length (full length), maximizing the number of matches and minimizes the number of gaps. Generally, the GAP default parameters are used, with a gap creation penalty = 50 (nucleotides) / 8 (proteins) and gap extension penalty = 3 (nucleotides) / 2 (proteins). For nucleotides the default scoring matrix used is nwsgapdna and for proteins the default scoring matrix is Blosom62 (Henikoff & Henikoff, 1992, PNAS 89, 915-919). It is clear than when RNA sequences are said to be essentially similar or have a certain degree of sequence identity with DNA sequences, thymine (T) in the DNA sequence is considered equal to uracil (U) in the RNA sequence.

Sequence alignments and scores for percentage sequence identity may be determined using computer programs, such as the GCG Wisconsin Package, Version 10.3, available from Accelrys Inc., 9685 Scranton Road, San Diego, CA 92121-3752 USA or the open-source software Emboss for Windows (e.g. version 2.10.0) using e.g.
5 the program “needle” (with the above mentioned GAP opening and extension penalties). Alternatively percent similarity or identity may be determined by searching against databases such as FASTA, BLAST, etc.

“Stringent hybridization conditions” can also be used to identify nucleotide sequences, which are essentially similar to a given nucleotide sequence. Stringent
10 conditions are sequence dependent and will be different in different circumstances. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point T_m for the specific sequences at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typically stringent conditions will
15 be chosen in which the salt concentration is about 0.02 molar at pH 7 and the temperature is at least 60°C. Lowering the salt concentration and/or increasing the temperature increases stringency. Stringent conditions for RNA-DNA hybridizations (Northern blots using a probe of e.g. 100nt) are for example those which include at least one wash in 0.2X SSC at 63°C for 20min, or equivalent conditions. Stringent
20 conditions for DNA-DNA hybridization (Southern blots using a probe of e.g. 100nt) are for example those which include at least one wash (usually 2) in 0.2X SSC at a temperature of at least 50°C, usually about 55°C, for 20 min, or equivalent conditions.

The term “comprising” is to be interpreted as specifying the presence of the stated parts, steps or components, but does not exclude the presence of one or more
25 additional parts, steps or components. A nucleic acid sequence comprising region X, may thus comprise additional regions, i.e. region X may be embedded in a larger nucleic acid region.

In addition, reference to an element by the indefinite article “a” or “an” does not exclude the possibility that more than one of the element is present, unless the context
30 clearly requires that there be one and only one of the elements. The indefinite article “a” or “an” thus usually means “at least one”.

Further, when reference is made to a nucleotide or amino acid “sequence” (as depicted in the sequence listing), it is understood that the physical molecule, i.e. the

nucleic acid molecule or protein molecule having that sequence of nucleic acids or amino acids is referred to.

A "substantially pure" BxNEP protein refers herein to a BxNEP protein purified by liquid chromatography, and which is therefore essentially free of contaminants, such as DNA, RNA, other proteins, etc.

Detailed description of the invention

The present invention relates to a novel necrosis and ethylene inducing proteins (NEP) from fungi of the genus *Botrytis* and variants thereof. Two novel NEPs, BcNEP1 and BcNEP2, were cloned from the haploid *Botrytis cinerea* strain B05.10. BcNEP1 and BcNEP2 alleles were also cloned from the *Botrytis cinerea* strain LB338. While the BcNEP2 allele of strain LB338 is identical to that of strain B05.10, the BcNEP1 allele differs in a single amino acid substitution from the B05.10 allele (at position 77 of BcNEP1 of strain LB338 Asparagine is replaced by Tyrosine). Orthologs of both BxNEP1 and BxNEP2 were cloned from other *Botrytis* species, in particular *B. elliptica* (BeNEP) and *B. tulipae* (BtNEP), and also from *B. aclada* (BaNEP), *B. byssoidae* (BbNEP), *B. calthae* (BcaNEP), *B. convoluta* (BcoNEP), *B. fabae* (BfNEP), *B. ficariarum* (BfiNEP), *B. galanthina* (BgalNEP), *B. gladiolorum* (BglaNEP), *B. globosa* (BgloNEP), *B. hyacinthi* (BhNEP), *B. paeoniae* (BpaeNEP), *B. pelargonii* (BpelNEP), *B. polyblastis* (BpolNEP), *B. porri* (BporNEP), *B. ranunculi* (BrNEP), *B. sphaerosperma* (BspNEP), *B. squamosa* (BsqNEP), *B. croci* (BcrNEP) and *B. narcissicola* (BnNEP). It was surprisingly found that these newly identified proteins BcNEP1 and BcNEP2 and their orthologs are important proteins in causing cell death and subsequent colonization of plant hosts by the *Botrytis* fungi. This activity of the *Botrytis* NEP1 and NEP2 proteins (including variants thereof and orthologs from other *Botrytis* species) is exploited in the present invention in various applications that involve determining the susceptibility of a plant or a plant's tissue for necrosis and/or ethylene production induced by *Botrytis* NEP proteins or nucleic acids encoding these NEP proteins and variants thereof. The proteins according to the invention are also herein referred to as BxNEP, especially BxNEP1 and BxNEP2, where Bx denotes the *Botrytis* species (e.g Bc denotes *B. cinerea*, Bt denotes *B. tulipae*, Be denotes *B. elliptica*, Ba denotes *B. aclada*, etc., as shown above) and wherein NEP1 refers to BcNEP1 and orthologs thereof and NEP2 refers to BcNEP2 and orthologs thereof.

Thus, in total BxNEP1 and BxNEP2 proteins (or in some cases partial proteins) of 22 *Botrytis* species are provided. For some of the NEP1 and NEP2 orthologs listed in SEQ ID NO: 16-36 (BxNEP1) and SEQ ID NO: 37-57 (BxNEP2), in some instances amino acids at the N-terminal and/or at the C-terminal are missing (as can be seen when
5 aligning the sequences with the full length BcNEP proteins). The N-terminal amino acids represent the putative signal peptide. A skilled person can easily determine the missing amino acids, using for example primers or probes based on the cDNAs provided for amplification or cloning, followed by sequencing.

10 One such application of the BxNEP proteins is e.g. the breeding and/or identification (and selection) of plants having genotypes that are resistant to at least one BxNEP protein, i.e. the plant cells do not die after exposure to the protein(s), and the plants are thereby also resistant to one or more species of the genus *Botrytis* itself. The term "resistance" includes herein not only the complete resistance of a host plant
15 species to *Botrytis* infection (no symptoms develop on the host tissue following pathogen exposure), but also to various degrees of resistance, such as intermediate or moderate resistance levels. A host plant with moderate resistance levels may for example still be infected by *Botrytis*, but the damage caused, especially the degree and extent of necrosis of the tissue, is significantly reduced compared to susceptible host
20 tissue.

Another application is the use of BxNEP proteins to manufacture (bio)herbicide compositions. Such compositions comprise at least one BxNEP protein, or mixtures of BxNEP proteins. In a preferred embodiment, host-specific herbicide compositions are provided.

25

Methods for determining a plant tissue's susceptibility

Thus in a first aspect the present invention relates to a method for determining the susceptibility of a plant's tissue for necrosis and/or ethylene production induced by a *Botrytis* necrosis and ethylene inducing protein (BxNEP) (as defined elsewhere herein).

30

The method preferably comprises the steps of: (a) bringing a tissue of the plant into contact with a *Botrytis* necrosis and ethylene inducing protein; and, (b) determining the amount of necrosis and/or ethylene production of the plant's tissue,

and/or damage to its cells by visual, bio-physical or biochemical means. Optionally a further step (c) is carried out, whereby those plants which have significantly reduced susceptibility (i.e. increased resistance) to the protein(s) are identified and selected. These plants can then be used in breeding programs in order to generate elite breeding
5 lines and cultivars with enhanced or complete *Botrytis* resistance. Steps (a) and (b), or steps (a) to (c), may be repeated for the same or for different BxNEP proteins according to the invention and/or for different plant tissues. In the method, the *Botrytis* necrosis and ethylene inducing protein preferably is as defined herein below.

The term "contact" in the above method refers to physical contact between the
10 protein and the plant's cells. This contact may be brought about using direct or indirect means. For example, a solution comprising at least one BxNEP protein may be infiltrated, injected or taken up by the plant's vascular tissue through aspiration (direct contact). Likewise, the plant roots may be dipped into in a solution comprising the protein, or seeds, seedlings or hypocotyls may be placed onto/into medium comprising
15 the protein, etc. Clearly, various methods of bringing the protein into direct contact with the plant cells are known in the art. Indirect contact is brought about by expressing the protein in a recombinant host cell or organism and contacting the recombinant cells with the plant's cells. Indirect means also include (transient or stable) expression of the protein in a plant cell, so that the contact is brought about following transcription and
20 translation (e.g. infiltration of recombinant *Agrobacterium* into the plant's tissue).

When referring to plant tissue in the above method, it is understood that one or more specific parts (tissues or organs such as whole leaves, or subsections thereof, such as one or more cm² of a tissue, or the roots) of the plant or, alternatively, the whole plant may be brought into contact with a protein according to the invention. Thus, for
25 example explants (leaf, stem, petiole, hypocotyls, flower, root, fruit, etc), seeds, pollen or microspores, protoplasts or a cell cultures may be contacted with the protein(s). Alternatively, the aerial part (whole or part) or the underground part (whole or in part) may be contacted with the protein. Thus, essentially any part or parts of a plant may be selected for contact with the protein(s). The plant tissue may be the tissue of any plant
30 species, dicotyledonous or monocotyledonous plants, wild or cultivated plants (cultivars), subspecies or varieties, etc. In a preferred embodiment, the plant species is a host species of one or more *Botrytis* species (e.g. *Botrytis cinerea*) such as fruit, including e.g. apple, pear, cherry, strawberry, raspberry, other berry types, orange,

- grapefruit, lemon, other citrus fruit, plum, nectarine, apricot, melon, grapevine, papaya, mango, passion fruit, kiwifruit, pineapple, custard apple, avocado, banana, walnut, hazelnut, and other nut types; vegetables, including e.g. carrot, cucumber, onion, leek, garlic, eschalot, other bulb vegetable types, tomato, pepper, eggplant, courgette, lettuce,
- 5 broad bean, other bean types, green peas, lentil, chickpea; spinach, endive, rhubarb, artichoke, cauliflower, Brussels sprouts, and other brassica types; ornamentals, including e.g. rose, gerbera, tulip, lily, gladiolus, Lisanthus, geranium, cyclamen, daffodil, hyacinth, crocus and other potted plants; herbs or medicinal plants, including e.g. rosemary, basil, ginkgo, neem tree; and non-food plant commodities, including e.g.
- 10 hemp, tobacco, flax, cotton, Arabidopsis and rapeseed. See Staats *et al.* 2005, Mol. Biol. Evolution 22: 333-346, especially Table 1 therein, for more detailed information on *Botrytis* species and their hosts.

- As already stated *B. cinerea* is considered to have a wide host range. All other *Botrytis*
- 15 species are specialists.

The table below shows typical host plant species.

Botrytis species	Typical host plant species (host family)	Comments
<i>B. cinerea</i> / <i>B. fuckeliana</i>	235 plant species	Polyphagous on eudicotyledons
<i>B. fabae</i>	<i>Vicia</i> spp. (Fabaceae)	According to Harrison, 1980, Ann. Appl. Biol 95, 63-71. Confirmed herein in Examples.
<i>B. calthae</i>	<i>Caltha palustris</i> (Ranunculaceae)	
<i>B. ranunculi</i>	<i>Ranunculus</i> spp. (Ranunculaceae)	
<i>B. ficariarum</i>	Buttercup, <i>Ficaria verna</i> (Ranunculaceae)	
<i>B. pelargonii</i>	<i>Pelargonium</i> spp. (Geraniaceae)	

B. paeoniae	Paeonia spp. (Paeoniaceae)	
B. hyacinthi	Hyacinthus spp. (Hyacinthaceae)	
B. tulipae	Tulipa spp. (Liliaceae)	
B. elliptica	Lilium spp. (Liliaceae)	
B. squamosa	Allium cepa (Alliaceae)	
B. aclada	Allium spp. (Alliaceae)	
B. byssoidea / B. allii	Allium spp. (Alliaceae)	
B. globosa	Allium ursinum. (Alliaceae)	
B. porri	Allium spp. (Alliaceae)	
B. sphaerosperma	Allium triquetrum (Alliaceae)	
B. narcissicola	Narcissus spp. (Amarylidaceae)	
B. polyblastis	Narcissus spp. (Amarylidaceae)	
B. galanthina	Galanthus spp. (Amarylidaceae)	
B. convolute	Iris spp. (Iridaceae)	
B. croci	Crocus spp. (Iridaceae)	
B. gladiolorum / B. draytonii	Gladiolus spp. (Iridaceae)	

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In the method of the invention the *Botrytis* necrosis and ethylene inducing protein may be brought into contact with the plant tissue by a variety of means well known in the art *per se*. Such means include e.g. infiltration of the intercellular space or injection (e.g. of the plant veins) of compositions comprising the protein into a plant's tissue, spraying or painting of the composition onto a plant's tissue, uptake of the composition through aspiration of the plant's tissue (for example by placing explants into a liquid medium comprising the protein or by placing the roots of whole seedlings or whole plants in such a medium), bringing a plant cell or suspension thereof into contact with the protein or by expression of a nucleic acid encoding the protein in a plant, plant cell or plant's tissue. Such methods are described in more details in the Examples herein and may be adapted from similar methods previously described for non-*Botrytis* NEP proteins in e.g. Jennings *et al.* (2000, Weed Science 48, 7-14) and Gronwald *et al.* (2004, Weed Science 52, 98-104). The method used for contacting the protein(s) with the plant's cells will to a large extent depend on the plant cells, tissues or organs chosen. Preferred are plant tissues which can be easily treated in large numbers, so that a plurality of tissues can be treated simultaneously and highly reproducible, and the amount of necrosis can be easily determined. For examples, trays of seedlings of one or more plant lines may be easily contacted with the protein by dipping their roots in a protein solution, by spraying and by infiltration. Also, detached leaves can be treated relatively easily through aspiration, by spraying and by infiltration. Other methods may be used. For example, seeds may be germinated on medium comprising the protein or pre-germinated seedlings may be placed onto/into protein comprising medium.

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Likewise, depending on the method used for bringing the plant tissue into contact with the protein(s), the formulation of the protein(s) according to the invention may vary. In one embodiment, liquid or semi-liquid compositions are used. These may either comprise a quantified amount of protein, or may comprise a recombinant microorganism transformed with an expression vector encoding the protein. Liquid compositions comprising the protein may for example contain at least about 50, 100, 200, 300 or 400ng NEP protein per millilitre solution, more preferably at least about

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800ng, 1µg, 5µg, 10µg, 20µg, 50µg, 100µg or more per millilitre, depending on the activity of the protein and the amount to be applied to the tissue. Clearly, the penetrability of the tissue will influence the extent and speed of the reaction. A fruit comprising a thick wax layer is less penetrable than tissue having a thin wax layer and/or thin cuticle. Addition of surfactants can modify penetrability. Likewise, a leaf surface comprising a high stomatal density is more penetrable than a surface with a lower density. In a preferred embodiment, the BxNEP protein is substantially purified, and this purified protein is used to make suitable compositions.

Thus, the absolute amounts of a functional NEP protein applied per cm² of tissue may range from about 5ng, 10ng, 20ng, 50ng, 100ng, 200ng, 400ng, 800ng per cm² to 1µg, 5µg, 10µg, 20µg, 50µg, 100µg or more per cm². As BxNEP1 proteins have a higher necrotizing activity than BxNEP2 proteins, the amount of functional BxNEP1 protein needed to result in a detectable response when brought in contact with susceptible host cells, is less than for BxNEP2. Also, the response of BxNEP1 proteins is detectable faster than that of BxNEP2 proteins, whereby the time of scoring can start earlier. As shown in the Examples, BcNEP1 infiltrated into tobacco leaves at a concentration of about 10ng to about 26ng protein per cm² of tissue resulted in a visible response within 24 hours and absolute necrosis within about 48 hours post infiltration. About 10ng (preferably of substantially pure) BxNEP1 per cm² of tissue may thus be used. This corresponds to 0.5 pmol protein/ per cm² of tissue. The necrotizing activity of BcNEP1 is, therefore estimated to be least about 5 to 10 times as active as the activity of FoNEP1 (Genbank Accession number AAC97382.1). In contrast, BcNEP2 resulted in a visible response within 48-72 hours using the same assay. Clearly, the amount of protein to be applied and the time point(s) which are optimal for scoring depend on various factors, such as the method of contact chosen, the activity of the protein and environmental factors, such as absorption or penetrability of the tissue, humidity and temperature post treatment and the presence of other compounds such as surfactants (e.g. Silwet 77 or Tween 20). A skilled person can easily determine the suitable amount of protein to be used in a particular method according to the invention and the best time point(s) for determining the amount of necrosis, using for example dose-response tests. This applies to both visual scoring methods and biophysical or biochemical scoring methods.

- “Determining the amount of necrosis” includes not only the determination of the actual amount of necrosis developed at one or more time points after contact between the plant’s cells and the protein, but includes also indirect and predictive determinations of necrosis developed or that will develop if later time points for scoring are chosen. For example, using non-destructive sensing techniques for determining the decrease in photosynthesis by measuring chlorophyll fluorescence as for example described by Chaerle *et al.* 2003 (Physiol. Plantarum 118: 613-619) or by Barbagallo *et al.* 2003 (Plant Physiol. 132: 485-493). See also further below.
- 10 In an alternative embodiment the protein(s) may be in a dry form (such as a powder, a gel such as agarose or a granulate). As mentioned above, it is another embodiment of the invention to express the protein in a recombinant microorganism, such as yeast or bacteria, as described elsewhere herein. The composition used in the above method may therefore comprise a suitable amount of the recombinant microorganism. In a further embodiment, a transient plant expression assay, such as 15 Potato Virus X (PVX) based assays or particle bombardement may be used (see Qutob *et al.* 2002, Plant Journal 32: 361-373; Mindrinoc *et al.* 1994, Cell 78, 1089-1099), followed by determining the amount of necrosis produced, as described below.
- 20 When the protein is expressed in the host cell, the “contact” does not take place by external application of a composition comprising the protein(s), but is determined by the transcriptional and translational activity of the chimeric gene(s). For example, when an inducible promoter is operably linked to the coding sequence of the protein, contact between the plant cell and the recombinant protein will only occur following induction 25 of the promoter (which may be regulated by external means or by developmental means). This embodiment is also described further below (e.g. agroinfiltration).

In the method of the invention the amount of necrosis and/or ethylene production of the plant’s tissue, and/or damage to its cells (or protoplasts) may be determined by a variety of methods that are well known in the art. Necrosis and/or damage to the plant's 30 tissue may easily be determined visually (see e.g. Figures 5 and 6). It may also be determined on intact plants, depending on which plant parts are being treated. Visual means comprise visually scoring the amount of necrosis present at one or more time

points following the contact between the plant cells and the protein. The scale and means used for visual scoring may vary with the experimental set-up. For example, when whole aerial parts are sprayed, plant death may be scored and the number of surviving plants indicates the susceptibility. Alternatively, if a leaf assay is used
5 whereby e.g. only parts of the leaf are treated with protein containing compositions and/or control compositions, a quantitative scoring of the size and severity of the necrotic area may be carried out. Scoring may thus be qualitative and/or quantitative. Positive controls, i.e. a tissue-protein combination resulting in a known and predictable phenotype, should obviously be included as a reference point. For example, when
10 scoring tobacco leaves from a plurality of tobacco plant lines or accessions for their susceptibility to BcNEP1, leaves of a tobacco plant line or cultivar resulting in a strong necrotic response following contact with BcNEP1 should be included. The amount of necrosis scored in relation (relative) to the positive control determines whether the susceptibility of the plant or plant part to the protein is modified (increased or
15 decreased compared to the positive controls). The scoring data may be analysed by statistical means (using known methods, such as analysis of variance, etc.) in order to determine whether a statistically significant difference in the tissue's susceptibility to the protein(s) exists. In step (c) of the method those plants with reduced susceptibility (i.e. enhanced resistance) to the protein(s) compared to the susceptible positive control
20 are selected for further use.

Preferably in order to select *Botrytis* resistant plants in step (c) above, dose-response experiments are carried out, with a plurality of tissues (or plants) being treated with various concentrations of a BxNEP protein according to the invention. Preferably at least 3, 4, 5 or 6 or more increasingly higher concentrations (as described above) are
25 contacted with a plurality of tissues each. When using leaf infiltration assays in dose-response tests, protein concentrations may for example be about 10, 20, 30, 40, 50, 100, 200, 300 or 400ng NEP protein per millilitre solution, or about 800ng, 1µg, 5µg, 10µg, 20µg, 50µg, 100µg or more per millilitre, depending on the activity of the protein. For BxNEP1 proteins the concentrations tested are preferably in a lower concentration
30 range than for BxNEP2 proteins.

The amount of necrosis is then determined at a specified time point, e.g. 24hr, 48hrs, 72hrs or more post contact. These data are then used to determine the LC50, LC90 and/or LC95 value (lethal concentration value), which is defined as the

concentration of a BxNEP protein causing necrosis of the tissue in 50%, 90% or 95% of the repetitions at the chosen time point and in a specific assay. As already mentioned, the lethal concentration value for a particular protein may be different in different assays (e.g. petiole uptake, leaf infiltration, etc.). It is, therefore, important to
5 establish the LC values for a NEP protein in a specific assay which is to be used subsequently.

The LC50 value is the concentration of protein causing necrosis in the tissue contacted with the protein in 50% of the repetitions. Likewise, the LC90 or LC95 value is the concentration of protein causing 90% of tissues (of the plurality of tissues tested)
10 contacted with the protein being necrotic at a defined time-point. For example the concentration of BcNEP1 protein needed to cause necrosis of 90% (or preferably 95%) of tissue infiltrated with BcNEP1 (and scored e.g. at 48 hrs post infiltration) is the LC90 (or LC95) value for BcNEP1. It is estimated that the LC50 value using a leaf infiltration assay is below 200ng BcNEP1 per 100µl volume, such as 100ng, 80ng,
15 60ng, 40ng or 20ng per 100µl for a susceptible tobacco plant. To determine the LC50, LC90 or LC95 value for a BxNEP protein according to the invention the tissue response to various concentrations of protein (as described above) can be tested using any of the assays described, using routine experimentation.

Preferably the assay is repeated independently two or more times, so that mean
20 LC50, LC90 or LC95 values can be calculated. LC50, LC90 and LC95 values can be calculated using, for example, Probit analysis (e.g. using the program POLO PC from LeOra Software, 1987, Berkeley, California) or as described by Bliss 1935 (Annals of Applied Biology 22: 134-167).

Plants or a plurality of plants are selected which exhibit a substantially higher
25 LC50, LC90 and/or LC95 value and optionally these are screened further to identify and select plants with even higher values than the susceptible control tissues (or starting tissues). Plants with a higher (mean) LC50, LC90 or LC95 value than susceptible controls or starting tissue population have a significantly reduced susceptibility (i.e. enhanced resistance) to the BxNEP protein, because a higher concentration is required
30 to cause the same response. See also Lecture 6 of Applied Environmental Toxicology, published by "Food and Environmental Quality Lab Teaching", <http://feql.wsu.edu/teaching.htm>.

Thus, in one embodiment step (c) comprises identifying and selecting (from a plurality of tissues tested) plants whose tissue have a higher LC50 value than the susceptible controls and/or the plurality of tissues started with. In a preferred embodiment plants are selected having a higher LC90 value, or even more preferred a higher LC95 value than susceptible controls and/or the plurality of tissues started with. The selected plants form thus a new population with an LC50, LC90 or LC95 value that is substantially higher than that of the original population.

Further, once the most discriminating concentration of a BxNEP protein has been determined for a particular assay, it is possible to use single-dose assays to screen plant tissues for resistance / susceptibility to a BxNEP protein (see further below). Thus, the LC50 concentration, LC90 or LC95 concentration may be used in all subsequent assays to discriminate between resistant and susceptible tissues/plants (see further below).

The reduced susceptibility to one or more proteins according to the invention preferably correlates with a reduced susceptibility to one or more species of *Botrytis*. This can be tested in bioassays in controlled environment tests, as described e.g. by Benito *et al.* 1998 (Eur. J. Plant Pathology 104:207-220), incorporated by reference. Optionally, other parts of the selected plants may be tested for susceptibility to the same BxNEP protein and/or to one or more other BxNEP proteins. Likewise, the selected plant(s) or parts thereof may be tested for resistance to one or more species of *Botrytis*, preferably at least to *Botrytis cinerea*.

Visual determination of the amount of necrosis may also be carried out using a microscope, such as a light microscope, Scanning Electron Microscope, Transmission Electron Microscope or others. Also, cell death (necrosis) may be determined using methods which determine cell viability, e.g. trypan blue staining of dead cells as described in Wang *et al.* 2004 (Applied and Environmental Microbiol. Vol 70, page 4989-4995) or chlorophyll fluorescence.

Alternatively the amount of necrosis may be determined by bio-physical or biochemical means. Many known methods exist. E.g. the effect of one or more BxNEP proteins on protoplast or protoplast viability may be determined by dye exclusion, changes in extracellular potassium or pH, production of H₂O₂ (see e.g. Jennings *et al.*, 2001, Plant Sci. 161: 891-899), production (i.e. biosynthesis) of ethylene (see e.g.

Jennings *et al.*, 2000, Weed Sci. 48: 7-14; Fellbrich *et al.* 2002, Plant J. 32: 375-390), viability staining of protoplasts (Fellbrich *et al.* 2002, Plant J. 32: 375-390) or the tetrazolium assay (Koch *et al.*, 1998, Planta, 206: 523-32). Necrosis and/or damage to the plant's tissue may also be determined by quantification of expression of the plant's genes and gene products (e.g. Pathogenesis related genes) involved in plant stress-responses and signal transduction (see e.g. Keates *et al.*, 2003, Plant Physiol. 132: 1610-22). Another method to detect cell death (apoptosis) is the detection of DNA laddering (see Veit *et al.* 2001, Plant Physiology 127: 832-841). It should be noted that some assays, such as ethylene production or hydrogen peroxide production precede the development of necrosis in time, as they are already induced minutes or hours following contact with the protein (see e.g. Jennings *et al.* 2001, Plant Science 161, 891-899). It is, therefore, possible to "determine the amount of necrosis" in a predictive way, i.e. before the necrosis is macroscopically visible. Activation or upregulation of genes involved in ethylene biosynthesis, such as ACC synthase and ACC oxidase, can therefore also already be detected at a time point before any visible symptoms develop (see Jennings *et al.* 2001, Plant Science 161, 891-899).

Methods for identifying plants with increased resistance to *Botrytis*

In another aspect, the invention provides a method for identifying and selecting, or breeding, a plant with increased resistance towards a fungus of the genus *Botrytis*, the method comprising the steps of:

- (a) determining the susceptibility for necrosis induced by a *Botrytis* necrosis and ethylene inducing protein (BxNEP) of tissue of a plurality of plants in a method as described above,
- (b) identifying and preferably selecting one or more plants whose tissue(s) have reduced susceptibility (or enhanced resistance) to the *Botrytis* necrosis and ethylene inducing protein as determined in (a); and,
- (c) optionally, further breeding using one or more plants selected in (b), i.e. especially using the selected plants to generate progeny and identifying progeny with enhanced resistance to the Botrytis NEP protein used in step (a), and/or to other NEP proteins (by repeating the above method). Obviously, also bioassays may be carried out, using one or more Botrytis species or strains from one species, in order to verify and/or quantify resistance.

Step (a) is carried out as described in the above embodiment. Thus, "tissue" does not only refer to the tissue of a whole plant, but also to tissues detached from the plants or cells grown in culture and the like. Step (b) is a selection step, whereby those plants whose tissue(s) as tested in step (a) have a reduced susceptibility to a BxNEP protein compared to the susceptible controls. Thus, the tissue shows less necrosis as determined by visual, biophysical or biochemical means as described. Preferably, those plants are selected which have a higher LC50 value for one or more proteins than the (susceptible) controls or starting population (as described above). More preferably those plants with a higher LC90 and/or higher LC95 value are selected.

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The selected plant can then be reproduced by normal breeding methods (selfing, crossing/backcrossing or clonal propagation methods) and the progeny can be tested for susceptibility/resistance to one or more species of the genus *Botrytis*. As pathogenicity of *Botrytis* strains may vary preferably several strains of a species are tested in the bioassays, so that plants with broad spectrum, durable resistance can be selected.

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Disease resistance assays for *Botrytis* species are well known in the art. In controlled environments, bioassays typically involve inoculation of leaf surfaces with conidiospore suspensions, followed by incubation and assessment of disease symptoms at regular intervals post inoculation, see Benito *et al.* 1998 (*supra*).

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The plants preferably have an enhanced resistance to one or more species and strains of *Botrytis*, especially *Botrytis cinerea*. Optionally, various rounds of screening and selection for reduced BxNEP protein sensitivity and/or screening and selection for reduced *Botrytis* susceptibility may be carried out.

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Further identification of progeny (further breeding of selected plants) in step (c) involves traditional breeding techniques as known in the art, combined with an assay for determining susceptibility to NEP and/or a *Botrytis* resistance assay (bioassay). The production of progeny depends on the reproductive strategy of the plant species, for example whether self incompatibility mechanisms exist, whether the plant reproduces mainly by outcrossing, selfing or non-sexual means, whether hybrid vigour exists, etc. See e.g. standard text books of plant breeding such as Allard, R.W., Principles of Plant

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Breeding (1960) New York, NY, Wiley; Simmonds, N.W., Principles of Crop Improvement (1979), London, UK, Longman, pp 408; Snee, J. et al., (1979) Tomato Breeding (p. 135-171) in: Breeding of Vegetable Crops, Mark J. Basset, (1986, editor), The Tomato crop: a scientific basis for improvement, by Atherton, J.G. & J. Rudich (editors), Plant Breeding Perspectives (1986); Fehr, Principles of Cultivar development—Theory and Technique (1987) New York, NY, MacMillan.

“Breeding” can be defined as crossing, selfing or backcrossing the selected plants and obtaining the progeny (F1, F2, F3, BC1, BC2, S1, etc. generations). The progeny may be analysed for agronomic characteristics and/or susceptibility to *Botrytis* or BxNEP proteins and progeny may be subjected to further selection. Thus, the selected plants may be used as male or female parent, may be used in intraspecific or interspecific crosses, may be used to develop inbred lines, hybrids etc. Preferably varieties or cultivars are developed which have high agronomic performance and which have an increased resistance to *Botrytis*.

The plants eventually identified and selected preferably have significantly enhanced *Botrytis* resistance compared to susceptible controls, such as a mean resistance which is at least about 2%, 5%, 10% or more (most preferably 100%; i.e. complete resistance) higher than the control. Apart from the plants obtainable according the methods of the invention, it is understood that progeny of the plants which retain the selected *Botrytis* resistance levels, and any derivatives of the plants, such as seeds and fruit, and any edible parts of the plants (broccoli heads, cauliflower heads, sprouts, cucumbers, fruit etc.) are also provided. Because *Botrytis* is also a serious post harvest pathogen, any harvested plant parts also have enhanced resistance to *Botrytis* and thus have enhanced shelf-life and storage capabilities. The reduction of post-harvest yield loss is therefore also an embodiment of the invention. Preferably, the plants and plant parts have other important agronomic characteristics, such as high yield, resistance to other pathogens, insects, herbivores, environmental stresses such as drought, salt, ozone, etc. Combining the *Botrytis* resistance phenotype with such other agronomical important characteristics can be done using routine breeding methods. Obviously, the plants according to the invention may also be further modified, e.g. by transformation.

The above BxNEP protein sensitivity assays may be used by regulatory authorities as standard tests and to set *Botrytis* resistance standards for plant varieties and breeding lines or for establishing and/or enforcing sanitary regulations (see below). For example, the BxNEP rating (e.g. the LC50 value or LC90 or LC95 value) may be used to define resistance standards.

- In yet another embodiment a method for identifying and discarding (or destroying) plants or plant parts which are susceptible to one or more NEP proteins and one or more species of *Botrytis* is provided. This method comprises the steps of:
- 10 (a) determining the susceptibility for necrosis induced by a *Botrytis* necrosis and ethylene inducing protein (BxNEP) of tissue of a plurality of plants in a method as described above, and further
 - (b) destroying any plants or tissues which show necrosis.

Thus, any plants or plant parts which are not resistant are discarded or destroyed.

15 Preferably, the tissue is discarded in such a way that *Botrytis* pathogens which may be present on or in the tissue are not given an opportunity to spread to other, healthy plants. The tissue may be placed into plastic bags or containers. Destruction may take place by burning.

In this method also non-*Botrytis* NEP proteins, as described below, may be used. Also

20 the other methods described herein below with respect to plant sanitary regulations can be applied to the selection criteria for tissue or plants which are to be destroyed.

Methods for enforcing plant sanitary regulations

In another embodiment a method for enforcing plant sanitary regulations in an area is provided. This method comprising the steps of:

- 25 (a) determining the susceptibility for necrosis induced by a necrosis and ethylene inducing protein of tissue of a plurality of plants in a method as described elsewhere herein,
- (b) barring from entry into the area plants whose tissues are susceptible to at least one
- 30 necrosis and ethylene inducing protein as determined in (a); and,
- (c) optionally, destruction of plants barred in (b).

In this method a BxNEP protein according to the invention may be used (defined elsewhere herein), or alternatively one or more (functional) non-Botrytis NEP proteins orthologs may be used. Non-Botrytis NEP proteins are any known and as yet unknown proteins, derived from other species than *Botrytis* ssp., which fall within the NEP protein family. The NEP protein family comprises proteins of various origin which have necrotizing activity when contacted with plant tissue (i.e. they are functional) and which comprise the conserved 7 amino acid GHRHDWE domain or a variant thereof. Variants of the GHRHDWE domain are domains which differ from this conserved domain by a single amino acid substitution. For example AHRHDWE or GHTHDWE are variants. Non-Botrytis NEP proteins comprise, therefore, proteins having at least about 25%, 30%, 35%, 40%, 50%, 60%, 79%, 80%, 90%, 95%, 98% or more amino acid sequence identity to a BxNEP protein according to the invention. Examples of non-Botrytis NEP proteins are those listed in the section "Background of the invention" above. Alternatively, non-Botrytis NEP proteins and genes encoding them may be isolated from various microorganisms, such as necrotrophic or hemibiotrophic plant pathogens, using standard molecular biology or biochemistry techniques. Functional hybrid or mutant NEP proteins may also be used.

This method allows an easy and quick way to prevent the transport of plants which are susceptible to *Botrytis* and/or a non-Botrytis pathogen (depending on the protein used) and therefore have the potential to spread the pathogen to other plants if brought into close proximity with un-infected plants. For examples, at country borders (airports, etc.) or at the borders of areas where many plants are brought together (e.g. markets, auctions, shops selling vegetables, flowers, plants, etc) one or more plants may be screened for the tissue's susceptibility to one BxNEP protein or to several BxNEP proteins (e.g. sequentially or as a mixture) or to one or more non-Botrytis NEP proteins. A disease assay would not be possible in such situations, as it is too slow and would carry the risk of introducing or spreading the pathogen in the first place.

Step (a) of the method is carried out as described in the above embodiments, which can also be used for non-Botrytis NEP proteins in an analogous way. Only routine methods will be required to determine the optimal protein concentration (dose-response analysis), tissue to be contacted, the time point(s) of determining necrosis, etc. For non-Botrytis NEP proteins preferably those plant tissues and species which are hosts of the

pathogen from which the NEP protein is derived are used. For example, when FoNEP1 is used in the method, plant species (or tissues thereof) which are hosts to *Fusarium oxysporum* are tested.

Step (b) involves barring susceptible plants from entry into the area. The cut-off level of the response which determines whether entry is allowed or is barred can be made more or less stringent, as the authority desires. For any given NEP protein, preferably only plants or plant tissues with a high LC50 value, more preferably a high LC90 or LC95 value, compared to susceptible controls, are allowed to pass because these plants are resistant and therefore reduce the risk of comprising infected tissues.

- 10 Preferably, only one or two discriminating concentrations of the protein need to be tested in order to determine the resistance level of the tissue. The ideal, discriminating concentration can be determined in dose-response reactions and by determining LC50, LC90 and/or LC95 values as described above. For example, a few leaves are harvested, placed into a few containers comprising a NEP protein of a suitable concentration, left to react for a certain number of minutes/hours and are then analysed for necrosis. Plants with less than a certain percentage of necrosis are allowed to pass, while plants with a higher percentage of necrosis are barred and/or destroyed.

- 15 A kit for screening may, for example, comprise solutions comprising a suitable amount of one or more BxNEP proteins or one or more non-Botrytis NEP proteins, a needleless syringe for tissue infiltration or a beaker or tube for placing a petiole into the solution, control samples (e.g. tissue of a susceptible plant), and instructions for carrying out the test. Only plants or plant parts which are not susceptible are allowed to be transported further, while other plants or plant parts are barred and returned or destroyed.

25 Proteins according to the invention

In one embodiment non-processed and mature (processed; lacking the putative signal peptide) BxNEP proteins and variants thereof are provided, as well as the nucleic acid sequence encoding these. These proteins can be used in any of the methods according to the invention.

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In particular, BxNEP1 and BxNEP2 proteins and variants thereof are provided. A BxNEP1 protein refers to a (full-length) *Botrytis* NEP protein comprising at least 51%, 52%, 53%, 54%, 55%, 60%, 62%, preferably at least 63%, 65%, 70%, 75%, 80%, 90%,

- 95%, 98%, 99% or 100% amino acid sequence identity with SEQ ID NO: 1 (BcNEP1 protein) using a GAP default parameters as defined above. This definition, thus, includes all *Botrytis* NEP proteins which are substantially similar to BcNEP1 (SEQ ID NO: 1). The following proteins are examples of this group of proteins: BcNEP1 (SEQ ID NO:1), the variant of BcNEP1 from strain LB338, having a point mutation at position 77 (Seq ID NO: 7) and BtNEP1 (SEQ ID NO: 10). Similarly, the BxNEP1 proteins of various *Botrytis* species, such as the (partial) amino acid sequences provided in SEQ ID NO: 16-36 are included.
- 10 When referring to %sequence identity herein, preferably the full length sequences are aligned using pairwise alignment, especially the Needleman and Wunsch algorithm (e.g. the program GAP or needle). Thus, unless indicated otherwise, the %sequence identity refers to the sequence identity over the full (entire) length of the SEQ ID NO. indicated.
- 15 A BxNEP2 protein refers to a (full length) *Botrytis* NEP protein comprising at least 66%, 67%, 68%, 69%, 70%, 72%, 73%, preferably at least 75%, 76%, 80%, 85%, 90%, 95%, 98%, 99% or 100% amino acid sequence identity with SEQ ID NO: 2 (BcNEP2 protein) using a GAP default parameters as defined above. This definition, thus,
- 20 includes all *Botrytis* NEP proteins which are substantially similar to BcNEP2 (SEQ ID NO: 2). The following proteins are examples of this group: BcNEP2 (SEQ ID NO: 2), BtNEP2 (Seq ID NO: 12) and BeNEP2 (SEQ ID NO: 14). Similarly, the BxNEP2 proteins of various *Botrytis* species, such as the (partial) amino acid sequences provided in SEQ ID NO: 37-57 are included.
- 25 Amino acids 1-20 of BcNEP1 and amino acids 1-21 of BcNEP2 encode putative signal peptides. The mature proteins thus comprise amino acids 21-246 for BcNEP1 and 22-244 for BcNEP2. Likewise, amino acids 1-20 of other BxNEP1 proteins and amino acids 1-21 of other BxNEP2 proteins appear to comprise a putative signal peptide.
- 30 BxNEP proteins can also be defined by the percentage sequence identity of the mature proteins. Thus, BxNEP1 proteins can also be defined as any (mature) *Botrytis* NEP protein comprising at least 51%, 52%, 53%, 54%, 55%, 60%, 64%, preferably at least 65%, 70%, 75%, 80%, 90%, 95%, 98%, 99% or 100% amino acid sequence identity

with amino acids 21 to 246 of SEQ ID NO: 1 (BcNEP1 protein) using a GAP default parameters as defined above. And a BxNEP2 protein refers to any (mature) *Botrytis* NEP protein comprising at least 66%, 67%, 68%, 69%, 70%, 72%, 75%, preferably at least 76%, 80%, 85%, 90%, 95%, 98%, 99% or 100% amino acid sequence identity

5 with amino acids 22-244 of SEQ ID NO: 2 (BcNEP2 protein) using a GAP default parameters as defined above. As for a number of BxNEP orthologs part of or all of the putative signal peptide amino acids are not depicted, this embodiment is particularly preferred for these (partial) proteins. The skilled person can easily determine where the putative signal peptide of SEQ ID NO: 16-36 and SEQ ID NO: 37-57 ends in the

10 sequence depicted and where the mature protein starts. For example, SEQ ID NO 18 comprises the N-terminal sequence AAAAVKKG, which corresponds to part of the putative signal peptide MHFSNAKFLSILAAAAVKKG of BcNEP1. The mature protein of SEQ ID NO: 18 thus starts at amino acid number 9.

15 In SEQ ID NO:s 16-36 (BxNEP1), the mature protein starts at the following amino acids, respectively: aa 21, 21, 9, 3, -4 (amino acids APIE are not depicted), 9, 3, 9, 9, 9, -1 (A is not depicted), 3, 3, 21, 3, 1, 3, 3, 9, 9, 15.

In SEQ ID NOs 37-57 (BxNEP2), the mature protein starts at the following amino

20 acids, respectively: 15, 15, 15, 15, 15, 15, 15, 15, 15, 15, 15, 15, 15, 15, 15, 14, 22, 22, 2, 22, 22,

Thus, the skilled person can easily determine where the mature protein would start and align the remaining part of the protein with the mature BcNEP1 or BcNEP2.

25 Alternatively, the skilled person can easily determine the full length amino acid sequence.

Putative signal peptides can be determined using computer program such as the program SignalP Version 3.0 (Jannick Dyrlov Bendtsen et al. J. Mol. Biol., 340:783-

30 795, 2004).

Also provided are functional variants of the above proteins, such as proteins having one or more amino acid deletions, insertions or mutations (replacements), but which retain

- a necrotizing and/or ethylene inducing activity when contacted with susceptible plant cells or tissue(s). Variants include for example proteins having conservative and/or non-conservative amino acid substitutions which do not substantially modify the functionality of the proteins. In one embodiment of the invention variants also include
- 5 functional truncated proteins (or protein fragments), which lack one or more parts or domains, but which retain the necrotizing and/or ethylene inducing properties. For example, in certain embodiments the putative signal peptide may be deleted without substantially modifying the protein's activity and use in the methods. Variants which are provided also include chimeric or hybrid BxNEP proteins, which for example
- 10 comprise a certain part (e.g. a functional domain) of BxNEP1 and another part of BxNEP2. In particular, the smallest functional BxNEP fragment is an embodiment herein. Such a fragment may for example comprise about 50, 60, 80, 100, 120, 150, 200, 210, 220, or more contiguous amino acids of a BxNEP protein as defined above.
- 15 Similarly, hybrid proteins may comprise one or more amino acids parts (or functional domains) of a non-Botrytis NEP protein or of other proteins. A hybrid protein may therefore have the combined functionality of the different domains, such as the domain responsible for inducing necrosis (originating from one NEP protein) and the ability to bind to a plant's NEP receptor(s) (originating from another NEP protein). A BxNEP
- 20 protein wherein the N-terminal amino acid sequence, comprising the putative signal peptide, has been replaced with a different signal peptide sequence is obviously also a hybrid protein according to the invention. The function of different domains can be determined using domain replacement, terminal deletion, mutation analysis and the like.
- 25 Using targeted mutagenesis one can also determine which amino acids or protein domains are responsible for host-specificity of the BxNEP proteins. Using this approach, proteins which are toxic to only one or a few plant species can be generated, and host specific herbicide compositions can be made, as explained elsewhere herein. Host specificity can be improved, and/or stability of the proteins can be modified.
- 30 In another embodiment variants of the NEP proteins according to the invention are provided, which lack the necrotizing properties (i.e. they are not phytotoxic to one or more plant species), but which retain the ability to bind to the plant's NEP receptors.

Thus, the protein domain(s) responsible for phytotoxicity is removed. These proteins are useful in generating (recombinant) plants, plant tissues and organs, which are resistant to one or more *Botrytis* species, especially *Botrytis cinerea*. They may also be used to generate compositions (e.g. foliage sprays) which are suitable for protecting plants and plant tissues from damage caused by one or more *Botrytis* species. This embodiment will be further described below. Proteins lacking the ability to induce necrosis, but which retain receptor binding activity, can be made using various known means, such as for example mutagenesis or deletion analysis, followed by functionality testing.

10

BxNEP1 and BxNEP2 proteins and variants thereof may be made synthetically *de novo*, using chemical synthesis (using e.g. a peptide synthesizer such as supplied by Applied Biosystems), may be isolated and/or (partially) purified from natural sources, such as from culture filtrates of the fungus or may be made using recombinant DNA technology, for example by expression in recombinant microorganisms, such as bacteria (for example *Pseudomonas*, *Bacillus* or *Escherichia*), fungi (yeast species, e.g. *Saccharomyces* ssp, *Hansenula*, *Pichia*, *Kluyveromyces*, *Candida*, *Aspergillus*, *Chrysosporium*, etc.), oomycetes, viruses or algae, etc.

NEP protein variants can be generated using known mutagenesis techniques or they can be isolated from fungal strains (e.g. any *Botrytis* species and strains thereof) comprising natural variants or from mutagenized fungal strains (treated for example with known mutagens such as UV or gamma-radiation, chemical mutagens such as EMS, etc.). Variants can also easily be made by making modifications to the nucleic acid sequence encoding the protein. Nucleic acid sequences of BxNEP proteins and protein variants can be easily isolated or made by various means, as described further below. Small modifications to a DNA sequence can be routinely made, i.e., by PCR-mediated mutagenesis (Ho *et al.*, 1989, Gene 77, 51-59., White *et al.*, 1989, Trends in Genet. 5, 185-189). More profound modifications to a DNA sequence can be routinely done by *de novo* DNA synthesis of a desired coding region using available techniques. Codon preference can be modified as described below. Likewise, gene shuffling techniques can be used to generate protein variants with modified functionalities, such as enhanced

25
30

or reduced phytotoxicity. Gene shuffling techniques are well known in the art, e.g. as described in US 5,811,238, W097/20078, US 6,180,406 and US 6,117,679.

“Functionality” or “functional proteins or variants” refers herein mostly to the ability to induce plant cell death (necrosis) and/or ethylene production when contacted with the plant’s tissue as described, especially host plant tissue. However, when referring to truncated or modified proteins lacking the ability to cause necrosis, those proteins are said to be “functional” when they do not have the ability to cause necrosis but retain the ability to bind to the plant’s BxNEP protein receptor(s). The meaning referred to is deemed clear from the context.

Functionality of the proteins and protein variants, and in particular the necrotizing and/or ethylene inducing properties, can be tested using various methods. In broadest terms, a BxNEP protein (or variant or fragment) is functional if it has necrosis inducing activity, wherein the activity is such that less than 100µg of the protein applied to (or contacted with) 1 cm² of a *Nicotiana tabacum* leaf (or another host plant tissue, i.e. a tissue of a host plant species of the *Botrytis* species from which the protein or protein fragment is obtainable) produces visible or absolute necrosis within about 48 hours, at least within about 48 to 72 hours after application or contact. The application is preferably by infiltration, but uptake by the vascular tissue, injection and other methods of contact may also be used. Less than 100µg comprises any value below 100µg, such as but not limited to 50µg, 10µg, 5µg, 1µg, 0.5µg, 400µg, 300µg, 200µg, 100µg, 50µg, 25µg, 10µg or even less. In other words, if at least a suitable minimal amount of protein (depending on the activity of the protein), e.g. about 10ng, 25ng, 30ng, 50ng, 100ng, 1µg, 5µg, 10µg, 15µg, 25µg, or more protein (but less than 100µg) is contacted per 1 cm² of a host plant tissue, and if visible or absolute necrosis of the tissue results within at least about 48, or about 48-72, hours, the protein has necrosis inducing activity and is, therefore, functional. For illustrative purposes, functionality of BxNEP1 proteins can be tested by infiltration (using e.g. a needle-less syringe) of less than about 100ng (e.g. about 10 to 25ng) of the (full length) protein per 1 cm² of a *Nicotiana tabacum* leaf (or any tissue of a host plant), which produces an absolute necrosis within 48 hours post infiltration if the protein is functional (see Examples). For BxNEP2 proteins functionality can be tested by infiltration of less than about 10µg (full length) protein

per 1 cm² of a *Nicotiana tabacum* leaf (or any tissue of a host plant), which results in visible necrosis within about 48 to 72 hours post infiltration (see Examples). The necrotic area corresponds largely to the infiltrated area. For infiltration of 3-4 cm² of tissue a volume of about 50-100µl of the protein comprising solution is required.

5 Suitable solutions have for example a BxNEP1 concentration of about 80ng/100µl solution.

Dose-response curves can be established to determine the minimal amount of protein required for producing visible or absolute necrosis within 48, or within about 48-72
10 hours on a host tissue (susceptible tissue), preferably leaf tissue of a host species of the *Botrytis* species from which the protein is derived.

Clearly, any method wherein the protein is contacted with a susceptible (host) plant tissue in suitable amounts and for a suitable period of time can be used to determine the
15 functionality of the protein or variant. See also Figures 9-12, showing that the BxNEP protein causes necrosis on host plant tissue. One protein, BfNEP2, only showed necrosis on a host species and not on non-host species.

The proteins according to the invention may be used to raise mono- or polyclonal
20 antibodies, which may for example be used for the detection of BxNEP proteins in samples (immunochemical analysis methods and kits). Further, the proteins according to the invention may comprise a peptide tag, such as a N-terminal His-tag (six histidine residues), facilitating the purification (using the HIS-tail) of the protein and/or a FLAG tag for detection (using anti-FLAG antibodies).

25

The BxNEP proteins according to the invention may also be characterized by the presence of a conserved amino acid domain (7 amino acids) having the sequence GHRHDWE, located at amino acids 134-140 in SEQ ID NO: 1 and at amino acids 130 to 136 in SEQ ID: 2. In other BxNEP proteins (see Sequence Listing) this position and
30 sequence may vary. For example, in some BxNEP proteins, the sequence is GHRHEWE (see SEQ ID NO: 40 and 41). One, two or three amino acids of the conserved region may be added, deleted or replaced. The function of this domain can

be determined by modification of the domains and functional analysis of the modified protein.

Nucleic acid sequences according to the invention

5 Also provided are nucleic acid sequences (genomic DNA, cDNA, RNA) encoding BxNEP proteins or variants as defined above. Due to the degeneracy of the genetic code various nucleic acid sequences may encode the same amino acid sequence. Any nucleic acid sequence encoding a BxNEP protein is herein referred to as "*BxNEP*". The nucleic acid sequences provided include naturally occurring, artificial or synthetic
10 nucleic acid sequences.

A BcNEP1 nucleic acid sequence refers herein to any nucleic acid sequence having at least 57% , 58%, 59%, 60%, 65%, or more, preferably at least 68%, 70%, 80%, 90%, 95%, 98%, 99% or more nucleic acid sequence identity to SEQ ID NO: 3 (genomic
15 DNA comprising introns) or SEQ ID NO: 5 (cDNA) over the entire length using a GAP default parameters as defined above. This definition, thus, includes all *Botrytis* NEP genes which are substantially similar to *BcNEP1* (SEQ ID NO: 3 or 5). The following nucleic acid sequences are examples of this group: *BcNEP1* (SEQ ID NO: 3 and 5), the nucleic acid sequence encoding the variant of BcNEP1 from strain LB338 (SEQ ID
20 NO: 8 and 9), and *BtNEP1* (SEQ ID NO: 11). Also included are the nucleic acid sequences which encode the mature proteins, wherein the sequence encoding the (putative) secretion signal peptide (e.g. nucleotides 1 to 60) is removed. Likewise, SEQ ID NO: 58-78 are encompassed herein.

25 A BcNEP2 nucleic acid sequence refers herein to any nucleic acid sequence having at least 60% or more, preferably at least 62%, 65%, 70%, 80%, 90%, 95%, 98%, 99% or more nucleic acid sequence identity to SEQ ID NO: 4 (genomic DNA) or SEQ ID NO: 6 (cDNA) using a GAP default parameters as defined above. This definition, thus, includes *Botrytis* NEP genes which are substantially similar to *BcNEP2* (SEQ ID NO: 4
30 and 6). The following nucleic acid sequences are examples of this group: *BcNEP2* (SEQ ID NO: 4 and 6) and *BtNEP2* cDNA (SEQ ID NO: 13) and *BeNEP2* cDNA (SEQ ID NO: 15). Also included are the nucleic acid sequences which encode the mature proteins, wherein the sequence encoding the (putative) secretion signal peptide

(e.g. nucleotides 1-63) is removed. Likewise, SEQ ID NO: 79-99 are encompassed herein.

5 It is understood that when sequences are depicted in as DNA sequences while RNA is referred to, the actual base sequence of the RNA molecule is identical with the difference that thymine (T) is replace by uracil (U).

Also included are variants and fragments of *BxNEP* nucleic acid sequences, such as nucleic acid sequences hybridizing to *BxNEP* nucleic acid sequences, e.g. to *BcNEP1*
10 and/or *BcNEP2*, under stringent hybridization conditions as defined. It is clear that many methods can be used to identify, synthesise or isolate variants or fragments of *BxNEP* nucleic acid sequences, such as nucleic acid hybridization, gene shuffling, PCR technology, *in silico* analysis and nucleic acid synthesis, mutagenesis and the like.

15 The nucleic acid sequence, particularly DNA sequence, encoding the BxNEP proteins of this invention can be inserted in expression vectors to produce high amounts of BxNEP proteins (or e.g. chimeric proteins). For optimal expression in a host the *BxNEP* DNA sequences can be codon-optimized by adapting the codon usage to that most preferred in the host cell in which it is to be expressed. E.g. if the host cell is a
20 plant cell, codon usage can be adapted to plant genes, particularly to genes native to the plant genus or species of interest (Bennetzen & Hall, 1982, J. Biol. Chem. 257, 3026-3031; Itakura *et al.*, 1977 Science 198, 1056-1063.) using available codon usage tables (e. g. more adapted towards expression in cotton, soybean corn or rice). For expression in microorganisms codon usage may be adapted to the preferred codons used in the
25 microorganism. Codon usage tables for various species are published for example by Ikemura (1993, In "Plant Molecular Biology Labfax", Croy, ed., Bios Scientific Publishers Ltd.) and Nakamura *et al.* (2000, Nucl. Acids Res. 28, 292.) and in the major DNA sequence databases (e.g. EMBL at Heidelberg, Germany). Accordingly, synthetic DNA sequences can be constructed so that the same or substantially the same
30 proteins are produced. Several techniques for modifying the codon usage to that preferred by the host cells can be found in patent and scientific literature. The exact method of codon usage modification is not critical for this invention.

As it was found that *B. cinerea*, *B. tulipae* and *B. elliptica* each only comprise two orthologous NEP genes within their genome, it is reasonable to expect that other *Botrytis* species also comprise two NEP genes in their genome and that different strains of a species comprise various alleles (variants) of these two genes. Also, the *BtNEP* and *BeNEP* genes and encoded proteins have high sequence identity to the *BcNEP* cDNA and *BcNEP* proteins (an amino acid sequence identity of at least about 89% and 90%). Thus, orthologous NEP genes from other *Botrytis* species can be easily cloned using the herein disclosed nucleic acid sequences using known methods, such as stringent or moderately stringent nucleic acid hybridization methods (Southern analysis), PCR amplification, and the like. Fragments of nucleic acid sequences according to the invention can be used as primers or probes in order to clone BxNEP encoding genes from other *Botrytis* species. For example, stretches of at least 12, 15, 18, 20, 22, 25, 30 or more contiguous nucleotides may be used as primers or probes or to design degenerate primers (see also below). Alternatively, the proteins may be purified from culture filtrates of the fungus using known methods, as described in the Examples and the amino acid sequence determined (this information may in turn be used to isolate the nucleic acid sequence encoding the protein).

As more and more sequence information becomes available, other BxNEP nucleic acid and/or amino acid sequences may be identified *in silico*, e.g. by identifying nucleic acid or protein sequences in existing nucleic acid or protein database (e.g. GENBANK, SWISSPROT, TrEMBL) and using standard sequence analysis software, such as sequence similarity search tools (BLASTN, BLASTP, BLASTX, TBLAST, FASTA, etc.).

Also, the *BxNEP* nucleic acid sequences can be modified so that the N-terminus of the BxNEP protein has an optimum translation initiation context, by adding or deleting one or more amino acids at the N-terminal end of the protein. Often it is preferred that the proteins of the invention to be expressed in plant cells start with a Met-Asp or Met-Ala dipeptide for optimal translation initiation. An Asp or Ala codon may thus be inserted following the existing Met, or the second codon, Val, can be replaced by a codon for Asp (GAT or GAC) or Ala (GCT, GCC, GCA or GCG). The DNA sequences may also be modified to remove illegitimate splice sites.

In a further embodiment the nucleic acid sequences may be used to make nucleic acid vectors, especially expression vectors, for overexpressing the encoded NEP protein in a recombinant host (see further below).

5

In yet another embodiment of the invention PCR primers and/or probes and kits for detecting the *BxNEP* DNA sequences are provided. Degenerate or specific PCR primer pairs to amplify *BxNEP* DNA from samples can be synthesized based on SEQ ID NO's 3 to 6, or any other *BxNEP* nucleic acid sequence as described, as known in the art (see 10 Dieffenbach and Dveksler (1995) PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratory Press, and McPherson et al. (2000) PCR-Basics: From Background to Bench, First Edition, Springer Verlag, Germany). Likewise, DNA fragments of SEQ ID NO's 3 to 6 (or any other *BxNEP* nucleotide sequence as described) can be used as hybridization probes. An *BxNEP* detection kit may comprise either *BxNEP1* and / or 15 *BxNEP2* specific primers and/or *BxNEP* specific probes, and an associated protocol to use the primers or probe to detect *BxNEP* DNA in a sample. Such a detection kit may, for example, be used to determine, whether a plant has been transformed with an *BxNEP* gene (or part thereof) of the invention. Because of the degeneracy of the genetic code, some amino acid codons can be replaced by others without changing the amino 20 acid sequence of the protein.

Further provided are antibodies that bind specifically to a BxNEP protein according to the invention. In particular monoclonal or polyclonal antibodies that bind to BcNEP1 and/or BcNEP2 or to fragments or variants thereof, are encompassed herein. An 25 antibody can be prepared by using a BxNEP protein according to the invention as an antigen in an animal using methods known in the art, as e.g. described in Harlow and Lane "Using Antibodies: A laboratory manual" (New York: Cold Spring Harbor Press 1998) and in Liddell and Cryer "A Practical Guide to Monoclonal Antibodies" (Wiley and Sons, 1991). The antibodies can subsequently be used to isolate, identify, 30 characterize or purify the BxNEP protein to which it binds, for example to detect the protein in a sample, allowing the formation of an immunocomplex and detecting the presence of the immunocomplex by e.g. ELISA (enzyme linked immunoassay) or immunoblot analysis. Also provided are immunological kits, useful for detecting the

proteins, protein fragments or epitopes in a sample provided. Samples may be cells, cell supernatants, cell suspensions, tissues, etc. Such a kit comprises at least an antibody that binds to a BxNEP protein and one or more immunodetection reagents. The antibodies can also be used to isolate/identify other BxNEP proteins, for example by
5 ELISA or Western blotting.

Chimeric genes, Vectors and recombinant host cells

In one embodiment of the invention nucleic acid sequences encoding BxNEP proteins (including fragments and variants), as described above, are used to make chimeric
10 genes, and vectors comprising these for transfer of the chimeric gene into a host cell and production of the BxNEP protein(s) in host cells, such as cells, tissues, organs or organisms derived from transformed cell(s). Host cells are preferably microbial hosts (bacteria, yeast, fungi, etc.), but in certain embodiments also recombinant plant cells and plants are provided. Other host cells are also feasible, such as viruses and animal
15 cells (insect cells, mammalian cells, human cells, etc.). Suitable expression systems, transformation (or transfection) methods and regeneration methods are available to the skilled person.

Recombinant microorganism

20 In one embodiment the host cell used for BxNEP production is a microorganism, such as a gram-positive or gram-negative bacterial host cell (e.g. of the genus *Escherichia*, *Rhodococcus*, *Bacillus*, *Mycobacterium*, *Corynebacterium*, *Arthrobacter*, *Staphylococcus*, etc.) or a fungal host, such as a yeast cell selected from the genera *Pichia* (e.g. *Pichia pastoris*), *Hansenula* (e.g. *Hansenula polymorpha*), *Saccharomyces*
25 (e.g. *S. cerevisiae*), *Kluyveromyces* (e.g. *K. lactis*), *Yarrowia* (e.g. *Y. lipolytica*), *Arxula* species (e.g. *Arxula adeninivorans*), *Candida* species and *Schizosaccharomyces* (e.g. *S. pombe*). In a preferred embodiment the host cell is a methylotrophic yeast, such as *Pichia*. In another embodiment the host cell is a filamentous fungal host selected from the genera *Aspergillus*, *Trichoderma*, *Fusarium*, *Penicillium*, *Neurospora*,
30 *Chrysosporium*, *Sporotrichum*, *Humicola*, *Sordaria* and *Acremonium*.

Preferably, the recombinant protein is secreted into the growth medium. To achieve optimal expression and protein secretion, the nucleic acid sequence encoding the

protein may need to be modified using standard molecular biology techniques (e.g. codon usage adaptation, N-terminal modifications, removal of splice sites, removal of intron sequences, etc.). It was found that for expression in *Pichia pastoris* expression of the complete cDNA (including the native signal peptide encoding region) resulted in secretion of the functional protein into the medium (see Examples). However, expression of the mature cDNA is equally envisaged, as is the replacement of the putative secretion signal with a signal sequence recognized by the host cell. In particular for *in planta* expression (e.g. via Agro-infiltration or other plant cell transformation techniques) the native signal sequence may be replaced with secretion signal sequence recognized by plant cells, such as the signal sequence of the tobacco PR-1a gene (see below).

Suitable expression vectors for expression of *BxNEP* coding sequence can be either generated using known methods or can be obtained commercially. The transcription regulatory sequence is preferably strongly active in the host cell, either constitutively or following induction. A variety of transcription regulatory sequences capable of directing transcription in microbial host cells are available to the skilled person (Goosen et al., 1992, In: Handbook of Applied Mycology" 4: "Fungal Biotechnology", and Romanos et al., 1992, Yeast 8: 423). Preferably the promoter sequence is derived from a highly expressed gene. Examples of preferred highly expressed genes from which promoters are preferably derived include but are not limited to genes encoding glycolytic enzymes such as triose-phosphate isomerases (TPI), glyceraldehyde-phosphate dehydrogenases (GAPDH), phosphoglycerate kinases (PGK), pyruvate kinases (PYK), alcohol dehydrogenases (ADH), as well as genes encoding amylases, glucoamylases, xylanases, cellobiohydrolases, beta-galactosidases, alcohol (methanol) oxidases, elongation factors and ribosomal proteins. Specific examples of suitable highly expressed genes include e.g. the LAC4 gene from *Kluyveromyces* sp., the methanol oxidase genes (AOX and MOX) from *Pichia* and *Hansenula*, respectively, the glucoamylase (glaA) genes from *A. niger* and *A. awamori*, the *A. oryzae* TAKA-amylase gene, the *A. nidulans* gpdA gene and the *T. reesei* cellobiohydrolase genes.

For expression in yeast species, such as *Pichia* or *Hansenula* species, for example the strong (methanol inducible) AOX1 promoter of the alcohol oxidase gene of *Pichia* (see

US 4,855,231), the *Pichia pastoris* alcohol oxidase II (AOX2 promoter) (Ohi *et al.*, Mol. Gen. Genet 243: 489-499, 1994) or the MOX1 promoter of *Hansenula* are suitable. Alternative promoters are the *Pichia* formaldehyde dehydrogenase promoter (FLD) as described in US 6,730,499 and by Shen *et al.* Gene 216: 93-102, 1998, other
5 yeast promoters, such as the 3-phosphoglycerate kinase promoter (PGK), glyceraldehyde-3-phosphate dehydrogenase (GAFDH or GAP) promoter, galactokinase (GAL1, GAL10) promoter, galactose epimerase promoter, and alcohol dehydrogenase (ADH1, ADRIII) promoter, the *Pichia pastoris* YPT1 promoter (Sears *et al.*, Yeast 14: 783-790, 1998). Similarly, the strong POX promoters (e.g. POX2) of *Y. lipolytica* may
10 be used (Pignede *et al.* 2000, Applied and Environmental Microbiology 66: 3283-3289).

A *Pichia pastoris* expression system is, for example, commercially available as a kit from Invitrogen, which uses the promoter and terminator from the AOX1 gene. Other,
15 analogous expression systems may be used. Various expression vectors are available, such as integrative and autonomously replicating vectors (comprising an autonomous replicating sequence or ARS, as for example described in US 4,837,148).

The expression vector preferably also comprises a selectable marker gene. The
20 selectable marker may be any gene which confers a selectable phenotype upon the host and allows transformed cells to be identified and selected from untransformed cells. Suitable selectable markers which can be used for selection of the transformed host cells are well known to the skilled person (Goosen *et al.*, 1992, In: Handbook of Applied Mycology" 4: "Fungal Biotechnology", and Romanos *et al.*, 1992, Yeast 8:
25 423). Preferred markers include but are not limited to e.g. versatile marker genes that can be used for transformation of most filamentous fungi and yeasts such as acetamidase genes or cDNAs (the amdS genes or cDNAs from *A. nidulans*, *A. oryzae*, or *A. niger*), or genes providing resistance to antibiotics like G418 or hygromycin or phleomycin. Alternatively, more specific selection markers can be used such as
30 auxotrophic markers which require corresponding mutant host strains: e.g. URA3 (from *S. cerevisiae* or analogous genes from other yeasts), pyrG (from *A. nidulans* or *A. niger*) or argB (from *A. nidulans* or *A. niger*).

The selectable marker system may include an auxotrophic mutant methylotrophic yeast strain and a wild type gene which complements the host's defect. Examples of such systems include the *Saccharomyces cerevisiae* or *Pichia pastoris* HIS4 gene which may be used to complement his4 *Pichia* strains, or the *S. cerevisiae* or *Pichia pastoris* ARG4 gene which may be used to complement *Pichia pastoris* arg mutants, or the *Pichia pastoris* URA3 and ADE1 genes, which may be used to complement *Pichia pastoris* ura3 resp. ade1 mutants. Other selectable marker genes which function in *Pichia pastoris* include the zeo resistance gene, the G418 resistance gene, blasticidin resistance gene, and the like. Integration of the chimeric gene into the genome can be achieved by insertion or a transplacement into the region of the chromosomal AOX1 locus or integration may be targeted to the HIS4 locus.

At the 3'end of the coding sequence a 3'nontranslated nucleic acid sequence (3'end) may be added, which may contain one or more transcription termination sites recognized by the host's transcription machinery. The origin of the 3'end is not very critical and various suitable 3'end sequences may be used. For example, the 3'end sequence may be the 3'end of the *Pichia* AOX1 gene, the *Pichia* HIS4 gene or the *Pichia* FLD1 gene. Preferably, for expression in yeast, a 3'end of a yeast gene is used, for example of a gene naturally found in the host cell.

In another embodiment of the invention a microorganism, which comprises a nucleic acid sequence which encodes a functional BxNEP protein according to the invention, under control of a suitable promoter is provided. Especially a methylotropic yeast, preferably *Pichia* (e.g. *Pichia pastoris* or another readily transformable *Pichia* species) or *Hansenula* is provided, which, under suitable growth conditions produces high levels of functional BxNEP protein according to the invention. The microorganisms can be made by transforming a host strain with a vector as described above and selecting transformed cells. Preferred *Pichia pastoris* host strains are strains GS115 (NRRL Y-15851), GS190 (NRRL Y-18014), PPF1 (NRRL Y-18017), KM71, PPY120H, YGC4, and strains derived therefrom. Protease recognition sites, which may be recognized by host cell proteases, may be removed from the sequence used known methods.

The vectors comprising the BxNEP encoding nucleic acid sequence under control of a suitable promoter can be introduced into the host cell using known methods. The chimeric gene may be integrated into the host cell genome or may remain in the nucleus, as a freely replicating unit. It is understood that the vector backbone preferably also comprises other elements required, such as an origin of replication, a selectable marker gene, etc. Transformation methods for yeast hosts include, for example, the spheroplast technique, described by Cregg *et al.* 1985, or the whole-cell lithium chloride yeast transformation system, Ito *et al.* (Agric. Biol. Chem. 48:341), or modified for use in *Pichia* as described in EP 312,934. Other published methods useful for transformation of the plasmids or linear vectors include US 4,929,555; Hinnen *et al.* Proc. Nat. Acad. Sci. USA 75:1929 (1978); Ito *et al.* J. Bacteriol. 153:163 (1983); US 4,879,231; Sreekrishna *et al.* Gene 59:115 (1987). Electroporation and PEG1000 whole cell transformation procedures may also be used, as described by Cregg and Russel, Methods in Molecular Biology: *Pichia* Protocols, Humana Press, Totowa, N.J., pp. 27-39 (1998). For filamentous fungi suitable transformation protocols are described in Goosen *et al.*, 1992, In: Handbook of Applied Mycology" 4: "Fungal Biotechnology", and in EP-A-0 635 574 and include for example protoplast transformation. Transformed host cells can be selected by using appropriate techniques including such as culturing auxotrophic cells after transformation in the absence of the biochemical product required (due to the cell's auxotrophy), selection for and detection of a new phenotype, or culturing in the presence of an antibiotic only allows growth of transformants comprising a resistance gene. Transformants can also be selected and/or verified by integration of the expression cassette into the genome, which can be assessed by, e.g., Southern Blot analysis or PCR.

25

The culturing conditions depend on the host strain and promoter used. Factors such as pH, temperature, nutrients, oxygen, co-factors etc. can be optimized as known in the art. Preferably, the BxNEP protein is secreted into the culture medium. The culture medium comprising the protein may be used as. Alternatively the protein may be purified or partially purified from the medium for further use. Protein purification methods are known in the art, such as using solid phase extraction, chromatography methods, solvent extraction methods, distillation, etc. If a HIS-tail has been added to the nucleic acid sequence encoding the protein, the tail may be used for purification.

30

The recombinant protein may then be used in the methods according to the invention.

5 Provided is a method for producing a protein or a protein fragment having necrosis inducing activity (as indicated herein above), the method comprising the steps of:

- (a) growing a recombinant host cell comprising a nucleic acid sequence encoding a functional BxNEP1 or BxNEP2 protein or protein fragment (as defined) operably linked to a promoter active in said host cell under conditions conducive to the expression of the protein or protein fragment, and, optionally
- 10 (b) recovering the protein or protein fragment.

Optionally the protein can be further purified, as described in the Examples.

Recombinant plant cells for use in the methods

In one embodiment *in planta* expression is provided using Agroinfiltration. For this purpose, a chimeric gene is generated, which comprises a transcription regulatory sequence active in plant cells (e.g. the CaMV 35S promoter) operably linked to a BxNEP cDNA, and optionally linked to a suitable 3'end sequence. The BxNEP cDNA is modified to comprise a nucleic acid sequence encoding a plant secretion signal peptide, such as the tobacco PR-1a gene secretion signal. Secretion peptides from other plant genes, which are secreted into the intracellular space of the plant tissue are equally suitable. The chimeric gene is placed into a suitable vector backbone (e.g. a binary vector), which is then used to transform *Agrobacterium tumefaciens*. Thus, in one embodiment, recombinant *A. tumefaciens* strains comprising a chimeric gene according to the invention is provided. This recombinant strain is suitable for infiltration of plant tissue and for determining the amount of necrosis of the plant's tissue in any of the methods according to the invention.

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

30

Recombinant *Botrytis* resistant plant cells and plants

In another embodiment recombinant plant cells and/or whole plants are provided which produce a truncated or modified BxNEP protein according to the invention, and which

have enhanced resistance to one or more *Botrytis* species. Preferably, the protein has been modified in such a way that it lacks the necrosis inducing activity, while, at the same time, it prevents BxNEP proteins produced by an infecting fungus to interact with the BxNEP protein receptor in the recombinant plant tissue. Without limiting the
5 invention, it is thought that the modified BxNEP protein blocks binding to the receptor by occupying the receptors in the tissue. The native toxin has therefore no target to bind to and no necrosis (or substantially less necrosis) develops following pathogen attack.

The BxNEP protein, therefore, preferably retains receptor binding activity and is
10 secreted into the intracellular space by the recombinant plant cells. To enable secretion, preferably the native N-terminal amino acids comprising the putative target peptide are replaced with a secretion target peptide of plant origin or at least functional in the plant cells to be transformed. Routine experimentation can be used to generate a protein having these properties. The nucleic acid sequence encoding the protein is used to
15 generate a recombinant plant cell, and preferably a recombinant plant derived from that cell.

The transgene is preferably stably integrated within the host genome. The recombinant cells or tissues or plants can be easily distinguished by the presence of the recombinant
20 DNA (detectable by PCR-based methods, nucleic acid hybridization based methods, etc.) or the RNA transcript levels (using e.g. quantitative RT-PCR), and by a enhanced resistance to *Botrytis*. "Enhanced resistance to *Botrytis*" refers herein to less visible necrosis developing when the plant tissue is contacted with one or more *Botrytis* species compared to susceptible controls. Standard disease assays can be carried out as
25 described by Benito *et al.* 1998 (*supra*). Preferably substantially no visible necrosis develops at all (the plant tissue being completely resistant).

The method for generating a recombinant, *Botrytis* resistant plant comprises the steps of: a) transforming a plant cell with a chimeric gene comprising a nucleic acid
30 sequence encoding a BxNEP protein having BxNEP receptor binding activity but lacking necrosis inducing activity, wherein the nucleic acid sequence is operably linked to a (constitutive, inducible, tissue specific or developmentally regulated) promoter

active in plant cells, and b) regenerating and selecting plants which express the chimeric gene.

The construction of chimeric genes and vectors for, preferably stable, introduction of BxNEP protein-encoding nucleic acid sequences into the genome of host cells is generally known in the art. To generate a chimeric gene the nucleic acid sequence encoding a BxNEP protein according to the invention is operably linked to a promoter sequence, suitable for expression in the host cells, using standard molecular biology techniques. The promoter sequence may already be present in a vector so that the *BxNEP* nucleic sequence is simply inserted into the vector downstream of the promoter sequence. The vector is then used to transform the host cells and the chimeric gene is inserted in the nuclear genome or into the plastid, mitochondrial or chloroplast genome and expressed there using a suitable promoter (e. g., Mc Bride *et al.*, 1995 Bio/Technology 13, 362; US 5,693, 507). In the present embodiment the chimeric gene comprises a suitable promoter for expression in plant cells, operably linked thereto a nucleic acid sequence encoding a functional BxNEP protein according to the invention, optionally followed by a 3' nontranslated nucleic acid sequence.

The *BxNEP* nucleic acid sequence can be stably inserted in a conventional manner into the nuclear genome of a single plant cell, and the so-transformed plant cell can be used in a conventional manner to produce a transformed plant that has an altered phenotype due to the presence of the protein in certain cells at a certain time. In this regard, a T-DNA vector, comprising a nucleic acid sequence encoding a BcNEP protein, in *Agrobacterium tumefaciens* can be used to transform the plant cell, and thereafter, a transformed plant can be regenerated from the transformed plant cell using the procedures described, for example, in EP 0 116 718, EP 0 270 822, PCT publication WO84/02913 and published European Patent application EP 0 242 246 and in Gould *et al.* (1991, Plant Physiol. 95,426-434). The construction of a T-DNA vector for *Agrobacterium* mediated plant transformation is well known in the art. The T-DNA vector may be either a binary vector as described in EP 0 120 561 and EP 0 120 515 or a co-integrate vector which can integrate into the *Agrobacterium* Ti-plasmid by homologous recombination, as described in EP 0 116 718.

Preferred T-DNA vectors contain a promoter operably linked to the *BxNEP* encoding nucleic acid sequence between T-DNA border sequences, or at least located to the left of the right border sequence. Border sequences are described in Gielen *et al.* (1984, EMBO J 3,835-845). Of course, other types of vectors can be used to transform the
5 plant cell, using procedures such as direct gene transfer (as described, for example in EP 0 223 247), pollen mediated transformation (as described, for example in EP 0 270 356 and WO85/01856), protoplast transformation as, for example, described in US 4,684, 611, plant RNA virus- mediated transformation (as described, for example in EP 0 067 553 and US 4,407, 956), liposome-mediated transformation (as described, for
10 example in US 4,536, 475), and other methods such as those described methods for transforming certain lines of corn (e. g., US 6,140, 553; Fromm *et al.*, 1990, Bio/Technology 8, 833-839; Gordon-Kamm *et al.*, 1990, The Plant Cell 2, 603-618) and rice (Shimamoto *et al.*, 1989, Nature 338, 274-276; Datta *et al.* 1990, Bio/Technology 8, 736-740) and the method for transforming monocots generally (PCT
15 publication WO92/09696). The most widely used transformation method for dicot species is *Agrobacterium* mediated transformation. Brassica species (e.g. cabbage species, broccoli, cauliflower, rapeseed etc.) can for example be transformed as described in US5750871 and legume species as described in US 5565346. Musa species (e.g. banana) may be transformed as described in US5792935. *Agrobacterium*-
20 mediated transformation of strawberry is described in Plant Science, 69, 79-94 (1990). Likewise, selection and regeneration of transformed plants from transformed cells is well known in the art. Obviously, for different species and even for different varieties or cultivars of a single species, protocols are specifically adapted for regenerating transformants at high frequency.

25

Preferred promoters include the strong constitutive 35S promoters or (double) enhanced 35S promoters (the "35S promoters") of the cauliflower mosaic virus (CaMV) of isolates CM 1841 (Gardner *et al.*, 1981, Nucleic Acids Research 9, 2871-2887), CabbB-S (Franck *et al.*, 1980, Cell 21, 285-294) and CabbB-JI (Hull and
30 Howell, 1987, Virology 86,482-493); the 35S promoter described by Odell *et al.* (1985, Nature 313, 810-812) or in US5164316, promoters from the ubiquitin family (e.g. the maize ubiquitin promoter of Christensen *et al.*, 1992, Plant Mol. Biol. 18,675-689, EP 0 342 926, see also Cornejo *et al.* 1993, Plant Mol.Biol. 23, 567-581), the *gos2* promoter

(de Pater *et al.*, 1992 Plant J. 2, 834-844), the emu promoter (Last *et al.*, 1990, Theor. Appl. Genet. 81,581-588), Arabidopsis actin promoters such as the promoter described by An *et al.* (1996, Plant J. 10, 107.), rice actin promoters such as the promoter described by Zhang *et al.* (1991, The Plant Cell 3, 1155-1165) and the promoter described in US 5,641,876 or the rice actin 2 promoter as described in WO070067; promoters of the Cassava vein mosaic virus (WO 97/48819, Verdaguer *et al.* 1998, Plant Mol. Biol. 37,1055-1067), the pPLEX series of promoters from Subterranean Clover Stunt Virus (WO 96/06932, particularly the S7 promoter), a alcohol dehydrogenase promoter, e.g., pAdh1S (GenBank accession numbers X04049, X00581), and the TR1' promoter and the TR2' promoter (the "TR1'promoter" and "TR2'promoter", respectively) which drive the expression of the 1' and 2' genes, respectively, of the T-DNA (Velten *et al.*, 1984, EMBO J 3, 2723-2730), the Figwort Mosaic Virus promoter described in US6051753 and in EP426641, histone gene promoters, such as the Ph4a748 promoter from Arabidopsis (PMB 8: 179-191), or others.

Alternatively, a promoter can be utilized which is not constitutive but rather is specific for one or more tissues or organs of the plant (tissue preferred / tissue specific, including developmentally regulated promoters), for example fruit (or fruit development or ripening) preferred, leaf preferred, epidermis preferred, root preferred, flower tissue preferred, seed preferred, pod preferred, stem preferred, whereby the *BxNEP* gene is expressed only in cells of the specific tissue(s) or organ(s) and/or only during a certain developmental stage. For example, the *BxNEP* gene(s) can be selectively expressed in green tissue / aerial parts of a plant by placing the coding sequence under the control of a light-inducible promoter such as the promoter of the ribulose-1, 5-bisphosphate carboxylase small subunit gene of the plant itself or of another plant, such as pea, as disclosed in US 5,254, 799 or Arabidopsis as disclosed in US5034322.

To modify Botrytis resistance of aerial plant parts a constitutive, a leaf specific, epidermis specific or light-inducible promoter would be suitable. Suitable epidermal specific promoters, such as for example the Arabidopsis *LTP1* promoter (Thoma *et al.*, 1994, Plant Physiol. 105(1):35-45.), the *CER1* promoter (Aarts *et al.* 1995. Plant Cell.

7:2115-27), and the *CER6* promoter (Hooker *et al* 2002, Plant Physiol 129:1568-80.) and the orthologous tomato *LeCER6* (Vogg *et al*, 2004, J. Exp Bot. 55: 1401-10), provide specific expression in above ground epidermal surfaces.

- 5 Another alternative is to use a promoter whose expression is inducible. The Botrytis resistance may thus only develop after induction of *BxNEP* gene expression. If an appropriate promoter is chosen, expression of the *BxNEP* gene may be switched on only prior and/or during early stages of Botrytis attack. Examples of inducible promoters are pathogen inducible promoters (Stuiver and Custers 2001, Nature 411
10 865-868), wound-inducible promoters, such as the MPI promoter described by Cordera *et al.* (1994, The Plant Journal 6, 141), which is induced by wounding (such as caused by insect or physical wounding), or the COMPTII promoter (WO0056897) or the promoter described in US6031151. Alternatively the promoter may be inducible by a chemical, such as dexamethasone as described by Aoyama and Chua (1997, Plant
15 Journal 11: 605-612) and in US6063985 or by tetracycline (TOPFREE or TOP 10 promoter, see Gatz, 1997, Annu Rev Plant Physiol Plant Mol Biol. 48: 89-108 and Love *et al.* 2000, Plant J. 21: 579-88). Other inducible promoters are for example inducible by a change in temperature, such as the heat shock promoter described in US 5,447, 858, by anaerobic conditions (e.g. the maize ADH1S promoter), by light
20 (US6455760), by pathogens (e.g. EP759085 or EP309862) or by senescence (SAG12 and SAG13, see US5689042). Obviously, there are a range of other promoters available.

- The *BxNEP* coding sequence is inserted into the plant genome so that the coding
25 sequence is upstream (i.e. 5') of suitable 3'end transcription regulation signals ("3'end") (i.e. transcript formation and polyadenylation signals). Polyadenylation and transcript formation signals include those of the CaMV 35S gene ("3' 35S"), the nopaline synthase gene ("3' nos") (Depicker *et al.*, 1982 J. Molec. Appl. Genetics 1, 561-573.), the octopine synthase gene ("3'ocs") (Gielen *et al.*, 1984, EMBO J 3, 835-845) and the
30 T-DNA gene 7 ("3' gene 7") (Velten and Schell, 1985, Nucleic Acids Research 13, 6981-6998), which act as 3'-untranslated DNA sequences in transformed plant cells, and others.

Preferably, for selection purposes but also for weed control options, the transgenic plants of the invention are also transformed with a DNA encoding a protein conferring resistance to herbicide, such as a broad-spectrum herbicide, for example herbicides based on glufosinate ammonium as active ingredient (e.g. Liberty® or BASTA; resistance is conferred by the PAT or *bar* gene; see EP 0 242 236 and EP 0 242 246) or glyphosate (e.g. RoundUp®; resistance is conferred by EPSPS genes, see e.g. EP 0 508 909 and EP 0 507 698). Alternatively, other selectable marker genes may be used, such as antibiotic resistance genes. As it is generally not accepted to retain antibiotic resistance genes in the transformed host plants, these genes can be removed again following selection of the transformants. Different technologies exist for removal of transgenes. One method to achieve removal is by flanking the chimeric gene with lox sites and, following selection, crossing the transformed plant with a CRE recombinase-expressing plant (see e.g. EP506763B1). Site specific recombination results in excision of the marker gene. Another site specific recombination systems is the FLP/FRT system described in EP686191 and US5527695. Site specific recombination systems such as CRE/LOX and FLP/FRT may also be used for gene stacking purposes. Further, one-component excision systems have been described, see e.g. WO9737012 or WO9500555).

When reference to “a transgenic plant cell” or “a recombinant plant cell” is made anywhere herein, this refers to a plant cell (or also a plant protoplast) as such in isolation or in tissue/cell culture, or to a plant cell (or protoplast) contained in a plant or in a differentiated organ or tissue, and these possibilities are specifically included herein. Hence, a reference to a plant cell in the description or claims is not meant to refer only to isolated cells in culture, but refers to any plant cell, wherever it may be located or in whatever type of plant tissue or organ it may be present. Also, parts removed from the recombinant plant, such as harvested fruit, seeds, cut flowers, pollen, etc. as well as cells derived from the recombinant cells, such as seeds derived from traditional breeding (crossing, selfing, etc.) which retain the chimeric BxNEP gene are specifically included.

30

Plant protective Compositions

The BxNEP proteins lacking the domains(s) responsible for inducing necrosis, but retaining the ability to bind the NEP receptor(s) may also be used to make

compositions suitable for protecting plants from *Botrytis* induced necrosis. For this purpose the protein may be produced in a recombinant microorganism. It may then be used as such or further purified for the manufacture of a composition. Apart from comprising a suitable amount of protein, other components may be present, such as surfactants or wetting agents (e.g. 0.2% v/v of Silwet 1-77), etc. The composition may be liquid for spraying aerial parts of plants, or it may be in solid or semi-solid form for application to the plant surface. Preferably, the protein is able to enter the intracellular space of the aerial tissue.

10 Herbicidal compositions

The phytotoxic proteins according to the invention can be used as bioherbicides, as described for non-*Botrytis* derived NEP proteins (Jennings *et al.* 2000, Weed Science 48, 7-14; Gronwald *et al.* 2004, Weed Science 52, 98-104). For this purpose compositions may be formulated as for known contact herbicides. Optionally, compositions may further comprise surfactants and/or other chemical herbicides (e.g. glyphosate, glufosinate ammonium, etc.) and/or other bioherbicides such as other NEP proteins.

Preferably, either broad spectrum herbicide compositions or host-specific herbicide compositions are made. Broad spectrum compositions are made by using as active ingredient at least one BxNEP protein, protein fragment or variant which is capable of causing cell death of a broad range of plant species, such as the BcNEP1 and/or BcNEP2 proteins. Also, mixtures of proteins having specific host-specificities may be made, to generate compositions which kill those specific species.

Host specific compositions are made by using as active ingredient at least a BxNEP protein, protein fragment or variant which is capable of causing cell death of a small number of specific host species (e.g. only one, two, three, four or five species). Suitable are the BxNEP proteins of *Botrytis* species which have a narrow or specific host range, as indicated in the Table provided herein above.

Also mixtures of various BxNEP proteins, fragments and variants are provided herein.

Methods for identifying compounds which reduce BxNEP protein activity

In another embodiment a method for screening and identification of a substance that reduces the necrosis and/or ethylene inducing activity of a *Botrytis* necrosis and ethylene inducing protein (BxNEP) is provided. The method comprising the steps of:

- (a) determining the susceptibility for necrosis induced by a *Botrytis* necrosis and ethylene inducing protein of tissue of a plant in a method as described above, in the presence of one or more different substances; and,
- (b) identifying a substance that reduces susceptibility of the plant to the *Botrytis* necrosis and ethylene inducing protein as determined in (a) (or, in other words, identifying a substance that reduces the activity of the Botrytis NEP protein as determined in step (a)).

This method is useful for identifying new compounds which inhibit or reduce effect of a BxNEP protein, for example substances which block the BxNEP receptor or reduce/prevent interaction with the receptor. In this method one or more test substances are applied to the plant tissue prior to or at the same time as the BxNEP protein(s). When the application is in sequence, the application of the test substance and BxNEP protein preferably is carried out using the same (identical) method, selected from one of the methods described, such as infiltration, injection, etc. The test substance(s) of step (a) may be selected from any kind of substance. It may be a (partially) purified protein, a protein composition, a protein hydrolysate, an extract (e.g. a plant tissue extract), natural or synthetic chemicals and minerals.

Clearly appropriate controls, especially the same tissue-BxNEP protein combination without additional substance (or with water replacing the substance), need to be included in the method, in order to detect differences between the extent of necrosis that develops in the tissue-BxNEP interaction and the modulation of the necrosis in the tissue-BxNEP-substance X interaction.

The substances selected can be used to make compositions suitable for protecting plants and plant parts from Botrytis damage (plant protection agents). These plant-protection agents can be formulated as liquids, solids, etc., using methods known in the art.

It is also an object of the invention to provide a method for screening and identification of a substance that reduces the susceptibility of a plant for necrosis induced by a *Botrytis* necrosis and ethylene inducing protein necrosis, the method comprising the steps of:

- 5 (a) bring a plant or plant part into contact with two or more different substances, one of which is a functional BxNEP protein (or fragment or variant);
- (b) determining the amount of necrosis induced by the *Botrytis* necrosis and ethylene inducing protein of tissue of a plant in a method as described herein above; and,
- (c) identifying a substance that reduces susceptibility of the plant or plant part to the
- 10 *Botrytis* necrosis and ethylene inducing protein as determined in (b).

Also provided is a plant protective substance obtained by these methods, and its use as plant protective agent, especially suitable for protecting plants and plant tissue from damage caused by one or preferably several necrotrophic fungal plant pathogens, such

15 as *Botrytis* spp., *Sclerotinia sclerotiorum*, *Cochliobolus heterostrophus*, *Alternaria* spp, *Monilinia* spp. and *Monilia* spp. Optionally, the compositions may also act as plant protective agents for protecting plant tissue against hemi-biotrophic fungal pathogens, such as *Pyrenopeziza brassicae*, *Magnaporthe grisea*, *Phytophthora infestans*, *Colletotrichum* spp., *Pythium* spp. and *Aphanomyces* spp.

20

In particular provided is a substance obtainable by the screening methods described above, wherein said substance is capable of reducing (*Botrytis* induced) necrosis, directly or indirectly, when contacted with a plant or plant part.

25 Sequences

SEQ ID NO 1: Amino acid sequence of non-processed BcNEP1 (246 amino acids) of strain B05.10

SEQ ID NO 2: Amino acid sequence of non-processed BcNEP2 (244 amino acids)

SEQ ID NO 3: genomic DNA of *BcNEP1* (854 nucleotides) of strain B05.10

30 SEQ ID NO 4: genomic DNA of *BcNEP2* (845 nucleotides)

SEQ ID NO 5: cDNA *BcNEP1* of strain B05.10

SEQ ID NO 6: cDNA *BcNEP2*

- SEQ ID NO 7: Amino acid sequence of non-processed BcNEP1, *Botrytis cinerea* strain LB338
- SEQ ID NO 8: genomic DNA of BcNEP1 of strain LB338
- SEQ ID NO 9: cDNA of BcNEP1 of strain LB338
- 5 SEQ ID NO 10: amino acid sequence of BtNEP1
- SEQ ID NO 11: cDNA of BtNEP1
- SEQ ID NO 12: amino acid sequence of BtNEP2
- SEQ ID NO 13: cDNA of BtNEP2
- SEQ ID NO 14: amino acid sequence of BeNEP2
- 10 SEQ ID NO 15: cDNA of BeNEP2
- SEQ ID NO 16 – SEQ ID NO 36: BxNEP1 proteins or partial proteins from various species of *Botrytis*. Sequence ID NO: 36 (*B. tulipae* NEP1 protein) is identical to sequence ID NO: 10, except that the N-terminal amino acids MHSFNA are not depicted.
- 15 SEQ ID NO 37 - 57: BxNEP2 proteins or partial proteins from various species of *Botrytis*. SEQ ID NO: 56 (*B. tulipae* NEP2 protein) is identical to sequence ID NO: 12, except that the amino acid no. 6 (Arginine) is a Lysine in SEQ ID NO: 12. Also, SEQ ID NO: 57 (*B. elliptica* NEP2 protein) is identical to SEQ ID NO: 14, except that the amino acid no. 6 (Arginine) is a Lysine in SEQ ID NO: 14 and amino acid no. 22
- 20 (Isoleucine) is a Leucine in SEQ ID NO: 14.
- SEQ ID NO 58 - 78: cDNA sequences encoding the proteins of SEQ ID NO 16 – 36.
- SEQ ID NO 79 - 99: cDNA sequences encoding the proteins of SEQ ID NO 37 – 57.

Description of the figures

- 25 Figure 1: Schematic representations of the *Bcnep1* and *Bcnep2* expression cassettes cloned in binary vector pMOG800 for Agro-infiltration.
- Figure 2: Schematic representations of the *Bcnep1* and *Bcnep2* expression cassettes generated for expression in *P. pastoris*.
- Figure 3: Schematic representation of BcNEP1 and BcNEP2 proteins.
- 30 Figure 4: Expression of *BcNep1* and *Bcnep2* genes as determined by Northern-blotting during infection of tomato at various hours post infection (h.p.i.).
- Figure 5: Agro-infiltration of *A. tumefaciens* containing different *Bcnep* gene expression constructs. Nomenclature of expression constructs is indicated in Example

1.7.1. *cf-4/avr4* refers to Agroinfiltration of *A. tumefaciens* comprising a Cf4 construct mixed with *A. tumefaciens* comprising an Avr4 expression construct.

Figure 6: Culture filtrates of *P. pastoris* expressing BcNEP1 or BcNEP2 protein infiltrated in tobacco leaves.

5 Fig. 7. Protein gel electrophoresis of *P. pastoris* culture filtrates expressing the various *Bcnep* gene constructs and their *in planta* activity.

Fig. 8. Uptake experiment of BcNEP proteins through aspiration.

Fig. 9. - Infiltration of 10x *ccf* of P-BfNEP2, containing BfNEP2 and 10x *ccf* of untransformed *P. pastoris* GS115 (control) on the left side, associated with *Botrytis fabae* isolate CBS 109.57 spore inoculation ($\sim 1.0 \times 10^4$ conidia/droplet) on the right side of the leaves of *Vicia faba* "3x Wit" (A), *Phaseolus vulgaris* "Processor" (B),
10 *Vigna unguiculata* "Black Eye" (C), *Phaseolus lunatus* "Sieva Pole" (D) and *Glycine max* "White Hilum" (E). Four days post infiltration/inoculation.

Fig. 10. - Infiltration of concentrated culture filtrate of BcNEP1 (Panel 1), BcNEP2
15 (Panel 2) and control (C) in leaves of *Vicia faba* "3x Wit" (a), *Phaseolus vulgaris* "Florine" (1b), *Phaseolus vulgaris* "Rondina" (2b), *Vigna unguiculata* "Black Eye" (c), *Phaseolus lunatus* "Sieva Pole" (d) and *Glycine max* "White Hilum" (e). Concentration of the culture filtrate is indicated above the infiltration area.

Fig. 11 - Infiltration of three times concentrated culture filtrate (3x *ccf*) of *P. pastoris*
20 *Bfnep2* transformants in *N. benthamiana* (A) and *V. faba* (B). A; the infiltrated area of *Bfnep2* transformant P-BfNEP2 became slightly necrotic. B; severe necrotic response of the culture filtrate from the same *Bfnep2* transformant. Infiltrated culture filtrates from untransformed *P. pastoris* gave no response (control).

Fig. 12 - Necrosis caused by BcNEP1 purified protein in *Nicotiana tabacum* and
25 *Arabidopsis thaliana*

Fig. 13 - Alignment of BxNEP1 and BxNEP2 proteins.

The following non-limiting Examples describe the identification and use of BxNEP genes according to the invention. Unless stated otherwise in the Examples, all
30 recombinant DNA techniques are carried out according to standard protocols as described in Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, and Sambrook and Russell (2001) Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor

Laboratory Press, NY; and in Volumes 1 and 2 of Ausubel et al. (1994) Current Protocols in Molecular Biology, Current Protocols, USA. Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfax (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, UK.

Examples

1 Materials and Methods

1.1 Gene searching and analysis

For identifying relevant genes the *Botrytis cinerea* strain B05.10 genome sequence was searched. Sequences of homologous genes from other fungi were used in an off-line TBLASTN search. The resulting sequences, including the flanking sequences of approximately 500 base pairs were obtained.

DNA and protein sequences were analyzed and compared using Vector NTI and the included sequence similarity analysis program according to CLUSTALW with the standard settings.

1.2 Fungal strains and growth procedures

Botrytis cinerea wild type haploid strain B05.10 (Büttner *et al.*, 1996) was used for analysis. As a reference *B. cinerea* strain LB338, isolated from lily leaves near Lisse, was analyzed. Isolates from other *Botrytis* species that were analyzed are: *B. tulipae* Bt0005, *B. elliptica* Be0004, *B. elliptica* Be9610, *B. elliptica* Be9401, *B. fabae* MUCL98, *B. fabae* MUCL7923, *B. pelargonii* MUCL1152, *B. calthae* MUCL2830, *B. byssoidae* MUCL94, *B. polyblastis* CBS287.38, *B. globosa* MUCL21514, *B. sphaerosperma* MUCL21481, *B. sphaerosperma* MUCL21482, *B. porri* MUCL3234, and *B. aclada* MUCL8415, *B. ranunculi* CBS178.63, *B. ficariarum* CBS176.63, *B. ficariarum* MUCL376, *B. paeoniae* 0003, *B. paeoniae* MUCL16084, *B. hyacinthi* 0001, *B. squamosa* PRI026, *B. squamosa* MUCL1107, *B. squamosa* MUCL9112, *B. narcissicola* MUCL2120, *B. galanthina* MUCL435, *B. galanthina* MUCL3204, *B. convoluta* 9801, *B. convoluta* MUCL11595, *B. croci* MUCL436, *B. gladiolorum* 9701, *B. gladiolorum* MUCL3865.

The conidia of all *Botrytis* species were stored in glycerol stocks. To grow mycelium or isolate conidia malt extract agar plates (Difco) were inoculated with

conidia and incubated at 20°C. Plates, which were completely covered with mycelium, were placed under near UV light for 16 hours to induce sporulation. Conidia were harvested from the sporulating plates 7 to 14 days later using 5 ml sterile water, containing 0.05% (v/v) Tween 80. The suspension was filtered through glass wool,
5 washed once by 5' centrifugation at 114xg and resuspended in sterile water.

Filter sterilised Gamborg's B5 medium including vitamins (Duchefa, Haarlem, The Netherlands), supplemented with 10mM sucrose and 10 mM NaH₂PO₄, were inoculated with 5×10⁷ conidia/100ml. After 2-4 hours of pre-incubation with occasional shaking, the germinating conidia were incubated for 24 to 48 hours at 20°C in a rotary
10 shaker at 180 rpm.

1.3 Genomic DNA isolation and Southern analysis

Mycelium from a liquid culture was harvested by filtration over Miracloth (Calbiochem) and freeze dried. The dried mycelium was homogenized in liquid nitrogen by placing a pestle in the tube while vortexing. 3 ml TES (100mM Tris-HCl
15 pH 8.0, 10 mM EDTA and 2% (w/v) SDS) and 60 µl proteinase K (20 µg/µl) were added and the suspension was incubated for one hour at 60°C. Subsequently, 840 µl 5 M NaCl and 130 µl 10% (w/v) N-cetyl-N,N,N-trimethylammonium bromide (CTAB) were added and incubation was continued for 20' at 65°C. Then the suspension was extracted by adding 4.2 ml chloroform/IAA (24:1) followed by vortexing shortly,
20 incubation for 30' on ice and centrifugation for 5' at 18000×g. The aqueous top phase was transferred and 1350 µl 7.5 M NH₄Ac was added, it was incubated on ice for one hour and centrifuged for 15' at 18000×g. To precipitate the DNA, 0.7 volume of isopropanol was added. The DNA was transferred from the liquid using a glass rod, washed in 70% (v/v) ethanol and dried. The genomic DNA was dissolved in 1 ml TE
25 (10 mM Tris-HCl pH 7.5 and 0.1 mM EDTA) containing 2.5 u RNase A, incubated for 30' at 50°C and precipitated with ethanol. The pellet was dissolved in 200 µl TE. 1 µg genomic DNA was digested to completion with 100 units of the desired restriction enzyme in a total volume of 100 µl. DNA fragments were separated on a 0.8% (w/v) agarose gel and subsequently blotted using the protocol for alkali blotting on
30 Hybond™-N⁺ membrane (Amersham). A capillary blot was set up according to Sambrook *et al.* (1989) using 0.4 M NaOH as blotting solution. After DNA transfer, the membrane was rinsed in 2×SSC (0.3 M NaCl and 0.03 M sodium citrate, pH 7) and dried. DNA was cross-linked to the membrane by UV treatment (312 nm, 0.6 J/cm²).

The Prime-A-Gene DNA Labelling System (Promega) was used to obtain radioactively labelled probes. 20 ng of the necessary DNA fragment was labelled according to manufacturers' instructions. Labelled DNA fragments were purified on Sephadex G50. Hybridisation was according to Church and Gilbert (1984). Autoradiograms were made using Kodak X-OMAT AR film.

1.4 Amplification of genomic sequences

The *Bxnep1* and *Bxnep2* genes of all different Botrytis species were amplified in a standard PCR, in which various combinations of 5'- and 3'-primers (see Table 1) were used to obtain the gene fragments.

Table 1: Primers Used for PCR amplification

Primer Name	Target region	Primer sequence (5'-3')	Position ^A
NEP1(-207)for	<i>NEP1</i>	CACCTTGTGGGAGATTGTATGGGTGGATATACATC	-207
NEP1(+1124)rev	<i>NEP1</i>	GGTCACCTAATTTTGGCTTTTCAGGGTC	+1124
NEP2(-200)for	<i>NEP2</i>	GAACCTTTGAATAGTGGGCAGTTGGG	-200
NEP2(+1147)rev	<i>NEP2</i>	GAGTTTCAGGTATATTCGTTTGGTGGA	+1147
NEP1for	<i>NEP1</i>	gtgactgtaaaacgacggccagtCCAACGCAAAATTCCTTTCTATCC	+11
NEP1revA	<i>NEP1</i>	gtgaccaggaaacagctatgaccCTTGGCGAGGTTGTTGTTGAAGTT	+842
NEP1revB	<i>NEP1</i>	gtgaccaggaaacagctatgaccGTTGGCGAAGTTGTGGTCATTGAA	+807
NEP2forD	<i>NEP2</i>	gtgactgtaaaacgacggccagtTTGCCTTCTCAAAATCATTACAGC	+5
NEP2revD	<i>NEP2</i>	gtgaccaggaaacagctatgaccTCTAGAAAGTAGCCTTCGCAAGAT	+846
NEP2forE	<i>NEP2</i>	gtgactgtaaaacgacggccagtTCATCATGGTTGCCTTCTCAAGAT	-5
NEP2revE	<i>NEP2</i>	gtgaccaggaaacagctatgaccAAGTAGCAGCTGCAAGATTGTTTG	+840
NEP2forF	<i>NEP2</i>	gtgactgtaaaacgacggccagtTTCGGTCTTGGCATCTACAGTCAT	+36

^A Base pair coordinates relative to the ATG start codon of the homologous *Bcnep1* or *Bcnep2* genes of *B. cinerea* strain B0510

1.5 Expression of *BcNep1* and *BcNep2* during infection of tomato leaves

Conidia of sporulating *B. cinerea* cultures, wild type strain B05.10, were harvested, resuspended in Gamborg's B5 medium (Duchefa), supplemented with 10 mM glucose and 10 mM potassium phosphate, pH 6.0, (10^6 conidia/ml) and sprayed onto detached tomato leaves (*Lycopersicon esculentum*). The inoculum was air-dried and the compound leaves were incubated with their stem inserted in wet florist's foam

oasis, in closed plastic boxes with a transparent lid to obtain a humidity of 100%. The boxes were placed at 18°C with a diurnal cycle of 16 hours light and 8 hours darkness. Leaves were harvested at 0, 8, 16, 24, 32, 40, 48, 64, 72, 96, 120 and 144 hours post inoculation and stored at -80°C until further use.

5 1.6 Electrophoresis, blotting and hybridization of total RNA

Isolation of total RNA from infected leaves and mycelium, electrophoresis under denaturing conditions, blotting and hybridisation were performed as described (Prins *et al.*, 2000, Mol. Plant Pathol. 1, 169-178).

1.7 Synthesis, cloning and characterisation of BcNEP cDNAs

- 10 The cDNA of both *Bcnep1* and *Bcnep2* was generated by using the One-Step RT-PCR with Platinum *Taq* kit from Invitrogen according to the manufacturer's protocol. As template, total RNA was used which was isolated from tomato leaves 72 hours after infection with *B. cinerea* strain B05.10. The *Bcnep1* cDNA was obtained by using the primers NEP1+1-for (5'-ACGCGTCGACATGCATTTCTCCAACGCA-3') and
- 15 NEP1+907-rev (5'-TCCCCGCGGCTGACAGGACAAACTTCCAGATTCTCC-3'). The NEP1+1-for forward primer starts directly at the ATG start codon, which is preceded by a *SalI* restriction site. The NEP1+907-rev reverse primer starts 53 bases downstream the TAA-stop codon and is preceded by a *SacII* restriction site. The *Bcnep2* cDNA was obtained by using the primers NEP2+1-for (5'-
- 20 ACGCGTCGACATGGTTGCCTTCTCAAAATC-3') and NEP2+898-rev (5'-TCCCCGCGGCCAATAGACTCCCAGAATATAGCCCCT-3'). The NEP2+1-for forward primer starts directly at the ATG start codon, which is preceded by a *SalI* restriction site. The NEP2+898-rev reverse primer starts 53 bases downstream the TAG-stop codon and is preceded by a *SacII* restriction site.
- 25 In both cases the reverse transcription reaction followed by the PCR amplification resulted in cDNA fragments of approximately 750 bp, which were subsequently cloned into the pCR4-TOPO vector, transformed to *E. coli* TOP10-F'cells according to the manufacturer's recommendations and sequenced.

30 1.8 Synthesis, cloning and characterisation of BfNEP2 cDNA

Bfnep cDNA fragments were obtained by the SuperScript™ III One-Step reverse transcriptase-polymerase chain reaction (RT-PCR) system with Platinum *Taq* high fidelity (Invitrogen Life Technologies) according to the manufacturer's recommended

conditions, using 0.1-0.01 µg of purified RNA, as determined by optical density measurement at 260 nm. The total RNA was isolated from primary leaves of Broad bean (*Vicia faba*) infected with *B. fabae* isolate CBS 109.57 and purified using RNeasy Mini Protocol for Isolation of Total RNA from Plant Cells and Tissues and Filamentous Fungi (Qiagen). In this amplification specific primers containing restriction sites (*Eco*RI and *Not*I; underlined respectively) for cloning in *Pichia pastoris* expression vector pPIC3.5: BfNEP2-cDNA(+1)-for-Pp (CCGGAATTCATGGTTGCCTTCTCAAAATC) and BfNEP2-cDNA(+845)-rev-Pp (ATAAGAATGCGGCCGCCTAGAAAGTAGCCTTCGCAAGATTATC). The obtained cDNA fragment of approximately 750 bp was initially cloned into vector pCR4-TOPO-TA, transformed to *E. coli* TOP10-F' cells according to the manufacturer's recommendations and sequenced.

1.8 Expression of *Bcnep1*, *Bcnep2* and *Bfnep2* in heterologous expression systems

1.8.1 *In planta* expression of *Bcnep1* and *Bcnep2* through agroinfiltration (ATTA)

For *in planta* expression of *BcNep1* and *BcNep2* two different sets of constructs were made (see Figure1).

In the first set the complete *Bcnep1* and *Bcnep2* cDNAs, which were cloned in pCR4-TOPO TA, were cloned into the pAT1 vector by means of the *Sa*I and *Sac*II restriction sites. The vector pAT1 is a modified pRH80 vector (van der Hoorn et al. 2000, Mol. Plant-Microbe Interact. 13, 439-446), in which a *Not*I site was introduced between the *Xho*I and *Pst*I site. This resulted in two vectors, in which either the *Bcnep1* or *Bcnep2* cDNA sequence was put under the control of the CaMV 35S promotor.

For the second set both *Bcnep1* and *Bcnep2* cDNA sequences, which were cloned in pCR4-TOPO TA, were amplified by using the primer combinations NEP1+65-for (ACGCGTCGACAATTGAGGAGAGCACCATTCAAGCTCGCGCC) and NEP1+907-rev for *Bcnep1* and NEP2+68-for (ACGCGTCGACTACACCATCACAACCTTGAGTCTCGGG) and NEP2+898-rev for *Bcnep2*. Both NEP1+65-for and NEP2+68-for primers contain a *Sa*I restriction preceding the priming site. As a result, both these cDNAs lacked the predicted signal sequence coding regions. They were generated by using the One-Step RT-PCR with Platinum *Taq* kit from Invitrogen according to the manufacturer's protocol and, as

template, total RNA isolated from tomato leaves 72 hours after infection with *B. cinerea* strain B05.10. The cDNA fragments were cloned in the pCR4-TOPO TA vector and verified by sequencing. These *Bcnep1*-Δs and *Bcnep2*-Δs cDNAs were cloned into the pAT2 vector by means of the *SalI* and *SacII* restriction sites. The vector

5 pAT2 was modified from pAT1 by introducing the tobacco PR1a signal sequence (Honée *et al.* 1998, Plant Physiol. 117, 809-820) into pAT1 using the *NcoI* and *SalI* restriction sites. This resulted in two vectors, in which either the *Bcnep1*-Δs or *Bcnep2*-Δs cDNA sequence was placed in frame with the tobacco PR-1a signal sequence coding region and put under the control of the CaMV 35S promoter.

10 The four expression cassettes were transferred as a whole to the binary vector pMOG800 by using the *EcoRI* and *XbaI* restriction sites present at the outside of the cassettes. The resulting binary vectors pMOG1-1 (*Bcnep1*), pMOG2-1 (*Bcnep1*-Δs), pMOG3-1 (*Bcnep2*) and pMOG4-1 (*Bcnep2*-Δs) were transformed into *Agrobacterium tumefaciens* strain GV3101 (+pSOUP). Together with the control, which was *A.*

15 *tumefaciens* transformed with the empty pMOG800 vector, the four *A. tumefaciens* transformants were cultured individually according to the protocol, resuspended in MMA at a final O.D. (at 600nm) of 2 and infiltrated into *Nicotiana tabacum* leaves by using a 1 ml syringe.

1.8.2 Heterologous expression of *Bcnep1*, *Bcnep2* and *Bfnep2* in *Pichia pastoris* and protein analysis

20 For heterologous expression in the yeast *Pichia pastoris*, *Bcnep1* and *Bcnep2* cDNA sequences had to be generated with suitable restriction sites. Therefore, the *Bcnep1* and *Bcnep2* cDNA initially cloned in pCR4-TOPO TA, was amplified by PCR using alternative primers and Expand polymerase (proof reading) (Roche).

25 For cloning of the complete P-*Bcnep1* cDNA the primers NEP1+1-for-Pp (CCGGAATTCATGCATTTCTCCAACGCA) and NEP1+854-rev-Pp (ATAAGAATGCGGCCGCTTAGATTCTTGCCTTGGCGAGGTTG) were used, containing an *EcoRI* and a *NotI* restriction site, respectively. For cloning of the *Bcnep1* cDNA lacking the signal sequence (P-*Bcnep1*-Δs) the NEP1+60-for-Pp

30 (GACTACAAGGACGACGATGACAAGGCTCCAATTGAGGAGAGCACCATTCAAGCTCGCGCC) and NEP1+854-rev-Pp primers were used. Primer NEP1+60-for-Pp was phosphorylated at the 5'-end and a FLAG-sequence coding region preceded the *Bcnep1* primer sequence in frame.

For cloning the complete P-*Bcnep2* cDNA the primers NEP2+1-for-Pp (CCGGAATTCATGGTTGCCTTCTCAAAATC) and NEP2+845-rev-Pp (ATAAGAATGCGGCCGCCTAGAAAAGTAGCCTTCGCAAGATTGTC) were used, containing an *EcoRI* and a *NotI* restriction site, respectively.

5 For cloning of the *Bcnep2* cDNA lacking the signal sequence (P-*Bcnep2*- Δ s) the NEP2+63-for-Pp (GACTACAAGGACGACGATGACAAG ATCCCTACACCATCACAACCTTGAGTCTCGGG) and NEP2+845-rev-Pp primers were used. Primer NEP2+63-for-Pp was phosphorylated at the 5' end and a FLAG-sequence coding region preceded the *Bcnep2* primer sequence.

10 The amplified *Bcnep1* and *Bcnep2* cDNA fragments were both digested with *EcoRI* and *NotI* and individually cloned into *EcoRI/NotI*-digested expression vector pPIC3.5 (Invitrogen) (see Figures 2).

The amplified *Bcnep1*- Δ and *Bcnep2*- Δ cDNA fragments were both digested with *NotI* and individually cloned into *SmaI*(blunt)/*NotI*-digested expression vector pPIC9-
15 HIS (Invitrogen) (Figures 2c and 2d). The vector pPIC9-HIS was modified from pPIC9 (Invitrogen), in which a sequence coding for six histidine residues is cloned in frame directly after the signal sequence cleavage site of the α -factor signal sequence for secretion and in front cloning site for the gene of interest. The resulting pPIC9HIS-
20 FLAG constructs can be of interest for detection (anti-FLAG antibodies) and protein purification (by means of HIS-tail). After purification the complete tail can be cleaved off exactly by the enzyme enterokinase, resulting in the native BcNEP1 and BcNEP2 proteins.

All four plasmid constructs were verified by sequencing and subsequently transformed into *P. pastoris* strain GS115 (Invitrogen) according to the electroporation
25 protocol provided by Invitrogen. Transformants were analyzed by growing 4 ml batch cultures in the presence of methanol to induce the *Bcnep* expression followed by an *in planta* screening method. Therefore, culture filtrates were filter-sterilized and infiltrated into tobacco leaves by using a 1 ml syringe. Alternatively, protein activity was analyzed by aspiration, in which petioles of detached tobacco leaves were placed in
30 sterilized culture filtrate and, after taking up approximately 350 μ l, subsequently transferred to tap water. In addition, the filter-sterilized culture filtrate was concentrated via a Microcon YC10 filter unit (Amicon) and the filter was washed with MMA

medium (lacking acetosyringone). This was used for infiltration into tobacco leaves and protein analysis through protein gel electrophoresis.

The cloned cDNA *Bfnep2* was isolated as *EcoRI/NotI* fragment and subsequently cloned into the *EcoRI/NotI*-digested expression vector pPIC3.5 (Invitrogen). The plasmid constructs was verified by sequencing and subsequently transformed into *P. pastoris* strain GS115 (Invitrogen) according to the electroporation protocol provided by Invitrogen. Transformants were analyzed by growing 4 ml batch cultures in the presence of methanol to induce the *Bcnep* expression followed by an *in planta* screening method. Therefore, culture filtrates were filter-sterilized and infiltrated into tobacco leaves by using a 1 ml syringe. In addition, the filter-sterilized culture filtrate was concentrated via a Microcon YC10 filter unit (Amicon) and the filter was washed with MMA medium (lacking acetosyringone). This was used for infiltration into tobacco and bean leaves and protein analysis through protein gel electrophoresis.

1.8.3 BcNEP1 and BcNEP2 purification and quantification

The BcNEPs proteins can be purified from the crude culture filtrates by affinity chromatography.

For BcNEP1 purification a column BioRad Econocolumn (1.5 x 10 cm) was loaded with 18 ml Streamline SP XL (Amersham) sludge, resulting in a 14 ml packed column, and equilibrated with 3 column volumes 10 mM KPi, pH 7.0. The column was subsequently loaded with 17 ml concentrated protein solution and rinsed two column volumes 10 mM KPi, pH 7.0. Bound proteins were recovered from the column by eluting with increasing salt concentrations in three steps: one column volume 0.25 M NaCl, one column volume 0.5 M NaCl and one column volume 1 M NaCl, all buffered with 10 mM KPi, pH 7.0. The eluted protein fractions were desalted and concentrated using Amicon Ultra-15 PGCL Centrifugal filters. The presence and activity of the proteins were analyzed protein gel electrophoresis and in planta screening through infiltration into tobacco leaves, respectively.

For BcNEP2 purification the same protocol was followed although in this case a BioRad Econocolumn (1.5 x 10 cm) loaded with 18 ml Streamline Q XL (Amersham) was used.

Protein concentrations were quantified using the BCA protein assay kit (Pierce) according to the manufacturer's protocol.

2. Results

2.1 Isolation and characterization of the *Bcnep1* and *Bcnep2* genes

The genomic sequence of *Botrytis cinerea* strain B05.10 was searched for protein sequences that are homologous to the necrosis and ethylene inducing peptide (NEP1; AAC97382.1) from *Fusarium oxysporum* f.sp *erythroxyli* by using a TBLASTN search. This resulted in two different homologous sequences. The gene with the highest homology to the *Fonep1* gene was designated *Bcnep1*, the second gene was designated *Bcnep2*.

The *Bcnep1* gene spans 854 bases from the ATG-start until the TAA-stop codon and two intron regions were predicted (SEQ ID NO. 3). The *Bcnep2* gene spans 845 bases from the ATG-start until the TAG-stop codon and two intron regions were predicted (SEQ ID NO. 4).

2.2 cDNA synthesis, cloning and chacterization

In both cases the reverse transcription reaction followed by the PCR amplification resulted in DNA fragments of approximately 750 bp, which were subsequently cloned in the pCR4-TOPO vector and transformed to *E. coli* TOP10-F'cells according to the manufacturer's recommendations.

Sequencing analysis of the cloned DNAs confirmed that both *Bcnep1* and *Bcnep2* cDNA were amplified. The *Bcnep1* cDNA contained an open reading frame of 741 bases (including stop codon) and coded for a 246 amino acid protein (SEQ ID NO. 1). The *Bcnep2* cDNA contained an open reading frame of 735 bases (including stop codon) and coded for a 244 amino acid protein (SEQ ID NO. 2). In both sequences the splicing of the introns had occurred as predicted from the genomic sequence.

2.3 BcNEP1 and BcNEP2 sequence homology

The predicted protein sequences of BcNEP1 and BcNEP2 have a sequence similarity of 58% and a sequence identity of about 39%. Both proteins are relatively hydrophobic. *Bcnep1* contains three cysteine residues and *Bcnep2* contains four cysteine residues. *Bcnep1* contains three putative sites for N-linked glycosylation and *Bcnep2* contains none (Figure 3).

2.4 Isolation and characterization of the *nep* genes from other *Botrytis* species

The primers used for amplification of the *Bcnep1* and *Bcnep2* genes were also used to amplify *nep* homologs from two other *Botrytis* species, *Botrytis tulipae* and *Botrytis elliptica*. This resulted in the amplification of two *nep* genes from *B. tulipae* (*Btnep1* and *Btnep2*) and one from *B. elliptica* (*Benep2*, annotation is based on the fact that this sequence is the ortholog of *Bcnep2*). Analysis predicts that all three orthologs have two introns. The predicted protein sequences have a sequence similarity of 88.6% (*Btnep1*), 88.9% (*Btnep2*) and 89.8% (*Benep2*) with their respective orthologs in *B. cinerea*.

2.5 Similarity with known orthologs in bacteria, oomycetes and other fungal species

Both BcNEP1 and BcNEP2 sequences (SEQ ID NO: 1 and 2) share the distinct domain of 7 amino acids (GHRHDWE) at approximately amino acid position 130 (depending on the sequence) of the non processed sequence, which is conserved in almost all NEP protein sequences,.

Next to the predicted *B. tulipae* and *B. elliptica* NEP proteins, the protein sequence of the closest related NEP protein has a sequence similarity of 56.7% (FoNEP1, *Fusarium oxysporum* f. sp. *erythroxyli*, Accession number AAC97382.1) for BcNEP1 and 59.6% (*A. nidulans*, acc. no. EAA62936.1) for BcNEP2 using global pairwise alignment (Needleman and Wunsch).

2.6 Genomic analysis by Southern hybridization

Genomic DNA from *B. cinerea* strain B05.10, *B. tulipae* and *B. elliptica* digested with different restriction enzymes, and hybridized with the individual *Bcnep* cDNAs, indicates that *Bcnep1* and *Bcnep2* are the only two 'necrosis and ethylene-inducing' gene homologues present in the genome *B. cinerea*. This confirms the results of the initial 'TBLASTN-search' performed on the genome. In addition, it also demonstrates that both *B. tulipae* and *B. elliptica* each contain two *nep* genes.

2.7 Expression of *BcNep1* and *BcNep2* during infection of tomato leaves

Tomato leaves were infected by spray inoculation with spores of *B. cinerea* strain B05.10. At different time points after inoculation, the total RNA was isolated from infected leaves, separated on agarose gel and blotted onto a Hybond N⁺ filter. Hybridization with the *Bcnep1* and *Bcnep2* cDNA fragments revealed that the genes

were differentially expressed (Figure 4). Transcripts of *Bcnep1* can already be detected at 8 hours post infection (h.p.i.). At this point spores are in the process of germination and, presumably, penetration. The relative amount of fungal biomass and fungal total RNA, in this very early sample is low. The amount of *Bcnep1* transcripts steadily
5 increases during the infection, reaches a maximum at 48 h.p.i. and then declines quickly to low levels at 144 h.p.i.. At this latter time point the whole leaf has become necrotic and fungus has completely colonized the tomato leaf. The total RNA from this sample comprises mostly fungal RNA.

Transcripts from *Bcnep2* are detectable at 40 h.p.i. and the amounts steadily
10 increase until 144 h.p.i.. Therefore, the accumulation of *Bcnep2* seems to be correlated with the accumulation of fungal biomass, which for these type of experiments increases rapidly from approximately 40 h.p.i onwards (Benito *et al.* 1998, Eur. J. Plant Pathol. 104, 207-220).

15 2.8 Expression of *Bcnep1* and *Bcnep2* in heterologous expression systems

2.8.1 In planta expression of *Bcnep1* and *Bcnep2* through agroinfiltration (ATTA)

Infiltration of tobacco (*Nicotiana tabacum*) leaves with *A. tumefaciens* transformants containing either the *Bcnep1*-ΔS or *Bcnep2*-ΔS expression constructs resulted in a response, which results in necrosis of the infiltrated area (Figure 5).
20 Typically, for the *Bcnep1*-ΔS expression constructs the onset of this response becomes already visible within 24 hrs after infiltration, whereas for the *Bcnep2*-ΔS expression constructs the response is initiated 48 hours after infiltration. Infiltration of both the *Bcnep1* or *Bcnep2* expression constructs (both containing the native signal sequence) gave no response, indicating that the use of a plant signal sequence is necessary in this
25 heterologous (plant) expression system.

2.8.2 Heterologous expression of *Bcnep1* and *Bcnep2* in *Pichia pastoris*, protein analysis and activity.

Infiltration of tobacco (*Nicotiana tabacum*) leaves with culture filtrates of *Pichia*
30 *pastoris* transformants containing either the *Bcnep1* or *Bcnep2* expression constructs resulted in a response, which results in necrosis of the infiltrated area (Figure 6). Typically, for the *Bcnep1* expression constructs the onset of this response becomes already visible within 24 hrs after infiltration, whereas for the *Bcnep2* expression

constructs the response is initiated 48-72 hours after infiltration. Generally, the phenotypes of both responses are identical to the necrotic responses obtained with the *Bcnep1*-ΔS or *Bcnep2*-ΔS expression constructs in the agroinfiltration experiments. Protein gel electrophoresis showed that a 25 kDa protein was visible in the culture filtrate (Figure 7) in cases a necrotic response occurred in tobacco leaves. Infiltration of culture filtrates of *Pichia pastoris* transformants containing either the *Bcnep1* or the *Bcnep2* expression constructs with α-factor signal sequence combined with the HIS- and Flag-tag gave no response (Figure 6). In this case protein gel electrophoresis did not show the presence of a 25 kDa protein in the culture filtrate (Figure 7).

Uptake experiments by detached tobacco leaves of sterilized culture medium resulted in a completely necrotizing leaf in case the BcNEP1 protein was present in the medium (Figure 8). The response to BcNEP2 was less pronounced but still necrotic spots were visible. The control medium, not containing BcNEP proteins gave, like the water control, no response. Clearly, the plant responds to the presence of the two different 25 kDa proteins, which corroborate with the sizes of both BcNEP1 and BcNEP2.

2.8.3 Heterologous expression of *Bfnep2* in *Pichia pastoris*, protein analysis and activity.

Infiltration of tobacco (*Nicotiana tabacum*) leaves with culture filtrates of *Pichia pastoris* transformants containing the *Bfnep2* expression construct resulted in a response, which results in slight necrosis of the infiltrated area (Fig. 10) 48-72 hours after infiltration. However, infiltration experiments in leaves of the host plant, *Vicia faba*, resulted in a severe necrotic response (Fig. 9). The responses to BfNEP2 after infiltration correlated with host range of *B. fabae* (see also Fig 9):

Vicia fabae: Necrosis with BfNEP2 – *B. fabae* does colonize

Phaseolus vulgaris: No necrosis with BfNEP2 – *B. fabae* does not colonize

Phaseolus lunatus: No necrosis with BfNEP2 – *B. fabae* does not colonize

Vigna unguiculata: Slight necrosis with BfNEP2 – *B. fabae* does not colonize

Glycine max: No necrosis with BfNEP2 – *B. fabae* does not colonize

All these species respond to infiltration with either BcNEP1 or BcNEP2 and can be colonized by *B. cinerea*.

2.8.4 Activity of purified BcNEP1 and BcNEP2.

The activity of the purified protein was tested in a tobacco leaf infiltration assay. By using the purified protein it was calculated that infiltration of 10 ng BcNEP1 or 1 µg BcNEP2 per cm² of leaf surface still results in necrosis of the infiltrated area in 24 hours. This corresponds to 0.5 pmol /cm² BcNEP1 or 50 pmol/cm² BcNEP2.

Claims

1. A method for determining the susceptibility of a plant's tissue for necrosis induced by a *Botrytis* necrosis and ethylene inducing protein, the method comprising
5 the steps of:
(a) bringing a tissue of the plant into contact with a *Botrytis* necrosis and ethylene inducing protein; and,
(b) determining the amount of necrosis of the plant's tissue, or damage to its cells, by visual, bio-physical or biochemical means.
10
2. A methods according to claim 1, wherein the *Botrytis* necrosis and ethylene inducing protein comprises an amino acid sequence that has at least 51% amino acid identity to SEQ ID NO: 1 or at least 66% amino acid identity to SEQ ID NO: 2.
- 15 3. The method according to claim 1 or 2, wherein the protein has necrosis inducing activity when contacted with susceptible plant tissue.
4. A method according to any one of the preceding claims, wherein the *Botrytis* necrosis and ethylene inducing protein is brought into contact with the plant tissue by
20 infiltration, injection, spraying or uptake of the protein through aspiration, or by expression of a nucleic acid encoding the protein in the plant's tissue.
5. A method according to any one of the preceding claims wherein, the plant tissue is one or more tissues comprised in the plant's roots, stems, leaves, tubers, flowers or
25 fruit.
6. The method according to any one of claims of claims 1-5, further comprising the step of discarding plants or plant parts whose tissues show necrosis.
- 30 7. A method for screening and identification of a substance that reduces the necrosis and ethylene inducing activity of a *Botrytis* necrosis and ethylene inducing protein or that reduces the susceptibility of a plant or plant part to necrosis induced by such a protein, the method comprising the steps of:

- (a) determining the susceptibility for necrosis induced by a *Botrytis* necrosis and ethylene inducing protein of tissue of the plant in a method as defined in any one of claims 1 – 5, in the presence of one or more different substances; and,
- (b) identifying a substance that reduces the activity of the *Botrytis* necrosis and ethylene inducing protein or that reduces the susceptibility of the plant or plant part to the *Botrytis* necrosis and ethylene inducing protein as determined in (a).
8. An isolated protein comprising an amino acid sequence that has at least 51% amino acid identity to SEQ ID NO: 1 or at least 66% amino acid identity to SEQ ID NO: 2, wherein said percentage amino acid identity is the percentage over the entire length of the amino acid sequence.
9. A fragment of at least 100 contiguous amino acids of a protein according to claim 8.
10. An isolated nucleic acid molecule encoding the protein according to claim 8 or 9.
11. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide having a necrosis inducing activity, wherein the nucleotide sequence is selected from:
- (a) a nucleotide sequence encoding a polypeptide comprising an amino acid sequence that has at least 51% sequence identity with the amino acid sequence of SEQ ID NO. 1, wherein said percentage amino acid identity is the percentage over the entire length of the amino acid sequence;
- (b) a nucleotide sequence that has at least 57% sequence identity with the nucleotide sequence of SEQ ID NO. 3 or 5, wherein said percentage nucleic acid identity is the percentage over the entire length of the nucleic acid sequence;
- (c) a nucleotide sequence the complementary strand of which hybridises to a nucleic acid molecule sequence of (a) or (b)
- (d) a fragment of any of the nucleotide sequences of (a)-(c), said fragment comprising at least 300 contiguous nucleotides of the nucleotide sequences of (a)-(c).

12. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide having a necrosis inducing activity, wherein the nucleotide sequence is selected from the group consisting of:
- (a) a nucleotide sequence encoding a polypeptide comprising an amino acid sequence
5 that has at least 66 % sequence identity with the amino acid sequence of SEQ ID NO: 2, wherein said percentage amino acid identity is the percentage over the entire length of the amino acid sequence;
 - (b) a nucleotide sequence that has at least 60% sequence identity with the nucleotide
10 sequence of SEQ ID NO: 4 or 6, wherein said percentage nucleic acid identity is the percentage over the entire length of the nucleic acid sequence;
 - (c) a nucleotide sequence the complementary strand of which hybridises to a nucleic acid molecule sequence of (a) or (b); and,
 - (d) a fragment of any of the nucleotide sequences of (a)-(c), said fragment comprising
15 at least 300 contiguous nucleotides of the nucleotide sequences of (a)-(c).
13. A vector comprising the nucleotide sequence according to any one of claims 10 to 12.
14. A vector according to claim 13, wherein the vector further comprises a
20 transcription regulatory element capable of regulating expression in a host cell and wherein the transcription regulatory element is operably linked to the nucleotide sequence.
15. A host cell comprising a vector as defined in claims 13 or 14.
25
16. A host cell according to claim 15, wherein the host cell is a microorganism.
17. A method for producing a protein or a protein fragment having a necrosis inducing activity, the method comprising the steps of:
- 30 (a) growing a host cell as defined in claims 15 or 16 under conditions conducive to the expression of the protein or protein fragment; and,
 - (b) optionally, recovery of the protein or protein fragment.

18. Use of a protein according to claim 8 or a fragment according to claim 9 for the manufacture of a herbicidal composition.

19. A herbicidal composition comprising a protein according to claim 8 or a fragment
5 according to claim 9.

Abstract

5 The present invention relates to necrosis and ethylene inducing proteins from *Botrytis* species and their use. Provided are also methods for determining the susceptibility of a plant's tissue to a necrosis and ethylene inducing protein and methods for selecting plants being resistant to one or more of these proteins and to *Botrytis* infection.

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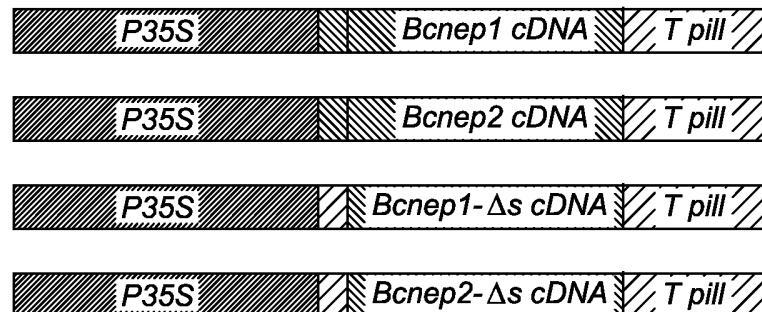
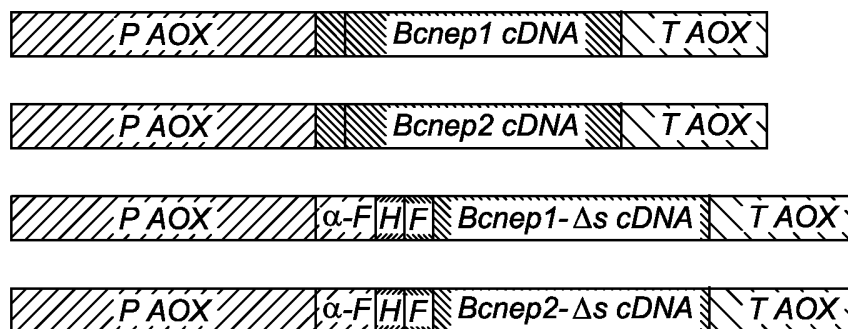
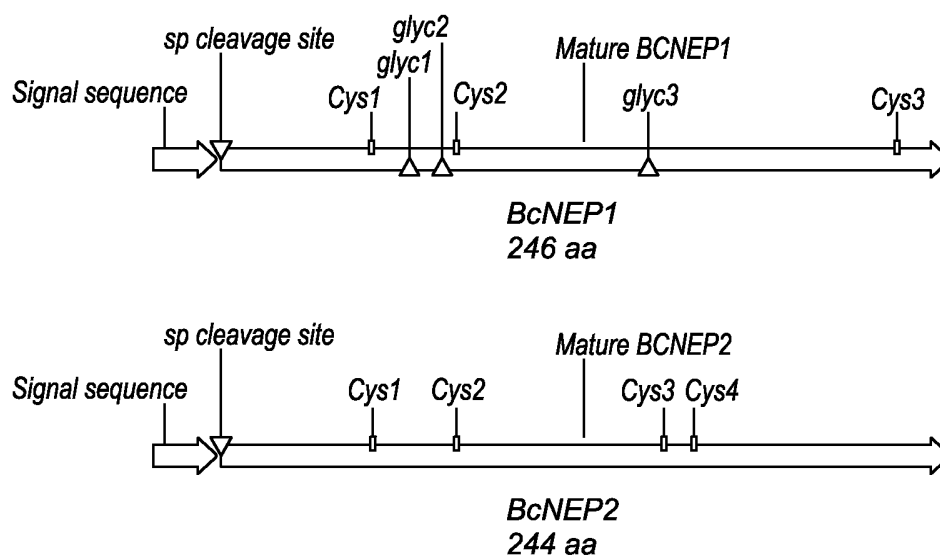
Fig 1*Fig 2**Fig 3*

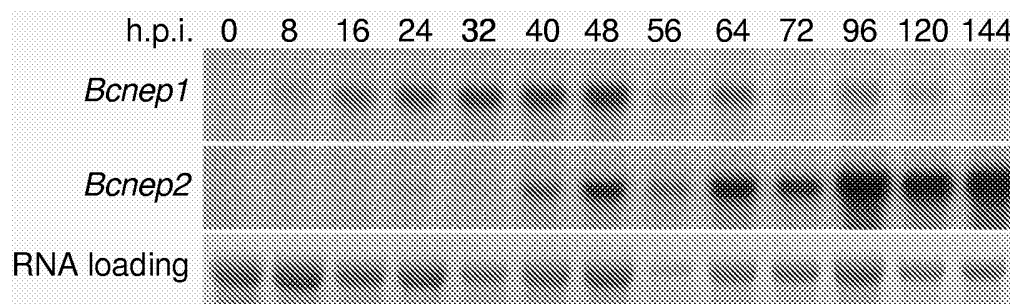
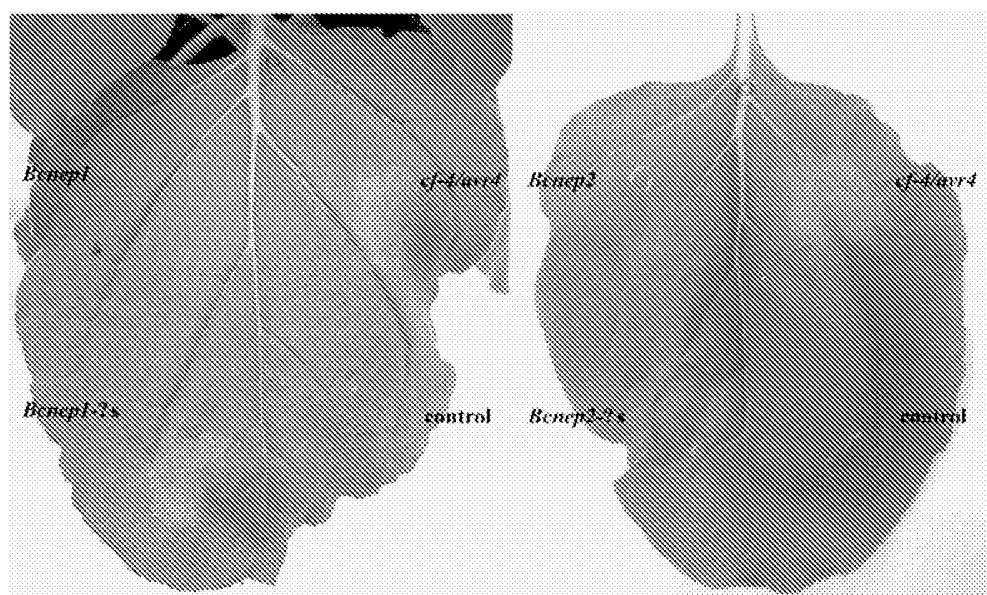
Fig 4*Fig 5*

Fig 6

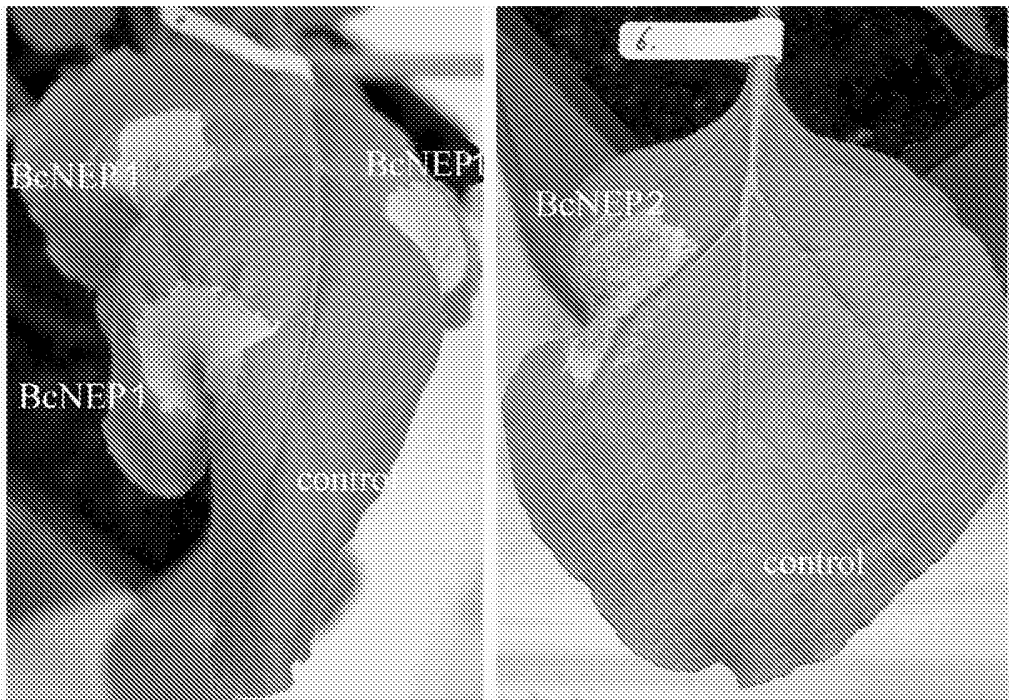


Fig 7

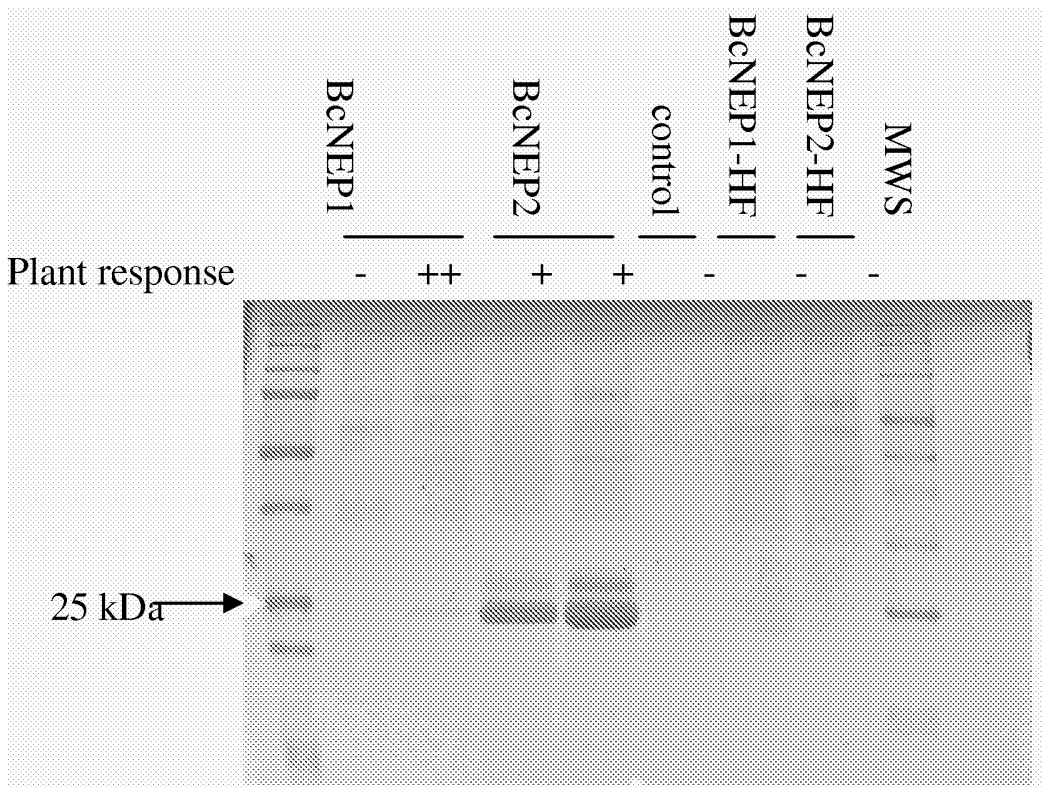


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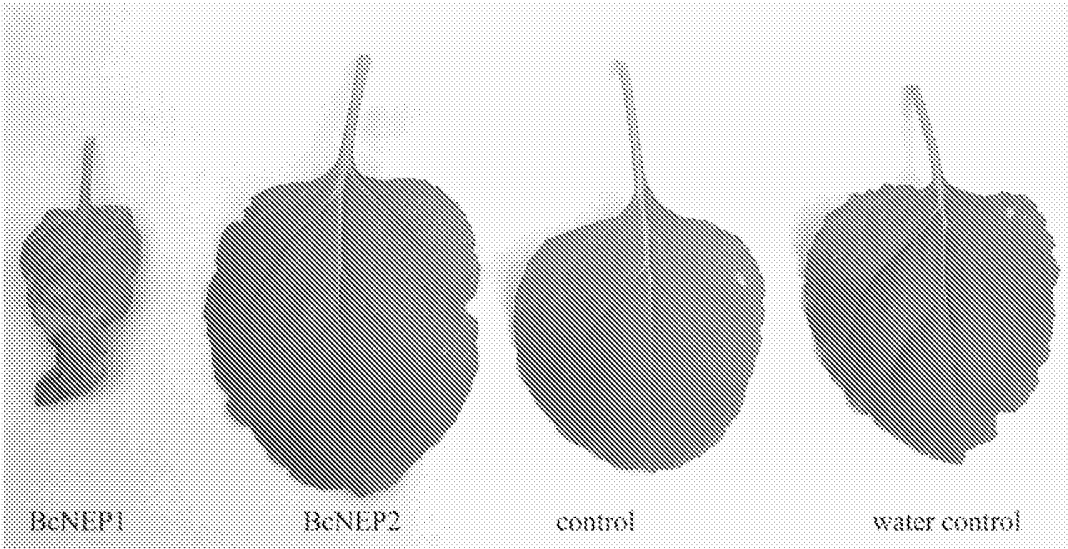


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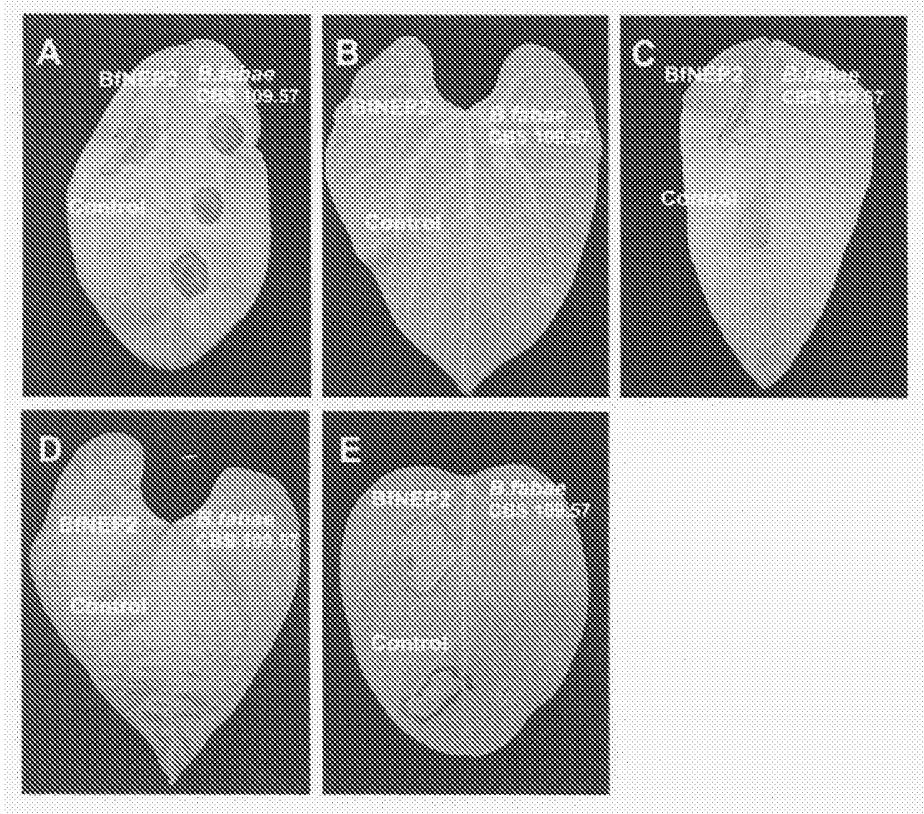
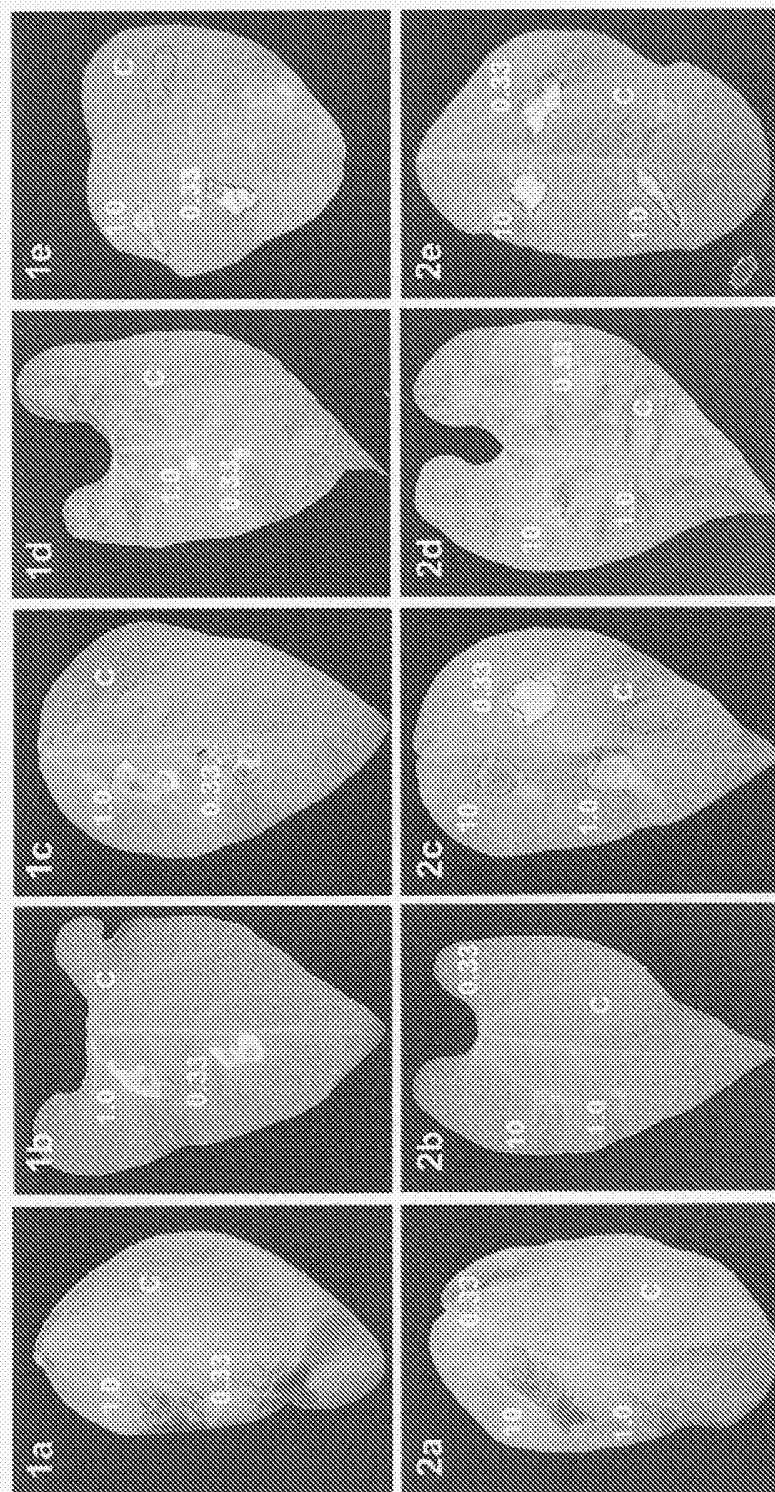
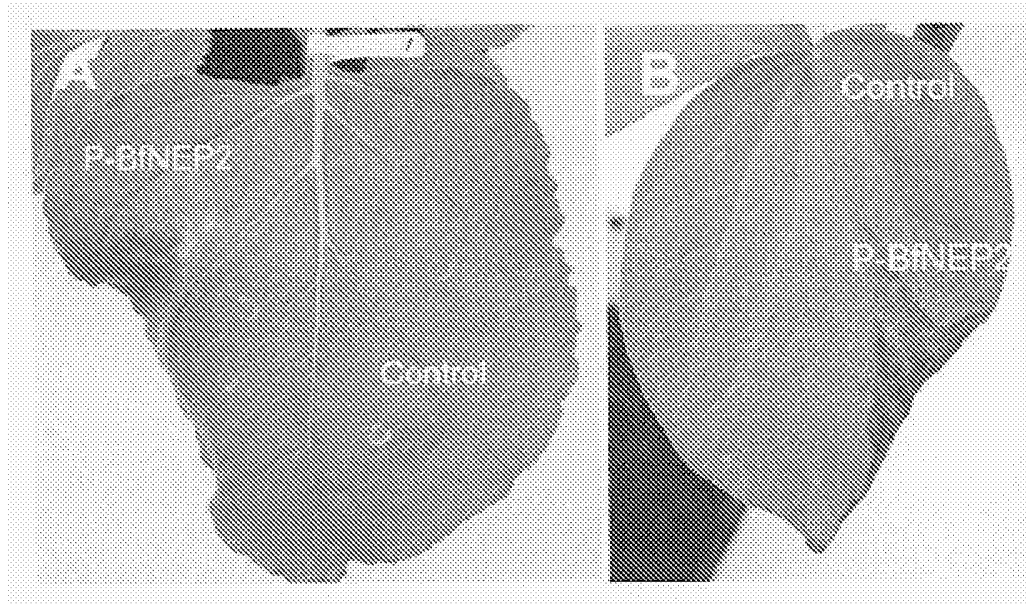
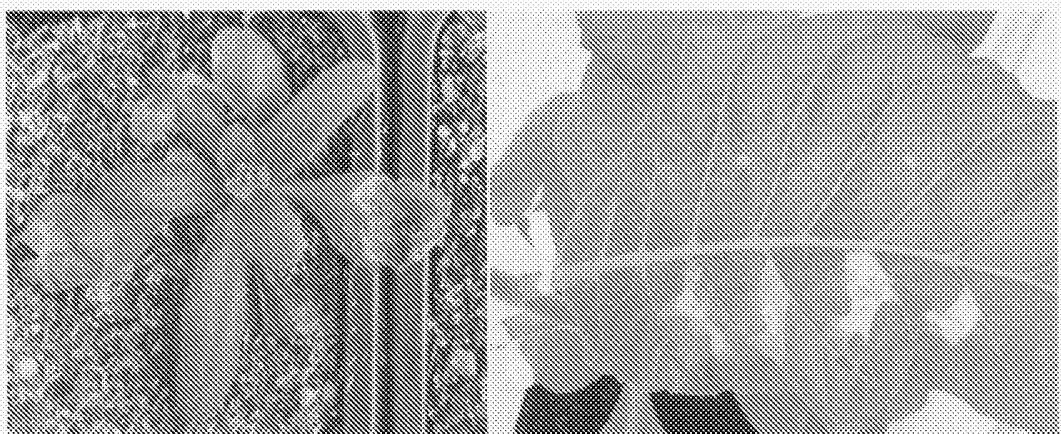


Fig 10

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Fig 11*Fig 12*

P6000092.ST251

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Ala His Gly Cys Gln Pro Tyr Ser Ala Val Asp Gly Asn Gly Asn Thr
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Ser Gly Gly Leu Gln Asp Thr Gly Asn Val Ser Ala Gly Cys Arg Asp
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Glu Leu Gln Phe Thr Asn Thr Leu Gly Arg Asp Leu Pro Met Met Trp
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Tyr Asp Phe Leu Pro Ala Val Ser Lys Thr Ala Leu Gln Asn Thr Asn
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<223> putative

<400> 12

Met Val Ala Phe Ser Lys Ser Leu Gln Leu Ser Leu Ser Val Leu Ala
 1 5 10 15

Ser Thr Val Ala Ala Ile Pro Thr Pro Ser Gln Leu Glu Ser Arg Ala
 20 25 30

Val Ile Ser His Asp Ala Val Val Gly Phe Pro Glu Thr Val Pro Ser
 35 40 45

Gly Thr Val Gly Thr Val Tyr Glu Thr Tyr Gln Pro Tyr Leu Lys Val
 50 55 60

Val Asn Gly Cys Val Pro Phe Pro Ala Val Asp Ala Ser Gly Asn Thr
 65 70 75 80

Gly Gly Gly Leu Ser Pro Thr Gly Ser Thr Asn Gly Met Cys Ser Ser
 85 90 95

Ser Thr Gly Gln Val Tyr Val Arg Gly Ala Gln Asn Gly Ser Tyr Tyr
 100 105 110

Gly Ile Met Tyr Ser Trp Tyr Met Pro Lys Asp Glu Pro Ser Thr Gly
 115 120 125

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Ile Gly His Arg His Asp Trp Glu Gly Val Ile Val Trp Leu Ser Arg
 130 135 140

Ser Thr Ala Thr Thr Ala Ser Asn Ile Val Ala Val Cys Pro Ser Ala
 145 150 155 160

His Gly Gly Trp Asp Cys Ser Thr Asp Gly Tyr Ser Leu Ser Gly Thr
 165 170 175

Ser Pro Leu Ile Lys Tyr Glu Ser Ile Trp Pro Ile Asp His Ser Met
 180 185 190

Gly Leu Thr Ser Thr Val Gly Gly Gln Gln Pro Met Ile Ala Trp Glu
 195 200 205

Ser Leu Pro Ala Ala Ala Gln Thr Ala Leu Glu Thr Thr Asp Phe Gly
 210 215 220

Ser Ala Asn Val Pro Phe Ile Pro Ser Ser Phe Ala Asn Asn Leu Ala
 225 230 235 240

Ala Ala Thr Phe

<210> 13

<211> 735

<212> DNA

<213> unknown

<220>

<223> cDNA of BtNEP2, Botrytis tulipae

<400> 13

atggtgcct tctcaaatc attacagctt tcccttcgg tttggcatc taccgtcgt 60

gccatcccta caccatcaca gcttgaatct cgtgccgta tcagtcacga tgccgttgta 120

ggatttcccg aaaccgttcc cagcgggaca gtaggaacag tttatgagac atatcaacca 180

tatcttaagg tcgtcaatgg ttgcgtacca ttcctgtctg tcgatgcac gggtaacaca 240

ggtggtggtt tgtaccaac tggcagtacc aatggtatgt gcagcagtag taccgtcaa 300

gtatacgtcc gaggagcaca gaacggatca tactacggta tcattgtact ctggtacatg 360

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ccaaaagatg agccttcaac cggatttggc caccgtcacg actgggaagg tgtaatcgtc 420
tggtctcca gatcaaccgc cacaaccgcc agcaacattg tagccgtctg tccttcgcc 480
catggaggct gggattgttc caccgatggt tattcccttt ctggcaccag ccctcttata 540
aagtagcaaa gtatctggcc tatgatcat tcaatgggtc ttactagtac tgttggtgga 600
caacaaccta tgattgcttg ggaatctcta cctgctgctg ctcaaactgc tcttgagacg 660
accgatttcg gctctgcgaa tgttccttc attccatctt ctctcgaaa caatcttgca 720
gctgctactt tctag 735

<210> 14

<211> 244

<212> PRT

<213> unknown

<220>

<223> Amino acid sequence of BeNEP2, Botrytis elliptica

<220>

<221> signal peptide

<222> (1)..(21)

<223> putative

<400> 14

Met Val Ala Phe Ser Lys Ser Leu Gln Leu Ser Leu Ser Val Leu Ala
1 5 10 15

Ser Thr Val Val Ala Leu Pro Thr Pro Ser Gln Leu Glu Ser Arg Ala
20 25 30

Val Ile Asn Ser Asp Ala Val Val Gly Phe Pro Glu Thr Val Pro Ser
35 40 45

Gly Ile Val Gly Thr Val Tyr Glu Thr Tyr Gln Pro Tyr Leu Lys Ile
50 55 60

Val Asn Gly Cys Val Pro Phe Pro Ala Val Asp Ala Ser Gly Asn Thr
65 70 75 80

Gly Gly Gly Leu Ser Pro Thr Gly Ser Ser Asn Gly Glu Cys Ser Ser
85 90 95

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Ser Thr Gly Gln Val Tyr Val Arg Gly Ala Gln Ser Gly Ser Tyr Tyr
100 105 110

Gly Ile Met Tyr Ser Trp Tyr Met Pro Lys Asp Glu Pro Ser Thr Gly
115 120 125

Ile Gly His Arg His Asp Trp Glu Gly Val Ile Val Trp Leu Ser Ser
130 135 140

Ser Thr Ala Thr Thr Ala Ser Asn Ile Val Ala Val Cys Pro Ser Ala
145 150 155 160

His Gly Gly Trp Asp Cys Ser Thr Asp Gly Tyr Ser Leu Ser Gly Thr
165 170 175

Ser Pro Leu Ile Lys Tyr Glu Ser Ile Trp Pro Ile Asp His Ser Met
180 185 190

Gly Leu Thr Ser Thr Val Gly Gly Gln Gln Pro Met Ile Ala Trp Glu
195 200 205

Ser Leu Pro Ala Ala Ala Gln Thr Ala Leu Glu Ser Thr Asp Phe Gly
210 215 220

Ala Ala Asn Val Pro Phe Ile Pro Ser Ala Phe Ala Asn Asn Leu Ala
225 230 235 240

Ala Ala Thr Phe

<210> 15

<211> 735

<212> DNA

<213> unknown

<220>

<223> cDNA of BeNEP2, Botrytis elliptica

<400> 15

atggtgcct tctcaaaatc actacaactt tccctttcgg ttttggcatc tacagtcgtt 60

ggcctcccta caccgtcaca gcttgaatct cgggccgtca tcaattccga tgccgttgta 120

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ggatttcccg aaaccgttcc cagcgggata gtaggaacag ttatgagac atatcaacca 180
 tatctcaaga tagtcaatgg ctgcgtacca ttcctgcag tcgatgcac gggaacaca 240
 ggtggtggtt tgtaccaac tggcagtagc aatggtgaat gcagcagtag taccggtcaa 300
 gtatagctcc gaggagcaca aagtggatca tactacggta tcatgtactc ctggtacatg 360
 ccaaaagatg agccctcaac cgggtattgt caccgtcacg actgggaagg tgtaatcgtc 420
 tggtttcca gctcaaccgc cacaaccgcc agcaacatcg tagccgtctg tccttcgcc 480
 catggagggtt gggattgttc caccgatggt tattcccttt ccggtaccag ccctctcgc 540
 aagtacgaaa gtatctggcc tatgatcat tcaatgggtc ttactagtagc tgttggcgga 600
 cagcaaccta tgattgcctg ggaatcttta cctgctgctg ctcaaactgc tcttgagtcg 660
 actgatttcg gcgctgcgaa tgttcattc attccatctg ctttcgcaa caatcttgca 720
 gctgctactt tctag 735

<210> 16
 <211> 246
 <212> PRT
 <213> unknown

<220>
 <223> B. fabae NEP1

<220>
 <221> signal peptide
 <222> (1)..(20)

<400> 16

Met His Phe Ser Asn Ala Lys Phe Leu Ser Ile Leu Ala Ala Ala Ala
 1 5 10 15

Ala Val Lys Gly Ala Pro Ile Glu Glu Ser Thr Ile Gln Ala Arg Ala
 20 25 30

Val Val Pro His Asp Ser Ile Asn Pro Trp Gly Glu Asn Val Pro Gly
 35 40 45

Asn Ala Leu Gly Asn Thr Leu Lys Arg Phe Glu Pro Tyr Leu His Ile
 50 55 60

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Ala His Gly Cys Gln Pro Tyr Ser Ala Val Asp Gly Asn Gly Asn Thr
65 70 75 80

Ser Gly Gly Leu Gln Asp Thr Gly Asn Val Ser Ala Gly Cys Arg Asp
 85 90 95

Gln Ser Lys Gly Gln Thr Tyr Val Arg Gly Gly Trp Ser Gly Gly Arg
 100 105 110

Tyr Gly Ile Met Tyr Ala Trp Tyr Phe Pro Lys Asp Gln Pro Ala Ala
 115 120 125

Gly Asn Val Ala Gly Gly His Arg His Asp Trp Glu Tyr Val Ile Ala
 130 135 140

Trp Val Asn Asn Pro Glu Val Ala Asn Pro Thr Leu Ile Gly Ala Gly
145 150 155 160

Ala Ser Gly His Gly Ser Ile Lys Leu Thr Thr Asn Pro Gln Arg Gln
 165 170 175

Gly Asp Arg Leu Lys Val Glu Tyr Phe Val Ser Phe Pro Thr Asn His
 180 185 190

Glu Leu Gln Phe Thr Asp Thr Leu Gly Arg Asp Leu Pro Met Met Trp
195 200 205

Tyr Asp Phe Leu Pro Ala Val Ser Lys Ala Ala Leu Gln Thr Thr Ser
210 215 220

Phe Gly Lys Ala Asn Cys Pro Phe Asn Asp Ala Asn Phe Asn Asn Asn
225 230 235 240

Leu Ala Lys Ala Lys Ile
 245

<210> 17

<211> 246

<212> PRT

<213> unknown

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<220>

<223> B. pelargonii NEP1 protein

<220>

<221> signal peptide

<222> (1)..(20)

<400> 17

Met His Phe Ser Asn Ala Lys Phe Leu Ser Ile Leu Ala Ala Ala Ala
1 5 10 15

Ala Val Lys Gly Ala Pro Ile Glu Glu Ser Thr Ile Gln Ala Arg Ala
20 25 30

Val Val Pro His Asp Ser Ile Asn Pro Trp Gly Glu Asn Val Pro Gly
35 40 45

Asn Ala Leu Gly Asn Thr Leu Lys Arg Phe Glu Pro Tyr Leu His Ile
50 55 60

Ala His Gly Cys Gln Pro Tyr Ser Ala Val Asp Gly Asn Gly Asn Ala
65 70 75 80

Ser Gly Gly Leu Gln Asp Thr Gly Asn Val Ser Ala Gly Cys Arg Asp
85 90 95

Gln Ser Lys Gly Gln Thr Tyr Val Arg Gly Gly Trp Ser Gly Gly Arg
100 105 110

Tyr Gly Ile Met Tyr Ala Trp Tyr Phe Pro Lys Asp Gln Pro Ala Ala
115 120 125

Gly Asn Val Val Gly Gly His Arg His Asp Trp Glu Tyr Val Val Ala
130 135 140

Trp Val Asn Asn Pro Glu Val Ala Asn Pro Thr Leu Ile Gly Ala Gly
145 150 155 160

Ala Ser Gly His Gly Ser Ile Lys Lys Thr Thr Asn Pro Gln Arg Gln
165 170 175

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Gly Asp Arg Leu Lys Val Glu Tyr Tyr Val Ser Phe Pro Thr Asn His
 180 185 190

Glu Leu Gln Phe Thr Asn Thr Leu Gly Arg Asp Leu Pro Met Met Trp
 195 200 205

Tyr Asp Phe Leu Pro Ala Val Ser Lys Thr Ala Leu Gln Asn Thr Asn
 210 215 220

Phe Gly Lys Ala Asn Cys Pro Phe Asn Asp Ala Asn Phe Asn Asn Asn
 225 230 235 240

Leu Ala Lys Ala Arg Ile
 245

<210> 18
 <211> 223
 <212> PRT
 <213> unknown

<220>
 <223> B. calthae NEP1

<220>
 <221> signal peptide
 <222> (1)..(8)
 <223> part of putative signal peptide

<400> 18

Ala Ala Ala Ala Ala Val Lys Gly Ala Pro Ile Glu Glu Asn Thr Ile
 1 5 10 15

Gln Ala Arg Ala Val Val Pro His Asp Ser Ile Asn Pro Trp Gly Glu
 20 25 30

Asn Val Pro Gly Asn Ala Leu Gly Asn Thr Leu Lys Lys Phe Glu Pro
 35 40 45

Tyr Leu His Ile Ala His Gly Cys Gln Pro Tyr Ser Ala Val Asp Gly
 50 55 60

Tyr Gly Asn Thr Ser Gly Gly Leu Gln Asp Thr Gly Asn Ile Ser Ala
 65 70 75 80

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Gly Cys Arg Asp Gln Ser Lys Gly Gln Thr Tyr Val Arg Gly Gly Trp
 85 90 95

Ser Gly Gly Arg Tyr Gly Ile Met Tyr Ala Trp Tyr Phe Pro Lys Asp
 100 105 110

Gln Pro Ala Ala Gly Asn Val Ile Gly Gly His Arg His Asp Trp Glu
 115 120 125

Tyr Val Val Ala Trp Val Asn Asn Pro Ser Val Ala Asn Pro Thr Leu
 130 135 140

Ile Gly Ala Gly Ala Ser Gly His Gly Ser Ile Lys Lys Thr Thr Asn
 145 150 155 160

Pro Gln Arg Gln Gly Asp Arg Leu Lys Val Glu Tyr Tyr Val Ser Phe
 165 170 175

Pro Thr Asn His Glu Leu Gln Phe Thr Asn Ser Leu Gly Arg Asp Leu
 180 185 190

Pro Met Met Trp Tyr Asp Phe Leu Pro Ala Val Ser Lys Thr Ala Leu
 195 200 205

Gln Asn Thr Ser Phe Gly Lys Ala Asn Cys Pro Phe Asn Asp Ala
 210 215 220

<210> 19

<211> 213

<212> PRT

<213> unknown

<220>

<223> B. paeoniae NEP1

<220>

<221> signal peptide

<222> (1)..(2)

<223> part

<400> 19

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Lys Gly Ala Pro Ile Glu Glu Ser Thr Ile Gln Ala Arg Ala Thr Val
1 5 10 15

Pro His Asp Ser Ile Asn Pro Trp Gly Glu Asn Val Pro Gly Asn Ala
20 25 30

Val Gly Asn Thr Leu Lys Arg Phe Glu Pro Phe Ile Arg Ile Ala His
35 40 45

Gly Cys Gln Pro Tyr Ser Ala Val Asp Gly Asn Gly Asn Thr Ser Gly
50 55 60

Gly Leu Gln Asp Thr Gly Asn Val Ser Ala Gly Cys Gly Asp Leu Ser
65 70 75 80

Lys Gly Gln Thr Tyr Val Arg Ala Gly Trp Ser Gly Gly Arg Tyr Gly
85 90 95

Ile Met Tyr Ala Trp Tyr Phe Pro Lys Asp Gln Pro Ala Ser Gly Asn
100 105 110

Val Val Gly Gly His Arg His Asp Trp Glu His Val Val Val Trp Val
115 120 125

Asn Asn Pro Ala Leu Pro Asn Pro Thr Leu Ile Gly Ala Ala Ala Ser
130 135 140

Gly His Gly Ser Leu Lys Lys Thr Thr Asn Pro Gln Arg Gln Gly Asp
145 150 155 160

Arg Val Lys Val Glu Tyr Tyr Val Ser Phe Pro Thr Asn His Glu Leu
165 170 175

Gln Phe Thr Asp Thr Leu Gly Arg Asp Leu Pro Met Met Trp Tyr Asp
180 185 190

Phe Leu Pro Ala Ala Ser Lys Thr Ala Leu Gln Asn Thr Ser Phe Gly
195 200 205

Lys Ala Val Cys Pro
210

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<210> 20

<211> 207

<212> PRT

<213> unknown

<220>

<223> B. convoluta NEP1, putative signal peptide and APIE missing

<400> 20

Glu Ser Thr Ile Gln Ala Arg Ala Asp Val Pro His Asp Ser Ile Asn
1 5 10 15

Pro Trp Gly Glu Asn Val Pro Gly Asn Ala Leu Gly Asn Thr Leu Lys
20 25 30

Lys Phe Glu Pro Phe Ile His Ile Ala His Gly Cys Gln Pro Tyr Ser
35 40 45

Ala Val Asp Gly Asn Gly Asn Thr Ser Gly Gly Leu Gln Asp Thr Gly
50 55 60

Asn Val Ser Ala Gly Cys Arg Asp Gln Ser Lys Gly Gln Thr Tyr Val
65 70 75 80

Arg Gly Gly Trp Ser Gly Gly Arg Tyr Gly Ile Met Tyr Ala Trp Tyr
85 90 95

Phe Pro Lys Asp Gln Pro Ala Ala Gly Asn Val Val Gly Gly His Arg
100 105 110

His Asp Trp Glu His Val Val Val Trp Val Asn Asn Pro Ser Val Ala
115 120 125

Asn Pro Thr Leu Ile Gly Ala Ala Ala Ser Gly His Gly Gly Leu Lys
130 135 140

Lys Thr Thr Asn Pro Gln Arg Gln Gly Asp Arg Leu Lys Val Glu Tyr
145 150 155 160

Tyr Val Thr Phe Pro Thr Asn His Glu Leu Gln Phe Thr Asn Thr Leu
165 170 175

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Gly Arg Asp Leu Pro Met Met Trp Tyr Asp Phe Leu Pro Ala Val Ser
180 185 190

Lys Thr Ala Leu Gln Asn Thr Asn Phe Gly Lys Ala Ile Cys Pro
195 200 205

<210> 21

<211> 219

<212> PRT

<213> unknown

<220>

<223> B. polyblastis NEP1

<220>

<221> signal peptide

<222> (1)..(8)

<223> part

<400> 21

Ala Ala Ala Ala Ala Val Lys Gly Ala Pro Val Glu Glu Asn Thr Ile
1 5 10 15

Gln Ala Arg Ala Val Ile Gly His Asp Ser Ile Asn Pro Trp Pro Glu
20 25 30

Asn Val Pro Gly Asn Ala Val Gly Asn Thr Leu Lys Lys Phe Glu Pro
35 40 45

Phe Ile His Ile Ala His Gly Cys Gln Pro Tyr Ser Ala Val Asp Gly
50 55 60

Tyr Gly Asn Thr Ser Gly Gly Leu Gln Asp Thr Gly Asn Val Ser Ala
65 70 75 80

Gly Cys Arg Asp Gln Ser Lys Gly Gln Thr Tyr Val Arg Gly Gly Trp
85 90 95

Ser Gly Gly Arg Tyr Gly Ile Met Tyr Ser Trp Tyr Phe Pro Lys Asp
100 105 110

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Gln Pro Ile Ala Gly Asn Val Ala Gly Gly His Arg His Asp Trp Glu
 115 120 125

Phe Val Val Val Trp Val Asn Asn Pro Ala Val Ala Asn Pro Thr Leu
 130 135 140

Ile Gly Ala Ala Ala Ser Ser His Gly Asn Leu Arg Lys Thr Thr Asn
 145 150 155 160

Pro Gln Arg Gln Gly Asn Arg Val Lys Val Glu Tyr Phe Thr Ser Phe
 165 170 175

Pro Thr Asn His Glu Leu Gln Phe Thr Asp Thr Leu Gly Arg Asp Leu
 180 185 190

Pro Met Met Trp Tyr Asp Phe Leu Pro Gly Val Ser Lys Thr Ala Leu
 195 200 205

Gln Asn Thr Asp Phe Gly Ser Ala Asn Cys Pro
 210 215

<210> 22

<211> 213

<212> PRT

<213> unknown

<220>

<223> B. gladiolorum NEP1

<220>

<221> signal peptide

<222> (1)..(2)

<223> part

<400> 22

Lys Gly Ala Pro Ile Glu Glu Asn Thr Ile Gln Ala Arg Ala Val Val
 1 5 10 15

Pro His Asp Ser Ile Asn Pro Trp Gly Glu Asn Val Pro Gly Asn Ala
 20 25 30

Leu Gly Asn Thr Leu Lys Lys Phe Glu Pro Phe Leu His Ile Ala His
 35 40 45

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Gly Cys Gln Pro Tyr Ser Ala Val Asp Gly Asn Gly Asn Thr Ser Gly
50 55 60

Gly Leu Gln Asp Thr Gly Asn Val Ser Ala Gly Cys His Asp Gln Ser
65 70 75 80

Lys Gly Gln Thr Tyr Val Arg Gly Gly Trp Ser Gly Gly Arg Tyr Gly
85 90 95

Ile Met Tyr Ala Trp Tyr Phe Pro Lys Asp Gln Pro Ala Ala Gly Asn
100 105 110

Leu Val Gly Gly His Arg His Asp Trp Glu Cys Val Val Ala Trp Val
115 120 125

Asn Asn Pro Ser Val Val Asn Pro Ser Leu Ile Gly Ala Ala Ala Ser
130 135 140

Gly His Gly Ser Val Lys Lys Thr Thr Asn Pro Gln Arg Gln Gly Asp
145 150 155 160

Arg Leu Lys Val Glu Tyr Tyr Val Ser Phe Pro Thr Asn His Glu Leu
165 170 175

Gln Phe Thr Asn Thr Leu Gly Arg Asp Leu Pro Met Met Trp Tyr Asp
180 185 190

Phe Leu Pro Ala Val Ser Lys Thr Ala Leu Gln Asn Thr Asn Phe Gly
195 200 205

Lys Ala Asn Cys Pro
210

<210> 23

<211> 126

<212> PRT

<213> unknown

<220>

<223> B. sphaerosperma NEP1

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<220>

<221> signal peptide

<222> (1)..(8)

<223> part

<400> 23

Ala Ala Ala Ala Ala Val Lys Gly Ala Pro Ile Glu Glu Asn Thr Ile
1 5 10 15

Gln Ala Arg Ala Val Val Pro His Asp Ser Ile Asn Pro Trp Gly Glu
20 25 30

Asn Val Pro Gly Asn Ala Leu Gly Asn Thr Leu Lys Lys Phe Glu Pro
35 40 45

Phe Leu His Ile Ala Leu Gly Cys Gln Pro Tyr Ser Ala Val Asp Gly
50 55 60

Asn Gly Asn Thr Ser Gly Gly Leu Gln Asp Thr Gly Asn Val Ser Asn
65 70 75 80

Gly Tyr Arg Asp Gln Ser Lys Gly Arg Thr Tyr Val Arg Gly Gly Trp
85 90 95

Ser Gly Gly Arg Tyr Gly Ile Met Tyr Ala Trp Tyr Phe Pro Lys Asp
100 105 110

Gln Pro Ala Ala Gly Asn Val Val Gly Gly His Arg Tyr Asp
115 120 125

<210> 24

<211> 126

<212> PRT

<213> unknown

<220>

<223> B. globos NEP1

<220>

<221> signal peptide

<222> (1)..(8)

<223> part

P6000092.ST251

<400> 24

Ala Ala Ala Ala Val Lys Gly Ala Pro Ile Glu Glu Asn Thr Ile
1 5 10 15

Gln Ala Arg Ala Val Val Pro His Asp Ser Ile Asn Pro Trp Gly Glu
20 25 30

Asn Val Pro Gly Asn Ala Leu Gly Asn Thr Leu Lys Lys Phe Glu Pro
35 40 45

Phe Leu His Ile Ala His Gly Cys Gln Pro Tyr Ser Ala Val Asp Gly
50 55 60

Asn Gly Asn Thr Ser Gly Gly Leu Gln Asp Thr Gly Asn Val Ser Thr
65 70 75 80

Gly Tyr Arg Asp Gln Ser Lys Gly Arg Thr Tyr Val Arg Gly Gly Ser
85 90 95

Ser Gly Gly Arg Tyr Gly Ile Met Tyr Ala Trp Tyr Phe Pro Lys Asp
100 105 110

Gln Pro Ala Ala Gly Asn Val Val Gly Gly His Arg Tyr Asp
115 120 125

<210> 25

<211> 219

<212> PRT

<213> unknown

<220>

<223> B. byssoidae NEP1

<220>

<221> signal peptide

<222> (1)..(8)

<223> part

<400> 25

Ala Ala Ala Ala Val Lys Gly Ala Pro Ile Glu Glu Asn Thr Ile
1 5 10 15

P6000092.ST251

Gln Ala Arg Ala Val Val Pro His Asp Ser Ile Asn Pro Trp Gly Glu
20 25 30

Asn Val Pro Gly Asn Ala Leu Gly Asn Thr Leu Lys Lys Phe Glu Pro
35 40 45

Phe Leu His Ile Ala His Gly Cys Gln Pro Tyr Ser Ala Val Asp Gly
50 55 60

Asn Gly Asn Thr Ser Gly Gly Leu Gln Asp Thr Gly Asn Val Ser Ala
65 70 75 80

Gly Cys Arg Asp Gln Ser Lys Gly Gln Thr Tyr Val Arg Gly Gly Trp
85 90 95

Ser Gly Gly Arg Tyr Gly Ile Met Tyr Ala Trp Tyr Phe Pro Lys Asp
100 105 110

Gln Pro Ala Ala Gly Asn Val Val Gly Gly His Arg His Asp Trp Glu
115 120 125

Tyr Val Val Ala Trp Val Asn Asn Pro Ser Val Ala Asn Pro Ser Leu
130 135 140

Ile Gly Ala Ala Ala Ser Gly His Gly Ser Val Lys Lys Thr Thr Asn
145 150 155 160

Pro Gln Arg Gln Gly Asp Arg Leu Lys Val Glu Tyr Tyr Val Ser Phe
165 170 175

Pro Thr Asn His Glu Leu Gln Phe Thr Asn Thr Leu Gly Arg Asp Leu
180 185 190

Pro Met Met Trp Tyr Asp Phe Leu Pro Ala Val Ser Lys Thr Ala Leu
195 200 205

Gln Asn Thr Asn Phe Gly Lys Ala Asn Cys Pro
210 215

<210> 26

<211> 210

P6000092.ST251

<212> PRT

<213> unknown

<220>

<223> B. narcissicola NEP1, putative signal peptide and A missing

<400> 26

Pro Ile Glu Glu Asn Thr Ile Gln Ala Arg Ala Val Val Pro His Asp
1 5 10 15

Ser Ile Asn Pro Trp Gly Glu Asn Val Pro Gly Asn Ala Leu Gly Asn
20 25 30

Thr Leu Lys Lys Phe Glu Pro Phe Leu His Ile Ala His Gly Cys Gln
35 40 45

Pro Tyr Ser Ala Val Asp Gly Tyr Gly Asn Thr Ser Gly Gly Leu Gln
50 55 60

Asp Thr Gly Asn Val Ser Ala Asp Cys Arg Asp Gln Ser Lys Gly Gln
65 70 75 80

Thr Tyr Val Arg Gly Gly Trp Ser Gly Gly Arg Tyr Gly Ile Met Tyr
85 90 95

Ala Trp Tyr Phe Pro Lys Asp Gln Pro Ala Ala Gly Asn Val Ile Gly
100 105 110

Gly His Arg His Asp Trp Glu Tyr Val Val Ala Trp Val Asn Asn Pro
115 120 125

Ser Val Ala Asn Pro Ser Leu Ile Gly Ala Ala Ala Ser Gly His Gly
130 135 140

Ser Val Lys Lys Thr Thr Asn Pro Gln Arg Gln Gly Asp Arg Leu Lys
145 150 155 160

Val Glu Tyr Tyr Val Ser Phe Pro Thr Asn His Glu Leu Gln Phe Thr
165 170 175

Asn Thr Leu Gly Arg Asp Leu Pro Met Met Trp Tyr Asp Phe Leu Pro
180 185 190

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Ala Val Ser Lys Thr Ala Leu Gln Asn Thr Ser Phe Gly Lys Ala Asn
195 200 205

Cys Pro
210

<210> 27
<211> 213
<212> PRT
<213> unknown

<220>
<223> B. croci NEP1

<220>
<221> singal peptide
<222> (1)..(2)
<223> part

<400> 27

Lys Gly Ala Pro Ile Glu Glu Asn Thr Ile Gln Ala Arg Ala Val Val
1 5 10 15

Pro His Asp Ser Ile Asn Pro Trp Gly Glu Asn Val Pro Gly Asn Ala
20 25 30

Leu Gly Asn Thr Leu Lys Arg Phe Glu Pro Phe Leu His Ile Ala His
35 40 45

Gly Cys Gln Pro Tyr Ser Ala Val Asp Gly Asn Gly Asn Thr Ser Gly
50 55 60

Gly Leu Gln Asp Thr Gly Asn Val Ser Ala Gly Cys Arg Asp Gln Ser
65 70 75 80

Lys Gly Gln Thr Tyr Val Arg Gly Gly Trp Ser Gly Gly Arg Tyr Gly
85 90 95

Ile Met Tyr Ala Trp Tyr Phe Pro Lys Asp Gln Pro Ala Ala Gly Asn
100 105 110

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Val Val Gly Gly His Arg His Asp Trp Glu His Ile Val Val Trp Val
115 120 125

Asn Asn Pro Glu Val Gly Asn Pro Thr Leu Ile Gly Ala Ala Ala Ser
130 135 140

Gly His Gly Ser Val Lys Lys Thr Thr Asn Pro Gln Arg Gln Gly Asp
145 150 155 160

Arg Leu Lys Val Glu Tyr Tyr Val Ser Phe Pro Thr Asn His Glu Leu
165 170 175

Gln Phe Thr Asn Thr Leu Gly Arg Asp Leu Pro Met Met Trp Tyr Asp
180 185 190

Phe Leu Pro Ala Val Ser Lys Thr Ala Leu Gln Asn Thr Ser Phe Gly
195 200 205

Lys Ala Asn Cys Pro
210

<210> 28

<211> 213

<212> PRT

<213> unknown

<220>

<223> B. hyacinthi NEP1

<220>

<221> signal peptide

<222> (1)..(2)

<223> part

<400> 28

Lys Gly Ala Pro Ile Glu Glu Asn Thr Ile Gln Ala Arg Ala Val Val
1 5 10 15

Pro His Asp Ser Ile Asn Pro Trp Gly Glu Asn Val Pro Gly Asn Ala
20 25 30

Leu Gly Asn Thr Leu Lys Arg Phe Glu Pro Phe Leu His Ile Ala His
35 40 45

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Gly Cys Gln Pro Tyr Ser Ala Val Asp Gly Asn Gly Asn Thr Ser Gly
50 55 60

Gly Leu Gln Asp Thr Gly Asn Val Ser Ala Gly Cys Arg Asp Gln Ser
65 70 75 80

Lys Gly Gln Thr Tyr Val Arg Gly Gly Trp Ser Gly Gly Arg Tyr Gly
85 90 95

Ile Met Tyr Ala Trp Tyr Phe Pro Lys Asp Gln Pro Ala Ala Gly Asn
100 105 110

Val Val Gly Gly His Arg His Asp Trp Glu His Ile Val Val Trp Val
115 120 125

Asn Asn Pro Glu Val Gly Asn Pro Thr Leu Ile Gly Ala Ala Ala Ser
130 135 140

Gly His Gly Ser Val Lys Lys Thr Thr Asn Pro Gln Arg Gln Gly Asp
145 150 155 160

Arg Leu Lys Val Glu Tyr Tyr Val Ser Phe Pro Thr Asn His Glu Leu
165 170 175

Gln Phe Thr Asn Thr Leu Gly Arg Asp Leu Pro Met Met Trp Tyr Asp
180 185 190

Phe Leu Pro Ala Val Ser Lys Thr Ala Leu Gln Asn Thr Ser Phe Gly
195 200 205

Lys Ala Asn Cys Pro
210

<210> 29

<211> 246

<212> PRT

<213> unknown

<220>

<223> B. elliptica NEP1

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<220>

<221> signal peptide

<222> (1)..(20)

<400> 29

Met Tyr Phe Ser Asn Ala Lys Phe Leu Ser Ile Leu Ala Ala Ala Ala
1 5 10 15

Ala Val Lys Gly Ala Pro Ile Glu Glu Asn Thr Ile Gln Ala Arg Ala
20 25 30

Val Val Asn His Asp Ser Ile Asn Pro Trp Gly Glu Asn Val Pro Gly
35 40 45

Asn Ala Leu Gly Asn Thr Leu Lys Arg Phe Glu Pro Phe Leu His Ile
50 55 60

Ala His Gly Cys Gln Pro Tyr Ser Ala Val Asp Gly Tyr Gly Asn Thr
65 70 75 80

Ser Gly Gly Leu Gln Asp Thr Gly Asn Val Ser Ala Gly Cys Arg Asp
85 90 95

Gln Ser Lys Gly Gln Thr Tyr Val Arg Gly Gly Trp Ser Gly Gly Arg
100 105 110

Tyr Gly Ile Met Tyr Ala Trp Tyr Phe Pro Lys Asp Gln Pro Ala Ala
115 120 125

Gly Asn Val Val Gly Gly His Arg His Asp Trp Glu His Ile Val Val
130 135 140

Trp Val Asn Asn Pro Ser Val Ala Asn Pro Thr Leu Ile Gly Ala Ala
145 150 155 160

Ala Ser Gly His Gly Ser Val Lys Lys Thr Thr Asn Pro Gln Arg Gln
165 170 175

Gly Asp Arg Leu Lys Val Glu Tyr Tyr Val Ser Phe Pro Thr Asn His
180 185 190

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Glu Leu Gln Phe Thr Asn Thr Leu Gly Arg Asp Leu Pro Met Met Trp
195 200 205

Tyr Asp Phe Leu Pro Ala Val Ser Lys Thr Ala Leu Gln Asn Thr Ser
210 215 220

Phe Gly Lys Ala Asn Cys Pro Phe Asn Asp His Asn Phe Ala Asn Asn
225 230 235 240

Leu Ala Lys Ala Ala Ile
245

<210> 30
<211> 213
<212> PRT
<213> unknown

<220>
<223> B. squamosa NEP1

<220>
<221> signal peptide
<222> (1)..(2)
<223> part

<400> 30

Lys Gly Ala Pro Ile Glu Glu Asn Thr Ile Gln Ala Arg Ala Val Val
1 5 10 15

Asn His Asp Ser Ile Asn Pro Trp Gly Glu Asn Val Pro Gly Asn Ala
20 25 30

Leu Gly Asn Thr Leu Lys Arg Phe Glu Pro Phe Leu His Ile Ala His
35 40 45

Gly Cys Gln Pro Tyr Ser Ala Val Asp Gly Tyr Gly Asn Thr Ser Gly
50 55 60

Gly Leu Gln Asp Thr Gly Asn Val Ser Ala Gly Cys Arg Asp Gln Ser
65 70 75 80

Lys Gly Gln Thr Tyr Val Arg Gly Gly Trp Ser Gly Gly Arg Tyr Gly

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85 90 95

Ile Met Tyr Ala Trp Tyr Phe Pro Lys Asp Gln Pro Ala Ala Gly Asn
100 105 110

Val Val Gly Gly His Arg His Asp Trp Glu His Ile Val Val Trp Val
115 120 125

Asn Asn Pro Ser Val Ala Asn Pro Thr Leu Ile Gly Ala Ala Ala Ser
130 135 140

Gly His Gly Ser Val Lys Lys Thr Thr Asn Pro Gln Arg Gln Gly Asp
145 150 155 160

Arg Leu Lys Val Glu Tyr Tyr Val Ser Phe Pro Thr Asn His Glu Leu
165 170 175

Gln Phe Thr Asn Thr Leu Gly Arg Asp Leu Pro Met Met Trp Tyr Asp
180 185 190

Phe Leu Pro Ala Val Ser Lys Thr Ala Leu Gln Asn Thr Ser Phe Gly
195 200 205

Lys Ala Asn Cys Pro
210

<210> 31

<211> 211

<212> PRT

<213> unknown

<220>

<223> B. ranunculi NEP1, without putative signal peptide sequence

<400> 31

Ala Pro Ile Glu Glu Asn Thr Ile Gln Ala Arg Ala Val Val Asn His
1 5 10 15

Asp Ser Ile Asn Pro Trp Gly Glu Asn Val Pro Gly Asn Ala Leu Gly
20 25 30

Asn Thr Leu Lys Lys Phe Glu Pro Phe Leu His Ile Ala His Gly Cys

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35 40 45

Gln Pro Tyr Ser Ala Val Asp Gly Asn Gly Asn Thr Ser Gly Gly Leu
50 55 60

Gln Asp Thr Gly Asn Val Ser Ala Gly Cys Arg Asp Gln Ser Lys Gly
65 70 75 80

Gln Thr Tyr Val Arg Gly Gly Trp Ser Gly Gly Arg Tyr Gly Ile Met
85 90 95

Tyr Ala Trp Tyr Phe Pro Lys Asp Gln Pro Ala Ala Gly Asn Val Val
100 105 110

Gly Gly His Arg His Asp Trp Glu His Ile Val Val Trp Val Asn Asn
115 120 125

Pro Asn Val Gly Asn Pro Thr Leu Ile Gly Ala Ala Ala Ser Gly His
130 135 140

Gly Ser Val Lys Lys Thr Thr Asn Pro Gln Arg Gln Gly Asp Arg Leu
145 150 155 160

Lys Val Glu Tyr Tyr Val Ser Phe Pro Thr Asn His Glu Leu Gln Phe
165 170 175

Thr Asn Thr Leu Gly Arg Asp Leu Pro Met Met Trp Tyr Asp Phe Leu
180 185 190

Pro Ala Val Ser Lys Thr Ala Leu Gln Asn Thr Ser Phe Gly Lys Ala
195 200 205

Asn Cys Pro
210

<210> 32

<211> 213

<212> PRT

<213> unknown

<220>

<223> B. ficariarum NEP1

P6000092.ST251

<220>

<221> signal peptide

<222> (1)..(2)

<223> part

<400> 32

Lys Gly Ala Pro Ile Glu Glu Asn Thr Ile Gln Ala Arg Ala Val Val
1 5 10 15

Asn His Asp Ser Ile Asn Pro Trp Gly Glu Asn Val Pro Gly Asn Ala
20 25 30

Leu Gly Asn Thr Leu Lys Arg Phe Glu Pro Phe Leu His Ile Ala His
35 40 45

Gly Cys Gln Pro Tyr Ser Ala Val Asp Gly Asn Gly Asn Thr Ser Gly
50 55 60

Gly Leu Gln Asp Thr Gly Asn Val Ser Ala Gly Cys Arg Asp Gln Ser
65 70 75 80

Lys Gly Gln Thr Tyr Val Arg Gly Gly Trp Ser Gly Gly Arg Tyr Gly
85 90 95

Ile Met Tyr Ala Trp Tyr Phe Pro Lys Asp Gln Pro Ala Ala Gly Asn
100 105 110

Val Val Gly Gly His Arg His Asp Trp Glu His Ile Val Val Trp Val
115 120 125

Asn Asn Pro Asn Val Gly Asn Pro Thr Leu Ile Gly Ala Ala Ala Ser
130 135 140

Gly His Gly Ser Val Lys Lys Thr Thr Asn Pro Gln Arg Gln Gly Asp
145 150 155 160

Arg Leu Lys Val Glu Tyr Tyr Val Ser Phe Pro Thr Asn His Glu Leu
165 170 175

Gln Phe Thr Asn Thr Leu Gly Arg Asp Leu Pro Met Met Trp Tyr Asp

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180 185 190

Phe Leu Pro Ala Val Ser Lys Thr Ala Leu Gln Asn Thr Ser Phe Gly
195 200 205

Lys Ala Asn Cys Pro
210

<210> 33
<211> 213
<212> PRT
<213> unknown

<220>
<223> B. galanthina NEP1

<220>
<221> signal peptide
<222> (1)..(2)
<223> part

<400> 33

Lys Gly Ala Pro Ile Glu Glu Asn Thr Ile Gln Ala Arg Ala Asp Ile
1 5 10 15

Asp His Asp Ser Ile Asn Pro Trp Gly Glu Asn Val Pro Gly Asn Ala
20 25 30

Leu Gly Asn Thr Leu Lys Arg Phe Glu Pro Phe Leu His Ile Ala His
35 40 45

Gly Cys Gln Pro Tyr Ser Ala Val Asp Gly Tyr Gly Asn Thr Ser Gly
50 55 60

Gly Leu Gln Asp Thr Gly Asn Val Ser Ala Ser Cys Arg Asp Gln Ser
65 70 75 80

Lys Gly Gln Thr Tyr Val Arg Gly Gly Trp Ser Gly Gly Arg Tyr Gly
85 90 95

Ile Met Tyr Ala Trp Tyr Phe Pro Lys Asp Gln Pro Thr Ala Gly Asn
100 105 110

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Val Ile Gly Gly His Arg His Asp Trp Glu Tyr Ile Val Val Trp Val
 115 120 125

Asn Asn Pro Glu Val Gly Asn Pro Thr Leu Ile Gly Ala Ala Ala Ser
 130 135 140

Gly His Gly Ser Val Lys Lys Thr Thr Asn Pro Gln Arg Gln Gly Asp
 145 150 155 160

Arg Leu Lys Val Glu Tyr Tyr Val Ser Phe Pro Thr Asn His Glu Leu
 165 170 175

Gln Phe Thr Asp Thr Leu Gly Arg Asp Leu Pro Met Met Trp Tyr Asp
 180 185 190

Phe Leu Pro Ala Val Ser Lys Thr Ala Leu Gln Asn Thr Ser Phe Gly
 195 200 205

Lys Ala Asn Cys Pro
 210

<210> 34
 <211> 219
 <212> PRT
 <213> unknown

<220>
 <223> B. aclada NEP1

<220>
 <221> signal peptide
 <222> (1)..(8)
 <223> part

<400> 34

Ala Ala Ala Ala Val Lys Gly Ala Pro Ile Glu Glu Asn Thr Ile
 1 5 10 15

Gln Ala Arg Ala Val Val Pro His Asp Ser Ile Asn Pro Trp Gly Glu
 20 25 30

Asn Val Pro Gly Asn Ala Leu Gly Asn Thr Leu Lys Lys Phe Glu Pro

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35 40 45

Phe Leu His Ile Ala His Gly Cys Gln Pro Tyr Ser Ala Val Asp Gly
50 55 60

Asn Gly Asn Thr Ser Gly Gly Leu Gln Asp Thr Gly Asn Val Ser Ala
65 70 75 80

Gly Cys Arg Asp Gln Ala Lys Gly Gln Thr Tyr Val Arg Gly Gly Trp
85 90 95

Ser Gly Gly Arg Tyr Gly Ile Met Tyr Ala Trp Tyr Phe Pro Lys Asp
100 105 110

Gln Pro Ala Ala Gly Asn Val Val Gly Gly His Arg His Asp Trp Glu
115 120 125

His Ile Val Val Trp Val Asn Asn Pro Ser Ile Ala Asn Pro Thr Leu
130 135 140

Ile Gly Ala Ala Ala Ser Gly His Gly Ser Val Lys Lys Thr Thr Asn
145 150 155 160

Pro Gln Arg Gln Gly Asp Arg Leu Lys Val Glu Tyr Tyr Val Ser Phe
165 170 175

Pro Thr Asn His Glu Leu Gln Phe Thr Asn Thr Leu Gly Arg Asp Leu
180 185 190

Pro Met Met Trp Tyr Asp Phe Leu Pro Ala Val Ser Lys Thr Ala Leu
195 200 205

Gln Asn Thr Ser Phe Gly Lys Ala Asn Cys Pro
210 215

<210> 35

<211> 223

<212> PRT

<213> unknown

<220>

<223> B. porri NEP1

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<220>

<221> signal peptide

<222> (1)..(8)

<223> part

<400> 35

Ala Ala Val Ala Val Val Lys Gly Ala Pro Val Glu Glu Asn Thr Ile
1 5 10 15

Gln Ala Arg Ala Val Val Ser His Asp Ser Ile Asn Pro Trp Pro Glu
20 25 30

Asn Val Pro Gly Asn Ala Leu Gly Asn Thr Leu Lys Lys Phe Glu Pro
35 40 45

Phe Leu His Ile Ala His Gly Cys Gln Pro Tyr Ser Ala Val Asp Gly
50 55 60

Tyr Gly Asn Thr Ser Gly Gly Leu Gln Asp Ser Gly Ser Pro Ser Gly
65 70 75 80

Gly Cys Arg Asp Gln Ser Lys Gly Gln Thr Tyr Val Arg Gly Gly Trp
85 90 95

Ser Gly Gly Arg Tyr Gly Ile Met Tyr Ala Trp Tyr Phe Pro Lys Asp
100 105 110

Gln Pro Ile Pro Gly Asn Val Val Gly Gly His Arg His Asp Trp Glu
115 120 125

His Val Val Val Trp Val Asn Asn Pro Ser Val Ala Asn Pro Val Leu
130 135 140

Ile Gly Ala Gly Ala Ser Gly His Gly Lys Met Lys Lys Thr Thr Asn
145 150 155 160

Pro Gln Arg Gln Gly Asp Arg Val Lys Val Glu Tyr Phe Thr Thr Phe
165 170 175

Pro Thr Asn His Glu Leu Gln Phe Thr Asp Thr Leu Gly Arg Asp Leu

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180 185 190

Pro Met Met Trp Tyr Asp Phe Leu Pro Ala Ala Ser Lys Thr Ala Leu
195 200 205

Gln Asn Thr Asp Phe Gly Lys Ala Val Cys Pro Leu Asn Asp Ala
210 215 220

<210> 36
<211> 240
<212> PRT
<213> unknown

<220>
<223> B. tulipae NEP1

<220>
<221> signal peptide
<222> (1)..(14)
<223> part

<400> 36

Lys Phe Leu Ser Ile Leu Ala Ala Ala Ala Val Lys Gly Ala Pro
1 5 10 15

Ile Glu Glu Asn Thr Ile Gln Ala Arg Ala Val Val Ala His Asp Ser
20 25 30

Ile Asn Pro Trp Pro Glu Asn Val Pro Gly Asn Ala Ile Gly Asn Thr
35 40 45

Leu Arg Lys Phe Glu Pro Phe Ile His Ile Ala His Gly Cys Gln Pro
50 55 60

Tyr Ser Ala Val Asp Gly Phe Gly Asn Thr Ser Gly Gly Leu Gln Asp
65 70 75 80

Thr Gly Asn Val Ser Ala Gly Cys Arg Asp Gln Ser Lys Gly Gln Thr
85 90 95

Tyr Val Arg Gly Gly Trp Ser Gly Gly Arg Tyr Gly Ile Met Tyr Ala
100 105 110

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Trp Tyr Trp Pro Lys Asp Gln Pro Thr Ala Gly Asn Val Ile Gly Gly
115 120 125

His Arg His Asp Trp Glu Tyr Val Val Ala Trp Val Asn Asn Pro Ala
130 135 140

Val Pro Asp Pro Val Leu Ile Gly Ala Ala Ala Ser Gly His Gly Ser
145 150 155 160

Val Lys Lys Thr Thr Asn Pro Gln Arg Gln Gly Asp Arg Leu Lys Val
165 170 175

Glu Tyr Phe Val Gln Phe Pro Thr Asn His Glu Leu Gln Phe Thr Asp
180 185 190

Thr Leu Gly Arg Asp Leu Pro Met Met Trp Tyr Asp Phe Leu Pro Gly
195 200 205

Val Ser Lys Ala Ala Leu Gln Asn Thr Asp Phe Gly Ser Ala Asn Cys
210 215 220

Pro Phe Asn Asp His Asn Phe Ala Asn Asn Leu Ala Lys Ala Gln Ile
225 230 235 240

<210> 37

<211> 228

<212> PRT

<213> unknown

<220>

<223> B. convoluta NEP2

<220>

<221> signal peptide

<222> (1)..(14)

<223> partial putative signal peptide

<400> 37

Leu Gln Leu Ser Leu Ser Phe Trp Ala Ser Thr Val Val Ala Ile Pro
1 5 10 15

Thr Pro Ser Gln Leu Glu Ser Arg Ala Val Ile Asn Ser Asp Ala Val

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20 25 30

Val Gly Phe Pro Glu Thr Val Pro Ser Gly Thr Val Gly Thr Val Tyr
35 40 45

Glu Thr Tyr Gln Pro Tyr Leu Tyr Val Val Asn Gly Cys Val Pro Phe
50 55 60

Pro Ala Val Asp Ala Ser Gly Asn Thr Gly Gly Gly Leu Ala Pro Thr
65 70 75 80

Gly Ser Ser Asn Gly Asp Cys Ser Ser Ser Thr Gly Gln Val Tyr Val
85 90 95

Arg Gly Ala Gln Ser Gly Ser Tyr Tyr Gly Ile Met Tyr Ser Trp Tyr
100 105 110

Met Pro Lys Asp Glu Pro Ser Thr Gly Ile Gly His Arg His Asp Trp
115 120 125

Glu Gly Val Ile Val Trp Leu Ser Ser Ser Thr Ala Thr Thr Ala Asp
130 135 140

Asn Ile Val Ala Val Cys Pro Ser Ala His Gly Gly Trp Asp Cys Ser
145 150 155 160

Thr Asp Gly Tyr Ser Leu Ser Gly Thr Ser Pro Leu Ile Lys Tyr Glu
165 170 175

Ser Ile Trp Pro Ile Asp His Ser Met Gly Leu Thr Ser Thr Val Gly
180 185 190

Gly Gln Gln Pro Met Ile Ala Trp Glu Ser Leu Pro Thr Ala Ala Gln
195 200 205

Thr Ala Leu Glu Thr Thr Asp Phe Gly Ser Ala Asn Val Pro Phe Ile
210 215 220

Pro Ser Ala Phe
225

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<210> 38
<211> 228
<212> PRT
<213> unknown

<220>
<223> B. porri NEP2

<220>
<221> signal peptide
<222> (1)..(14)
<223> part

<400> 38

Leu Gln Leu Ser Phe Ser Phe Trp Ala Ser Thr Val Val Ala Ile Pro
1 5 10 15

Thr Pro Ser Gln Leu Glu Ser Arg Ala Val Ile Asn Ser Asp Ala Val
20 25 30

Val Gly Phe Pro Glu Thr Val Pro Ser Gly Thr Val Gly Thr Val Tyr
35 40 45

Glu Thr Tyr Gln Pro Tyr Leu Lys Ile Val Asn Gly Cys Val Pro Phe
50 55 60

Pro Ala Val Asp Ala Ser Gly Asn Thr Gly Gly Gly Leu Ala Pro Thr
65 70 75 80

Gly Ser Ser Asn Gly Asp Cys Ser Ser Ser Thr Gly Gln Val Tyr Val
85 90 95

Arg Gly Ala Gln Ser Gly Ser Tyr Tyr Gly Ile Met Tyr Ser Trp Tyr
100 105 110

Met Pro Lys Asp Glu Pro Ser Thr Gly Leu Gly His Arg His Asp Trp
115 120 125

Glu Gly Val Ile Val Trp Leu Ser Ser Ser Thr Gly Thr Thr Ala Asp
130 135 140

Asn Ile Val Ala Val Cys Pro Ser Ala His Gly Asp Trp Asp Cys Ser

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145 150 155 160

Thr Asp Glu Phe Ser Leu Ser Gly Thr Ser Pro Leu Ile Gln Tyr Glu

 165 170 175

Ser Ile Trp Pro Ile Asp His Ser Met Gly Leu Thr Ser Thr Val Gly

 180 185 190

Gly Gln Gln Pro Met Ile Ala Trp Glu Ser Leu Pro Thr Ala Ala Gln

 195 200 205

Thr Ala Leu Glu Thr Thr Asp Phe Gly Ser Ala Asn Val Pro Phe Ile

 210 215 220

Pro Ser Ala Phe

225

<210> 39

<211> 228

<212> PRT

<213> unknown

<220>

<223> B. paeoniae NEP2

<220>

<221> signal peptide

<222> (1)..(14)

<223> part

<400> 39

Leu Gln Leu Ser Phe Arg Phe Trp Ala Ser Thr Val Val Ala Ile Pro

1 5 10 15

Thr Pro Ser Gln Leu Glu Ser Arg Ala Val Ile Ser Ser Asp Ala Val

 20 25 30

Val Gly Phe Pro Glu Thr Val Pro Ser Gly Thr Val Gly Thr Val Tyr

 35 40 45

Glu Thr Tyr Gln Pro Tyr Leu Asp Val Val Asn Gly Cys Val Pro Phe

 50 55 60

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Pro Ala Val Asp Ala Ser Gly Asn Thr Gly Gly Gly Leu Ala Pro Thr
65 70 75 80

Gly Ser Ser Asn Gly Gly Cys Ser Ser Ser Thr Gly Gln Val Tyr Val
 85 90 95

Arg Gly Ala Gln Ser Gly Ser Tyr Tyr Gly Ile Met Tyr Ser Trp Tyr
 100 105 110

Met Pro Lys Asp Glu Pro Ser Thr Gly Ile Gly His Arg His Asp Trp
 115 120 125

Glu Gly Val Ile Val Trp Leu Ser Ser Ser Thr Ala Thr Thr Ala Asp
 130 135 140

Asn Ile Val Ala Val Cys Pro Ser Ala His Gly Gly Trp Asp Cys Ser
 145 150 155 160

Thr Asp Gly Tyr Ser Leu Ser Gly Thr Ser Pro Leu Ile Lys Tyr Glu
 165 170 175

Ser Ile Trp Pro Ile Asp His Ser Met Gly Leu Thr Ser Thr Val Gly
 180 185 190

Gly Gln Gln Pro Met Ile Ala Trp Glu Ser Leu Pro Thr Ala Ala Gln
 195 200 205

Thr Ala Leu Glu Thr Thr Asp Phe Gly Ser Ala Asn Val Pro Phe Ile
 210 215 220

Pro Ser Val Phe
225

<210> 40
<211> 228
<212> PRT
<213> unknown

<220>
<223> B. croci NEP2

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<220>

<221> signal peptide

<222> (1)..(14)

<223> part

<400> 40

Leu Gln Leu Ser Leu Ser Ile Leu Ala Ser Thr Val Val Ala Ile Pro
1 5 10 15

Thr Pro Ser Gln Leu Glu Ser Arg Ala Val Ile Asp His Asp Ala Val
20 25 30

Val Gly Phe Pro Glu Thr Val Pro Ser Gly Thr Val Gly Thr Val Tyr
35 40 45

Glu Thr Tyr Gln Pro Tyr Leu Lys Ile Val Asn Gly Cys Val Pro Phe
50 55 60

Pro Ala Val Asp Ala Ser Gly Asn Thr Gly Gly Gly Leu Ala Pro Thr
65 70 75 80

Gly Ser Glu Asn Gly Asp Cys Ser Ser Ser Thr Gly Gln Val Tyr Val
85 90 95

Arg Gly Ala Gln Ser Gly Ser Tyr Tyr Gly Ile Met Tyr Ser Trp Tyr
100 105 110

Met Pro Lys Asp Glu Pro Ser Asn Gly Leu Gly His Arg His Glu Trp
115 120 125

Glu Gly Val Ile Val Trp Leu Ser Ser Ser Thr Ala Thr Thr Ala Gly
130 135 140

Asn Ile Val Ala Val Cys Pro Ser Ala His Gly Gly Trp Asp Cys Ser
145 150 155 160

Thr Asp Gly Tyr Ser Leu Ser Gly Thr Ser Pro Leu Ile Lys Tyr Glu
165 170 175

Ser Ile Trp Pro Ile Asn His Ser Met Gly Leu Thr Ser Thr Val Gly
180 185 190

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Gly Gln Gln Pro Met Ile Ala Trp Glu Ser Leu Pro Ala Ala Ala Gln
195 200 205

Thr Ala Leu Glu Thr Thr Asp Phe Gly Ser Ala Asn Val Pro Phe Ile
210 215 220

Pro Ser Ala Phe
225

<210> 41
<211> 228
<212> PRT
<213> unknown

<220>
<223> B. hyacinthi NEP2

<220>
<221> signal peptide
<222> (1)..(14)
<223> part

<400> 41

Leu Gln Leu Ser Leu Ser Ile Leu Ala Ser Thr Val Val Ala Ile Pro
1 5 10 15

Thr Pro Ser Gln Leu Glu Ser Arg Ala Val Ile Asp His Asp Ala Val
20 25 30

Val Gly Phe Pro Glu Thr Val Pro Ser Gly Thr Val Gly Thr Val Tyr
35 40 45

Glu Thr Tyr Gln Pro Tyr Leu Lys Ile Val Asn Gly Cys Val Pro Phe
50 55 60

Pro Ala Val Asp Ala Ser Gly Asn Thr Gly Gly Gly Leu Ala Pro Thr
65 70 75 80

Gly Ser Glu Asn Gly Asp Cys Ser Ser Ser Thr Gly Gln Val Tyr Val
85 90 95

Arg Gly Ala Gln Ser Gly Ser Tyr Tyr Gly Ile Met Tyr Ser Trp Tyr

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100 105 110

Met Pro Lys Asp Glu Pro Ser Asn Gly Leu Gly His Arg His Glu Trp
 115 120 125

Glu Gly Val Ile Val Trp Leu Ser Ser Ser Thr Ala Thr Thr Ala Gly
 130 135 140

Asn Ile Val Ala Val Cys Pro Ser Ala His Gly Gly Trp Asp Cys Ser
 145 150 155 160

Thr Asp Gly Tyr Ser Leu Ser Gly Thr Ser Pro Leu Ile Lys Tyr Glu
 165 170 175

Ser Ile Trp Pro Ile Asn His Ser Met Gly Leu Thr Ser Thr Val Gly
 180 185 190

Gly Gln Gln Pro Met Ile Ala Trp Glu Ser Leu Pro Ala Ala Ala Gln
 195 200 205

Thr Ala Leu Glu Thr Thr Asp Phe Gly Ser Ala Asn Val Pro Phe Ile
 210 215 220

Pro Ser Ala Phe
 225

<210> 42
 <211> 228
 <212> PRT
 <213> unknown

<220>
 <223> B. galanthina NEP2

<220>
 <221> signal peptide
 <222> (1)..(14)
 <223> part

<400> 42

Leu Gln Leu Ser Leu Ser Val Leu Ala Ser Thr Val Val Ala Ile Pro
 1 5 10 15

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Thr Pro Ser Gln Leu Glu Ser Arg Ala Val Ile Asp Ser Asp Ala Val
20 25 30

Val Gly Phe Pro Glu Thr Val Pro Ser Gly Thr Val Gly Thr Val Tyr
35 40 45

Glu Thr Tyr Gln Pro Tyr Leu Lys Ile Val Asn Gly Cys Val Pro Phe
50 55 60

Pro Ala Val Asp Ala Ser Gly Asn Thr Gly Gly Gly Leu Ala Pro Thr
65 70 75 80

Gly Ser Ser Asn Gly Glu Cys Ser Ser Ser Thr Gly Gln Val Tyr Val
85 90 95

Arg Gly Ala Gln Ser Gly Ser Tyr Tyr Gly Ile Met Tyr Ser Trp Tyr
100 105 110

Met Pro Lys Asp Glu Pro Ser Thr Gly Ile Gly His Arg His Asp Trp
115 120 125

Glu Gly Val Ile Val Trp Leu Ser Ser Ser Thr Ala Thr Thr Ala Ser
130 135 140

Asn Ile Val Ala Val Cys Pro Ser Ala His Gly Gly Trp Asp Cys Ser
145 150 155 160

Thr Asp Asp Tyr Ser Leu Ser Gly Thr Ser Pro Leu Ile Lys Tyr Glu
165 170 175

Ser Ile Trp Pro Ile Asp His Ser Met Gly Leu Thr Ser Thr Val Gly
180 185 190

Gly Gln Gln Pro Met Ile Ala Trp Glu Ser Leu Pro Ala Ala Ala Gln
195 200 205

Thr Ala Leu Glu Thr Thr Asp Phe Gly Ala Ala Asn Val Pro Phe Ile
210 215 220

Pro Ser Ala Phe

P6000092.ST251

225

<210> 43

<211> 228

<212> PRT

<213> unknown

<220>

<223> B. sphaerosperma NEP2

<220>

<221> signal peptide

<222> (1)..(14)

<223> part

<400> 43

Leu Gln Leu Ser Leu Ser Val Leu Ala Ser Thr Val Ile Ala Ile Pro
1 5 10 15

Thr Pro Ser Gln Leu Glu Ser Arg Ala Val Ile Asp Ser Asp Ala Val
20 25 30

Val Gly Phe Pro Glu Thr Val Pro Ser Gly Thr Val Gly Thr Val Tyr
35 40 45

Glu Thr Tyr Gln Pro Tyr Leu Lys Ile Val Asn Gly Cys Val Pro Phe
50 55 60

Pro Ala Val Asp Ala Ala Gly Asn Thr Gly Gly Gly Leu Ala Pro Thr
65 70 75 80

Gly Ser Ser Asn Gly Met Cys Ser Ser Ser Thr Gly Gln Val Tyr Val
85 90 95

Arg Gly Ala Gln Ser Gly Ser Tyr Tyr Gly Ile Met Tyr Ser Trp Tyr
100 105 110

Met Pro Lys Asp Glu Pro Ser Thr Gly Ile Gly His Arg His Asp Trp
115 120 125

Glu Gly Val Ile Val Trp Leu Ser Ser Ser Ala Ala Thr Thr Ala Ser
130 135 140

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Asn Ile Val Ala Val Cys Pro Ser Ala His Gly Gly Trp Asp Cys Ser
145 150 155 160

Thr Asp Gly Tyr Ser Leu Ser Gly Thr Ser Pro Leu Ile Lys Tyr Gln
165 170 175

Ser Ile Trp Pro Ile Asp His Ser Met Gly Leu Thr Ser Ser Val Gly
180 185 190

Gly Gln Gln Pro Val Ile Ala Trp Glu Ser Leu Pro Ala Ala Ala Gln
195 200 205

Thr Ala Leu Glu Thr Thr Asp Phe Gly Ala Ala Asn Val Pro Phe Ile
210 215 220

Pro Ser Ala Phe
225

<210> 44
<211> 228
<212> PRT
<213> unknown

<220>
<223> B. globosa NEP2

<220>
<221> signal peptide
<222> (1)..(14)
<223> part

<400> 44

Leu Gln Leu Ser Phe Ser Ala Leu Ala Ser Thr Val Ile Ala Ile Pro
1 5 10 15

Thr Pro Ser Gln Leu Glu Ser Arg Ala Val Ile Asp Ser Asp Ala Val
20 25 30

Val Gly Phe Pro Glu Thr Val Pro Ser Gly Thr Val Gly Thr Val Tyr
35 40 45

Glu Thr Tyr Gln Pro Tyr Leu Lys Ile Val Asn Gly Cys Val Pro Phe

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50 55 60

Pro Ala Val Asp Ala Ala Gly Asn Thr Gly Gly Gly Leu Ala Pro Thr
 65 70 75 80

Gly Ser Ser Asn Gly Met Cys Ser Ser Ser Thr Gly Gln Val Tyr Val
 85 90 95

Arg Gly Ala Gln Ser Gly Ser Tyr Tyr Gly Ile Met Tyr Ser Trp Tyr
 100 105 110

Met Pro Lys Asp Glu Pro Ser Thr Gly Ile Gly His Arg His Asp Trp
 115 120 125

Glu Gly Val Ile Val Trp Leu Ser Ser Ser Ala Ala Thr Thr Ala Ser
 130 135 140

Asn Ile Val Ala Val Cys Pro Ser Ala His Gly Gly Trp Asp Cys Ser
 145 150 155 160

Thr Asp Gly Tyr Ser Leu Ser Gly Thr Ser Pro Leu Ile Lys Tyr Gln
 165 170 175

Ser Ile Trp Pro Ile Asp His Ser Met Gly Leu Thr Ser Ser Val Gly
 180 185 190

Gly Gln Gln Pro Met Ile Ala Trp Glu Ser Leu Pro Ala Ala Ala Gln
 195 200 205

Thr Ala Leu Glu Thr Thr Asp Phe Gly Ala Ala Asn Val Pro Phe Ile
 210 215 220

Pro Ser Ala Phe
 225

<210> 45
 <211> 228
 <212> PRT
 <213> unknown

<220>
 <223> B. gladiolorum NEP2

P6000092.ST251

<220>

<221> signal peptide

<222> (1)..(14)

<223> part

<400> 45

Leu Gln Leu Ser Leu Ser Val Leu Ala Ser Thr Val Ile Ala Ile Pro
1 5 10 15

Thr Pro Leu Gln Leu Glu Ser Arg Ala Val Ile Asp Ser Asp Ala Val
20 25 30

Val Gly Phe Pro Glu Thr Val Pro Ser Gly Thr Val Gly Thr Val Tyr
35 40 45

Glu Thr Tyr Gln Pro Tyr Leu Lys Ile Val Asn Gly Cys Val Pro Phe
50 55 60

Pro Ala Val Asp Ala Ala Gly Asn Thr Gly Gly Gly Leu Ala Pro Thr
65 70 75 80

Gly Ser Ser Asn Gly Met Cys Ser Ser Ser Thr Gly Gln Val Tyr Val
85 90 95

Arg Gly Ala Gln Ser Gly Ser Tyr Tyr Gly Ile Met Tyr Ser Trp Tyr
100 105 110

Met Pro Lys Asp Glu Pro Ser Thr Gly Ile Gly His Arg His Asp Trp
115 120 125

Glu Gly Val Ile Val Trp Leu Ser Ser Ser Thr Ala Thr Thr Ala Ser
130 135 140

Asn Ile Val Ala Val Cys Pro Ser Ala His Gly Gly Trp Asp Cys Ser
145 150 155 160

Thr Asp Gly Tyr Ser Leu Ser Gly Thr Ser Pro Leu Ile Lys Tyr Glu
165 170 175

Ser Ile Trp Pro Ile Asp His Ser Met Gly Leu Thr Ser Ala Val Gly

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180 185 190

Gly Gln Gln Pro Met Ile Ala Trp Glu Ser Leu Pro Ala Ala Ala Gln
195 200 205

Thr Ala Leu Glu Thr Thr Asp Phe Gly Ala Ala Asn Val Pro Phe Ile
210 215 220

Pro Ser Ala Phe
225

<210> 46
<211> 228
<212> PRT
<213> unknown

<220>
<223> B. polyblastis NEP2

<220>
<221> signal peptide
<222> (1)..(14)
<223> part

<400> 46

Leu Gln Leu Ser Leu Ser Val Leu Ala Ser Thr Val Ile Ala Ile Pro
1 5 10 15

Thr Pro Ser Gln Leu Glu Ser Arg Ala Val Ile Asp Ser Asp Ala Val
20 25 30

Val Gly Phe Pro Glu Thr Val Pro Ser Gly Ile Val Gly Thr Val Tyr
35 40 45

Glu Thr Tyr Gln Pro Phe Leu Lys Ile Val Asn Gly Cys Val Pro Phe
50 55 60

Pro Ala Val Asp Ala Ala Gly Asn Thr Gly Gly Gly Leu Ala Pro Thr
65 70 75 80

Gly Ser Ser Tyr Gly Met Cys Ser Ser Ser Thr Gly Gln Val Tyr Val
85 90 95

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Arg Gly Ala Gln Ser Gly Ser His Tyr Gly Ile Met Tyr Ser Trp Tyr
100 105 110

Met Pro Lys Asp Glu Pro Ser Thr Glu Ile Gly His Arg His Asp Trp
115 120 125

Glu Gly Val Ile Val Trp Leu Ser Ser Ser Thr Ala Thr Thr Ala Ser
130 135 140

Asn Ile Leu Ala Val Cys Pro Ser Ala His Gly Gly Trp Asp Cys Ser
145 150 155 160

Thr Asp Gly Tyr Ser Leu Ser Gly Thr Ser Pro Leu Ile Lys Tyr Glu
165 170 175

Ser Ile Trp Pro Ile Asp His Ser Met Gly Leu Thr Ser Ala Ile Gly
180 185 190

Val Gln Gln Pro Met Ile Ala Trp Glu Ser Leu Pro Ala Ala Ala Gln
195 200 205

Lys Ala Leu Glu Thr Thr Asp Phe Gly Ser Ala Asn Val Pro Phe Ile
210 215 220

Pro Ser Ala Phe
225

<210> 47
<211> 228
<212> PRT
<213> unknown

<220>
<223> B. narcissicola NEP2

<220>
<221> signal peptide
<222> (1)..(14)
<223> part

<400> 47

Leu Gln Leu Ser Leu Ser Val Leu Ala Ser Thr Val Ile Ala Ile Pro

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1 5 10 15

Thr Pro Ser Gln Leu Glu Ser Arg Ala Val Ile Asp Ser Asp Ala Val
20 25 30

Val Gly Phe Pro Glu Thr Val Pro Ser Gly Thr Val Gly Thr Val Tyr
35 40 45

Glu Thr Tyr Gln Pro Tyr Leu Lys Ile Val Asn Gly Cys Val Pro Phe
50 55 60

Pro Ala Val Asp Ala Ser Gly Asn Thr Gly Gly Gly Leu Ala Pro Thr
65 70 75 80

Gly Ser Ser Asn Gly Met Cys Ser Ser Ser Ile Gly Gln Val Tyr Val
85 90 95

Arg Gly Ala Gln Ser Gly Ser Tyr Tyr Gly Ile Met Tyr Ser Trp Tyr
100 105 110

Met Pro Lys Asp Glu Pro Ser Thr Gly Ile Gly His Arg His Asp Trp
115 120 125

Glu Gly Val Ile Val Trp Leu Ser Ser Ser Thr Ala Thr Thr Ala Ser
130 135 140

Asn Ile Val Ala Val Cys Pro Ser Ala His Gly Gly Trp Asp Cys Ser
145 150 155 160

Thr Asp Gly Tyr Ser Leu Ser Gly Thr Ser Pro Leu Ile Lys Tyr Glu
165 170 175

Ser Ile Trp Pro Ile Asp His Ser Met Gly Leu Thr Ser Ser Val Gly
180 185 190

Gly Gln Gln Pro Met Ile Ala Trp Glu Ser Leu Pro Ala Ala Ala Gln
195 200 205

Thr Ala Leu Glu Thr Thr Asp Phe Gly Ala Ala Asn Val Pro Phe Ile
210 215 220

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Pro Ser Ala Phe
225

<210> 48
<211> 228
<212> PRT
<213> unknown

<220>
<223> B. byssoidae NEP2

<220>
<221> signal peptide
<222> (1)..(14)
<223> part

<400> 48

Leu Gln Leu Ser Leu Ser Val Leu Ala Ser Thr Val Val Ala Ile Pro
1 5 10 15

Thr Pro Ser Gln Leu Glu Ser Arg Ala Val Ile Asp Ser Asp Ala Val
20 25 30

Val Gly Phe Pro Glu Thr Val Pro Ser Gly Ser Val Gly Thr Val Tyr
35 40 45

Glu Thr Tyr Gln Pro Tyr Leu Lys Ile Val Asn Gly Cys Val Pro Phe
50 55 60

Pro Ala Val Asp Ala Ser Gly Asn Thr Gly Gly Gly Leu Ala Pro Thr
65 70 75 80

Gly Ser Ser Asn Gly Met Cys Ser Ser Ser Thr Gly Gln Val Tyr Val
85 90 95

Arg Gly Ala Gln Ser Gly Ser Tyr Tyr Gly Ile Met Tyr Ser Trp Tyr
100 105 110

Met Pro Lys Asp Glu Pro Ser Thr Gly Ile Gly His Arg His Asp Trp
115 120 125

Glu Gly Val Ile Val Trp Leu Ser Ser Ser Thr Ala Thr Thr Ala Ser

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130 135 140

Asn Ile Val Ala Val Cys Pro Ser Ala His Gly Gly Trp Asp Cys Ser
 145 150 155 160

Thr Asp Gly Tyr Ser Leu Ser Gly Thr Ser Pro Leu Ile Lys Tyr Glu
 165 170 175

Ser Ile Trp Pro Ile Asp His Ser Met Gly Leu Thr Ser Ser Val Gly
 180 185 190

Gly Gln Gln Pro Met Ile Ala Trp Glu Ser Leu Pro Ala Ala Ala Gln
 195 200 205

Thr Ala Leu Glu Thr Thr Asp Phe Gly Ala Ala Asn Val Pro Phe Ile
 210 215 220

Pro Ser Ala Phe
 225

<210> 49
 <211> 228
 <212> PRT
 <213> unknown

<220>
 <223> B. squamosa NEP2

<220>
 <221> signal peptide
 <222> (1)..(14)
 <223> part

<400> 49

Leu Gln Leu Ser Leu Ser Val Leu Ala Ser Thr Val Val Ala Ile Pro
 1 5 10 15

Thr Pro Ser Gln Leu Glu Ser Arg Ala Val Ile Asn Ser Asp Ala Val
 20 25 30

Val Gly Phe Pro Glu Thr Val Pro Ser Gly Thr Val Gly Thr Val Tyr
 35 40 45

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Glu Ala Tyr Gln Pro Tyr Leu Lys Ile Val Asn Gly Cys Val Pro Phe
50 55 60

Pro Ala Val Asp Ala Ser Gly Asn Thr Gly Gly Gly Leu Ser Pro Thr
65 70 75 80

Gly Ser Ser Asn Gly Glu Cys Ser Ser Ser Thr Gly Gln Val Tyr Val
85 90 95

Arg Gly Ala Gln Ser Gly Ser Tyr Tyr Gly Ile Met Tyr Ser Trp Tyr
100 105 110

Met Pro Lys Asp Glu Pro Ser Thr Gly Ile Gly His Arg His Asp Trp
115 120 125

Glu Gly Val Ile Val Trp Leu Ser Ser Ser Thr Ala Thr Thr Ala Ser
130 135 140

Asn Ile Val Ala Val Cys Pro Ser Ala His Gly Gly Trp Asp Cys Ser
145 150 155 160

Thr Asp Gly Tyr Ser Leu Ser Gly Thr Ser Pro Leu Ile Lys Tyr Glu
165 170 175

Ser Ile Trp Pro Val Asp His Ser Met Gly Leu Thr Ser Thr Val Gly
180 185 190

Gly Gln Gln Pro Met Ile Ala Trp Glu Ser Leu Pro Ala Ala Ala Gln
195 200 205

Thr Ala Leu Glu Ser Thr Asp Phe Gly Ala Ala Asn Val Pro Phe Ile
210 215 220

Pro Ser Ala Phe
225

<210> 50

<211> 228

<212> PRT

<213> unknown

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<220>

<223> B. ficariarum NEP2

<220>

<221> signal peptide

<222> (1)..(14)

<223> part

<400> 50

Leu Gln Leu Ser Leu Ser Val Met Ala Ser Thr Val Val Ala Ile Pro
1 5 10 15

Thr Pro Ser Gln Leu Glu Ser Arg Ala Val Ile Asn Ser Asp Ala Val
20 25 30

Val Gly Phe Pro Glu Thr Val Pro Ser Gly Thr Val Gly Thr Val Tyr
35 40 45

Glu Thr Tyr Gln Pro Tyr Leu Lys Ile Val Asn Gly Cys Val Pro Phe
50 55 60

Pro Ala Val Asp Ala Ser Gly Asn Thr Gly Gly Gly Leu Ser Pro Thr
65 70 75 80

Gly Ser Ser Asn Gly Gly Cys Ser Ser Ser Thr Gly Gln Val Tyr Val
85 90 95

Arg Gly Ala Gln Ser Gly Ser Tyr Tyr Gly Ile Met Tyr Ser Trp Tyr
100 105 110

Met Pro Lys Asp Glu Pro Ser Thr Gly Ile Gly His Arg His Asp Trp
115 120 125

Glu Gly Val Ile Val Trp Leu Ser Ser Ser Thr Ala Lys Thr Ala Ser
130 135 140

Asn Ile Val Ala Val Cys Pro Ser Ala His Gly Gly Trp Asp Cys Ser
145 150 155 160

Thr Asp Gly Tyr Ser Leu Ser Gly Thr Ser Pro Leu Ile Lys Tyr Glu
165 170 175

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Ser Ile Trp Pro Val Asp His Ser Met Gly Leu Thr Ser Ala Val Gly
180 185 190

Gly Gln Gln Pro Met Ile Ala Trp Glu Ser Leu Pro Thr Ala Ala Gln
195 200 205

Thr Ala Leu Glu Pro Thr Asp Phe Gly Ala Ala Asn Val Pro Phe Ile
210 215 220

Pro Ser Ala Phe
225

<210> 51
<211> 228
<212> PRT
<213> unknown

<220>
<223> B. ranunculi NEP2

<220>
<221> signal peptide
<222> (1)..(14)
<223> part

<400> 51

Leu Gln Leu Ser Leu Ser Val Leu Ala Ser Thr Val Val Ala Ile Pro
1 5 10 15

Thr Pro Ser Gln Leu Glu Ser Arg Ala Val Ile Asn Ser Asp Ala Val
20 25 30

Val Gly Phe Pro Glu Thr Val Pro Ser Gly Thr Val Gly Thr Val Tyr
35 40 45

Glu Thr Tyr Gln Pro Tyr Ile Lys Ile Val Asn Gly Cys Val Pro Phe
50 55 60

Pro Ala Val Asp Ala Ser Gly Asn Thr Ser Gly Gly Leu Ser Pro Thr
65 70 75 80

Gly Ser Ser Ser Gly Gly Cys Ser Ser Ser Thr Gly Gln Val Tyr Val

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85 90 95

Arg Gly Ala Gln Ser Gly Ser Tyr Tyr Gly Ile Met Tyr Ser Trp Tyr
100 105 110

Met Pro Lys Asp Glu Pro Ser Thr Gly Ile Gly His Arg His Asp Trp
115 120 125

Glu Gly Val Ile Val Trp Leu Ser Ser Ser Thr Ala Thr Thr Ala Ser
130 135 140

Asn Ile Val Ala Val Cys Pro Ser Ala His Gly Gly Trp Asp Cys Ser
145 150 155 160

Thr Asp Gly Tyr Ser Leu Ser Gly Thr Ser Pro Leu Ile Lys Tyr Glu
165 170 175

Ser Ile Trp Pro Val Asp His Ser Met Gly Leu Thr Ser Thr Val Gly
180 185 190

Gly Gln Gln Pro Met Ile Ala Trp Glu Ser Leu Pro Thr Ala Ala Gln
195 200 205

Thr Ala Leu Glu Ser Thr Asp Phe Gly Ala Ala Asn Val Pro Phe Ile
210 215 220

Pro Ser Val Phe
225

<210> 52
<211> 227
<212> PRT
<213> unknown

<220>
<223> B. aclada NEP2

<220>
<221> signal peptide
<222> (1)..(13)
<223> part

<400> 52

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Gln Leu Ser Leu Ser Val Leu Ala Ser Thr Val Ile Ala Ile Pro Thr
1 5 10 15

Pro Ser Gln Leu Glu Ser Arg Ala Val Ile Asp Ser Asp Ala Val Val
20 25 30

Gly Phe Pro Glu Thr Val Pro Ser Gly Thr Val Gly Lys Val Tyr Glu
35 40 45

Ala Tyr Lys Pro His Leu Tyr Val Val Asn Gly Cys Val Pro Phe Pro
50 55 60

Ala Val Asp Ala Ser Gly Asn Thr Gly Gly Gly Leu Ser Pro Thr Gly
65 70 75 80

Ser Ser Asn Gly Gly Cys Ser Ser Ser Thr Gly Gln Val Tyr Val Arg
85 90 95

Gly Ala Gln Ser Gly Ser Tyr Tyr Gly Ile Met Tyr Ser Trp Tyr Met
100 105 110

Pro Lys Asp Glu Pro Ser Thr Gly Ile Gly His Arg His Asp Trp Glu
115 120 125

Gly Ala Ile Val Trp Leu Lys Ser Ala Thr Ala Thr Thr Ala Asp Asn
130 135 140

Ile Ala Ala Val Cys Pro Ser Ala His Gly Gly Trp Asp Cys Ser Thr
145 150 155 160

Asp Gly Phe Ser Leu Ser Gly Thr Ser Pro Leu Ile Lys Tyr Glu Ser
165 170 175

Ile Trp Pro Ile Asp His Ser Met Gly Leu Thr Ser Thr Val Gly Gly
180 185 190

Leu Gln Pro Met Ile Ala Trp Glu Ser Leu Pro Ala Ala Ala Gln Thr
195 200 205

Ala Leu Glu Thr Thr Asp Phe Gly Ser Ala Asn Val Asn Phe Ile Pro

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210 215 220

Ser Val Phe
225<210> 53
<211> 244
<212> PRT
<213> unknown<220>
<223> B. fabae NEP2<220>
<221> signal peptide
<222> (1)..(21)

<400> 53

Met Val Ala Phe Ser Lys Ser Leu Gln Leu Ser Leu Ser Val Leu Ala
1 5 10 15Ser Thr Val Ile Ala Ile Pro Thr Pro Ser Gln Leu Glu Ser Arg Ala
20 25 30Val Ile Asp Ser Asp Ala Val Val Gly Phe Ala Glu Thr Val Pro Ser
35 40 45Gly Thr Val Gly Thr Val Tyr Glu Ala Tyr Lys Pro Phe Leu Lys Val
50 55 60Val Asn Gly Cys Val Pro Phe Pro Ala Val Asp Ala Ser Gly Asn Thr
65 70 75 80Gly Gly Gly Leu Ser Pro Thr Gly Ser Ser Asn Gly Gly Cys Ser Ser
85 90 95Ser Thr Gly Gln Val Tyr Val Arg Gly Gly Gln Ser Gly Ser Asn Tyr
100 105 110Ala Ile Met Tyr Ser Trp Tyr Met Pro Lys Asp Glu Pro Ser Thr Gly
115 120 125

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Ile Gly His Arg His Asp Trp Glu Gly Val Ile Val Trp Leu Ser Ser
 130 135 140

Ala Thr Ala Thr Thr Ala Asp Asn Ile Leu Ala Val Cys Pro Ser Ala
 145 150 155 160

His Gly Gly Trp Asp Cys Ser Thr Asp Gly Tyr Ser Leu Ser Gly Thr
 165 170 175

Ser Pro Leu Ile Lys Tyr Glu Ser Ile Trp Pro Val Asp His Ser Met
 180 185 190

Gly Leu Thr Ser Thr Val Gly Gly Lys Gln Pro Leu Ile Ala Trp Glu
 195 200 205

Ser Leu Pro Thr Ala Ala Gln Thr Ala Leu Glu Asn Thr Asp Phe Gly
 210 215 220

Ala Ala Asn Val Pro Phe Ile Pro Ala Val Phe Thr Asp Asn Leu Ala
 225 230 235 240

Lys Ala Thr Phe

<210> 54

<211> 244

<212> PRT

<213> unknown

<220>

<223> B. pelargonii NEP2

<220>

<221> signal peptide

<222> (1)..(21)

<400> 54

Met Val Ala Phe Ser Lys Ser Leu Gln Leu Ser Leu Ser Val Leu Ala
 1 5 10 15

Ser Thr Val Ile Ala Ile Pro Thr Pro Ser Gln Leu Glu Ser Arg Ala
 20 25 30

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Val Ile Asp Ser Asp Ala Val Val Gly Phe Ala Glu Thr Val Pro Ser
35 40 45

Gly Thr Val Gly Thr Val Tyr Glu Ala Tyr Lys Pro Phe Leu Lys Val
50 55 60

Val Asn Gly Cys Val Pro Phe Pro Ala Val Asp Ala Ser Gly Asn Thr
65 70 75 80

Gly Gly Gly Leu Ser Pro Thr Gly Ser Ser Asn Gly Gly Cys Ser Ser
85 90 95

Ser Thr Gly Gln Val Tyr Val Arg Gly Gly Gln Ser Gly Ser Asn Tyr
100 105 110

Ala Ile Met Tyr Ser Trp Tyr Met Pro Lys Asp Glu Pro Ser Thr Gly
115 120 125

Ile Gly His Arg His Asp Trp Glu Gly Val Ile Val Trp Leu Ser Ser
130 135 140

Ala Thr Ala Thr Thr Ala Asp Asn Ile Leu Ala Val Cys Pro Ser Ala
145 150 155 160

His Gly Gly Trp Asp Cys Ser Thr Asp Gly Tyr Ser Leu Ser Gly Thr
165 170 175

Ser Pro Leu Ile Lys Tyr Glu Ser Ile Trp Pro Val Asp His Ser Met
180 185 190

Gly Leu Thr Ser Thr Val Gly Gly Lys Gln Pro Met Ile Ala Trp Glu
195 200 205

Ser Leu Pro Thr Ala Ala Gln Thr Ala Leu Glu Asn Thr Asp Phe Gly
210 215 220

Ala Ala Asn Val Pro Phe Ile Pro Ala Val Phe Thr Asp Asn Leu Ala
225 230 235 240

Lys Ala Thr Phe

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<210> 55
<211> 217
<212> PRT
<213> unknown

<220>
<223> B. calthae NEP2

<220>
<221> signal peptide
<222> (1)..(1)
<223> part

<400> 55

Ala Ile Pro Thr Pro Ser Gln Leu Glu Ser Arg Ala Val Ile Ser Ser
1 5 10 15

Asp Ala Val Val Gly Phe Ala Glu Thr Val Pro Ser Gly Thr Val Gly
20 25 30

Thr Val Tyr Glu Ala Tyr Lys Pro Phe Leu Lys Val Val Asn Gly Cys
35 40 45

Val Pro Phe Pro Ala Val Asp Ala Ser Gly Asn Thr Gly Gly Gly Leu
50 55 60

Ser Pro Thr Gly Ser Thr Asn Gly Gly Cys Ser Ser Ser Thr Gly Gln
65 70 75 80

Val Tyr Val Arg Gly Gly Gln Ser Gly Ser Lys Tyr Ala Ile Met Tyr
85 90 95

Ser Trp Tyr Met Pro Lys Asp Glu Pro Ser Thr Gly Ile Gly His Arg
100 105 110

His Asp Trp Glu Gly Val Ile Val Trp Leu Ser Ser Ala Thr Ala Thr
115 120 125

Thr Ala Asp Asn Ile Leu Ala Val Cys Pro Ser Ala His Gly Gly Trp
130 135 140

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Asp Cys Ser Thr Asp Gly Tyr Ser Leu Ser Gly Thr Arg Pro Leu Ile
145 150 155 160

Lys Tyr Glu Ser Ile Trp Pro Ile Asp His Ser Met Gly Leu Thr Ser
165 170 175

Thr Val Gly Gly Asn Gln Pro Met Ile Ala Trp Glu Ser Leu Pro Thr
180 185 190

Ala Ala Gln Thr Ala Leu Glu Asn Thr Asp Phe Gly Ala Ala Asn Val
195 200 205

Pro Phe Ile Pro Ala Ala Phe Thr Ser
210 215

<210> 56

<211> 244

<212> PRT

<213> unknown

<220>

<223> B. tulipae NEP2

<220>

<221> signal peptide

<222> (1)..(21)

<400> 56

Met Val Ala Phe Ser Arg Ser Leu Gln Leu Ser Leu Ser Val Leu Ala
1 5 10 15

Ser Thr Val Ala Ala Ile Pro Thr Pro Ser Gln Leu Glu Ser Arg Ala
20 25 30

Val Ile Ser His Asp Ala Val Val Gly Phe Pro Glu Thr Val Pro Ser
35 40 45

Gly Thr Val Gly Thr Val Tyr Glu Thr Tyr Gln Pro Tyr Leu Lys Val
50 55 60

Val Asn Gly Cys Val Pro Phe Pro Ala Val Asp Ala Ser Gly Asn Thr
65 70 75 80

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Gly Gly Gly Leu Ser Pro Thr Gly Ser Thr Asn Gly Met Cys Ser Ser
85 90 95

Ser Thr Gly Gln Val Tyr Val Arg Gly Ala Gln Asn Gly Ser Tyr Tyr
100 105 110

Gly Ile Met Tyr Ser Trp Tyr Met Pro Lys Asp Glu Pro Ser Thr Gly
115 120 125

Ile Gly His Arg His Asp Trp Glu Gly Val Ile Val Trp Leu Ser Arg
130 135 140

Ser Thr Ala Thr Thr Ala Ser Asn Ile Val Ala Val Cys Pro Ser Ala
145 150 155 160

His Gly Gly Trp Asp Cys Ser Thr Asp Gly Tyr Ser Leu Ser Gly Thr
165 170 175

Ser Pro Leu Ile Lys Tyr Glu Ser Ile Trp Pro Ile Asp His Ser Met
180 185 190

Gly Leu Thr Ser Thr Val Gly Gly Gln Gln Pro Met Ile Ala Trp Glu
195 200 205

Ser Leu Pro Ala Ala Ala Gln Thr Ala Leu Glu Thr Thr Asp Phe Gly
210 215 220

Ser Ala Asn Val Pro Phe Ile Pro Ser Ser Phe Ala Asn Asn Leu Ala
225 230 235 240

Ala Ala Thr Phe

<210> 57

<211> 244

<212> PRT

<213> unknown

<220>

<223> B. elliptica NEP2

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<220>

<221> signal peptide

<222> (1)..(21)

<400> 57

Met Val Ala Phe Ser Arg Ser Leu Gln Leu Ser Leu Ser Val Leu Ala
1 5 10 15

Ser Thr Val Val Ala Ile Pro Thr Pro Ser Gln Leu Glu Ser Arg Ala
20 25 30

Val Ile Asn Ser Asp Ala Val Val Gly Phe Pro Glu Thr Val Pro Ser
35 40 45

Gly Ile Val Gly Thr Val Tyr Glu Thr Tyr Gln Pro Tyr Leu Lys Ile
50 55 60

Val Asn Gly Cys Val Pro Phe Pro Ala Val Asp Ala Ser Gly Asn Thr
65 70 75 80

Gly Gly Gly Leu Ser Pro Thr Gly Ser Ser Asn Gly Glu Cys Ser Ser
85 90 95

Ser Thr Gly Gln Val Tyr Val Arg Gly Ala Gln Ser Gly Ser Tyr Tyr
100 105 110

Gly Ile Met Tyr Ser Trp Tyr Met Pro Lys Asp Glu Pro Ser Thr Gly
115 120 125

Ile Gly His Arg His Asp Trp Glu Gly Val Ile Val Trp Leu Ser Ser
130 135 140

Ser Thr Ala Thr Thr Ala Ser Asn Ile Val Ala Val Cys Pro Ser Ala
145 150 155 160

His Gly Gly Trp Asp Cys Ser Thr Asp Gly Tyr Ser Leu Ser Gly Thr
165 170 175

Ser Pro Leu Ile Lys Tyr Glu Ser Ile Trp Pro Ile Asp His Ser Met
180 185 190

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Gly Leu Thr Ser Thr Val Gly Gly Gln Gln Pro Met Ile Ala Trp Glu
 195 200 205

Ser Leu Pro Ala Ala Ala Gln Thr Ala Leu Glu Ser Thr Asp Phe Gly
 210 215 220

Ala Ala Asn Val Pro Phe Ile Pro Ser Ala Phe Ala Asn Asn Leu Ala
 225 230 235 240

Ala Ala Thr Phe

<210> 58

<211> 741

<212> DNA

<213> unknown

<220>

<223> B. fabae NEP1 cDNA

<400> 58

atgcatttct ccaacgcaaa attcctttct atcctcgtcg ccgctgcagc agtcaagggt 60
 gtcctaattg aggagagcac cattcaagct cgcgccgtcg ttctcatga ctccatcaac 120
 ccatggggag aaaacgtacc tggtaacgcc ttgggtaaca cctgaagag attcgagcca 180
 tatcttcaca ttgctcatgg ttgtcaaccg tacagtgccg ttgatggtaa tggtaacacc 240
 agtgggtggac tccaagatac tggcaatgtc tcagccggct gccgtgatca gagcaagggc 300
 cagacctatg tccgaggtgg ctggtctggg ggtcgctatg gaatcatgta cgcctggtac 360
 ttcccaagg atcaaccagc cgccggaaac gtcgccggag gtcaccgtca cgactgggag 420
 tacgtcatcg ctggggttaa caaccccgag gttgccaacc ccacctaat cggcgccggc 480
 gcatccggtc acggaagcat caagtgacc aaaaaccccc aacgccaggc cgacagattg 540
 aaggtcgaat actttgtctc ctccaacc aatcacgagt tgcagtttac cgacacctg 600
 ggcagagatt tgccaatgat gtggtatgac ttcttgccag ctgtagcaa ggcagcttta 660
 caaacacca gtttggaag ggcaaaactgc ccattcaatg acgcaaactt caacaacaac 720
 ctcgccaagg caaagatcta a 741

<210> 59

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<211> 741

<212> DNA

<213> unknown

<220>

<223> B. pelargonii NEP1 cDNA

<400> 59

atgcattct ccaacgcaaa attccttct atcctcgctg ccgctgcagc agtcaagggt 60

gctccaattg aggagagcac cattcaagct cgcgccgtcg ttctcatga ctccatcaac 120

ccatggggag aaaacgtacc tggtaacgcc ttgggtaaca ccttgaagag attcgagcca 180

tatcttcaca ttgctcatgg ttgtcaacca tacagtccg ttgatggtaa tggtaacgcc 240

agtgggtggac tccaagatac tggcaatgtc tcagccggct gccgtgatca gagcaagggc 300

caaacctatg tccgaggtgg ctggtctgga ggtcgctatg gaatcatgta cgcctgttac 360

ttcccaagg atcaaccagc cgctggaaac gtcgtcggag gtcaccgtca cgactgggag 420

tatgtcgtcg ctgggtcaa caaccccgag gttgccaacc ccacctaata cggcgccggc 480

gcacccgtc acggaagcat caagaagacc acaaaccccc aacgccaggg cgacagattg 540

aaggtcgaat actatgtctc ctcccaacc aaccacgagt tgcagttcac caacacctg 600

ggcagagatt tgccaatgat gtggtatgac ttcttgccag ctgttagcaa gacagcttta 660

caaaacacca actttggaaa ggcaactgc ccattcaatg acgcaactt caacaacaac 720

ctcgccaagg caagaatcta a 741

<210> 60

<211> 671

<212> DNA

<213> unknown

<220>

<223> B. calthae NEP1 cDNA

<400> 60

tcgctgccgc tgcagcagtc aagggtgctc caattgagga gaacaccatt caagctcgcg 60

ccgtcgttcc tcattgactcc atcaacccat ggggagaaaa cgtacctggt aacgccttgg 120

gtaacacctt gaagaaattc gagccataac ttacattgc tcattggtgt caaccataca 180

gtgccgttga tggttatggt aacaccagtg gtgggtcca agatactggc aatatctcag 240

ccggtgcccg tgatcagagc aagggccaaa cctatgtccg aggtggctgg tctggaggtc 300

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gctatggaat catgtacgcc tggatttcc ccaaggatca accagctgcc ggaaacgtca 360
 tcggaggtca tcgtcacgac tgggagtacg tcgtgcctg ggtcaacaac ccctcggttg 420
 ccaacccac cttaatggc gccggtgcat ccggccacgg aagcatcaag aagaccacaa 480
 atccccaacg ccaggcgac agattgaagg tcgaatacta tgtctcctc ccaactaacc 540
 acgagtgca attaccaac agcttgggca gagatttgc aatgatgtg tatgacttct 600
 tgccagctgt tagcaagaca gctttacaaa acaccagctt tggaaggca aactgccc 660
 tcaatgacgc a 671

<210> 61
 <211> 640
 <212> DNA
 <213> unknown

<220>
 <223> B. paeoniae NEP1 cDNA

<400> 61
 caagggtgct ccattgagg agagcaccat ccaagctgc gctacgtcc ctcacgattc 60
 gatcaacca tggggagaaa acgtacctgg taacgccgtg ggtaacacct tgaagagatt 120
 tgagccattc attcgattg ctcatggtg ccaaccatac agtgcaattg atgtaattg 180
 taacaccagt ggtggactcc aagatactgg caatgtctca gccggctgcg gtgatctaag 240
 caaaggtaa acctatgtcc gagctggctg gtctggaggt cgctatggtc tcatgtacgc 300
 ctggtatttc cccaaggatc aaccgcac cggaacgtc gttggaggtc atcgtcacga 360
 ctgggagcac gtcgtcgtc tggtaacaa ccccgccctc cccaaccca ccttaatcgg 420
 tgccgccga tctggccacg gaagtctca gaaaacgaca aatcccaac gccagggcga 480
 tagagtgaag gtcgaatact atgtctctt ccaaccaac cagagttgc agttaccga 540
 caccttgggc agagatttgc caatgatgtg gtatgattc ttgccagctg ccagcaagac 600
 agctttacag aacaccagct tcggaaggc cgtctgtcca 640

<210> 62
 <211> 621
 <212> DNA
 <213> unknown

<220>
 <223> B. convoluta NEP1 cDNA

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<400> 62

gagagcacia tccaagctcg cgccgacgtt cctcatgact ccatcaaccc atggggagaa 60
 aacgtacctg gtaacgcctt gggtaacacc ttgaagaaat tcgagccatt cattcacatt 120
 gctcatgggt gtcaaccata cagtgcagtt gatggtaatg gtaacaccag tgggggactc 180
 caagatactg gcaatgtctc agccggctgc cgtgatcaga gcaaaggta aacatatgtc 240
 cgtgggtggt ggtctggagg tcgctatggt atcatgtacg ctgggtattt cccaaggat 300
 caaccgccc cggaacgt cgtcggaggt catcgtcacg actgggagca cgtcgtcgtc 360
 tgggtcaaca accctccgt tgccaacccc acctgatcg gtccgcccgc atctggtcac 420
 ggaggtctca agaaacgac aaatcccaa cgccagggcg atagattgaa ggtcgaatac 480
 tatgtcactt tccaaccaa ccacgagttg cagttacca acacctggg cagagatttg 540
 ccaatgatgt ggtacgactt ctgccagct gttagcaaga cagctttaca gaaccaaac 600
 ttcgaaagg cgatctgtcc a 621

<210> 63

<211> 659

<212> DNA

<213> unknown

<220>

<223> B. polyblastis NEP1 cDNA

<400> 63

tcgtgccgc tgcagcagtc aagggtgctc ccgttgagga gaacaccatc caggctcgtg 60
 ccgtcattgg tcatgactcc atcaacccat ggccagaaaa cgtacctggt aacgccgtgg 120
 gtaacacttt gaagaaattc gagccattca ttacattgc tcatggttgt caaccataca 180
 gtgcagttga tggttatggt aacaccagtg gtggactcca agatactgga aatgtctcag 240
 ccggctgccg tgatcagagc aaaggtaaaa cctatgtccg aggtggctgg tctggaggtc 300
 gctatggtat catgtactcc tggattttcc ccaaggatca accaatagct ggaaacgtcg 360
 ctggaggcca tcgtcagcagc tgggagttcg tcgtcgtctg ggtcaacaac cctgccgtcg 420
 ctaacccac ctgatcgggt gccgccgcat ctctcacgg aaatttgagg aagacgacca 480
 atcccaacg ccagggtaat agagtgaagg tcgaatactt tacctcttc ccaaccaacc 540
 acgagtgca gttcaccgac acattgggca gagatttgcc aatgatgtgg tacgacttct 600

P6000092.ST251

tgccgggtgt tagcaagaca gctttacaga acaccgactt cggaagcgcc aactgtcct 659

<210> 64

<211> 640

<212> DNA

<213> unknown

<220>

<223> B. gladiolorum NEP1 cDNA

<400> 64

caagggtgct cccattgaag agaacacat ccaagctcgt gccgtgttc ctcatgactc 60

catcaacca tggggagaaa acgtacctgg taacgccttg ggtaacactt tgaaaaaatt 120

cgagccattc cttcacattg ctcatggttg tcaaccatac agtgcagttg atggtaatgg 180

taataccagt ggtggactcc aagatactgg aatgtctca gccggctgcc atgatcagag 240

caagggtcaa acctatgtcc gaggtggctg gtctggaggt cgctatggta tcatgtacgc 300

ctggtatttc cccaaggatc aaccagcagc tggaaacctc gttggaggcc atcgtcacga 360

ctgggagtgc gtcgttcct gggtaacaa cccttcctgc gtaaccctt ccttgatcgg 420

tgccgccgca tctggtcacg gaagtgtgaa gaagacgacc aatccccaac gccagggtga 480

tagattgaag gtcgaatact atgtctctt cccaaccaac cagcagttgc agttcaccaa 540

cacattgggc agagatttgc caatgatgtg gtacgacttc ttgcccgctg ttagcaagac 600

agctttacag aacaccaact tcggaaaggc caactgtcct 640

<210> 65

<211> 383

<212> DNA

<213> unknown

<220>

<223> B. sphaerosperma NEP1 cDNA

<400> 65

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ccgtcgttcc tcatgactcc atcaacccat ggggagaaaa cgtacctggt aacgccttgg 120

gtaacacttt gaagaaatc gagccattcc ttcacatcgc tcttggttgt caaccataca 180

gtgcagttga tgtaatggt aacaccagtg gtggactcca agatactgga aatgtctcaa 240

acggctaccg tgatcagagc aagggtcgaa cctatgtccg aggtggctgg tctggaggtc 300

P6000092.ST251

gctatggtat catgtacgcc tggatttcc ccaaggatca accagcagct ggaaacgtcg 360

ttggaggcca tcgctacgac tga 383

<210> 66

<211> 383

<212> DNA

<213> unknown

<220>

<223> B. globosa NEP1 cDNA

<400> 66

tcgctgccgc tgcagcagtc aaagggtgctc ccattgagga gaacacaatc caagctcgtg 60

ccgtcgttcc tcattgactcc atcaacccat ggggagaaaa cgtacctggt aacgccttgg 120

gtaaaccttt gaagaaatc gagccattcc ttcacatgc tcattggtgt caaccataca 180

gtgcagttga tggtaatggt aacaccagtg gtggactcca agatactgga aatgtctcaa 240

ccggctaccg tgatcagagc aagggtcgaa cctatgtccg aggtggctcg tctggaggtc 300

gctatggtat catgtacgcc tggatttcc ccaaggatca accagcagct ggaaacgtcg 360

ttggaggcca tcgctacgac tga 383

<210> 67

<211> 659

<212> DNA

<213> unknown

<220>

<223> B. byssoidae NEP1 cDNA

<400> 67

tcgctgccgc tgcagcagtc aagggtgctc ccattgagga gaacaccatc caagctcgtg 60

ccgtcgttcc tcattgactcc atcaacccat ggggagaaaa cgtacctggt aacgccttgg 120

gtaaaccttt gaagaaatc gagccattcc ttcatttgc tcattggtgt caaccataca 180

gtgcagttga tggtaatggt aacaccagtg gtggactcca agatactgga aatgtctcag 240

ccggctgccg tgatcagagc aagggtcaaa cctatgtccg aggtggctgg tctggaggtc 300

gctatggtat catgtacgcg tggatttcc ccaaggatca accagcagct ggaaacgtcg 360

ttggaggcca tcgtacgac tgggagtac tcgtgcctg ggtcaacaac cttccgtcg 420

ccaaccctc cttgatcggg gccgccgcat ctggtcacgg aagtgtgaag aagacgacca 480

P6000092.ST251

atccccaacg ccagggtgat agattgaagg tcgaatacta tgtctctttc ccaaccaacc 540
 acgagttgca gttcaccaac acattgggca gagatttgcc aatgatgtgg tacgacttct 600
 tgccagctgt tagcaagaca gctttacaga acaccaactt cggaagagcc aactgtcct 659

<210> 68

<211> 632

<212> DNA

<213> unknown

<220>

<223> B. narcissicola NEP1 cDNA

<400> 68

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 catggggaga aaacgtacct ggtaacgctt tgggtaacac ttgaagaaa ttgagccat 120
 tccttcacat tgctcatggt tgtcaacct acagtgcagt tgatggttat ggtaacacca 180
 gtggtggact ccaagatact ggaaatgtct cagccgactg ccgtgatcag agcaagggcc 240
 aaacctatgt ccgaggtggc tggctggag gtcgctatgg tatcatgtac gcctgttatt 300
 tcccaagga tcaaccagca gctggaaacg tcattggagg ccatcgtcac gactgggagt 360
 acgtcgtcgc ctgggtcaac aacccttccg tcgctaacc ctccttgatc ggtgccgccg 420
 catctggta cggaggtgtg aagaagacga ccaatccca acgccagggt gatagattga 480
 aggtcgaata ctatgtctct ttccaacca accacgagtt gcagttcacc aacacattag 540
 gcagagattt gccaatgatg tggtagact tcttgccagc tgtagcaag acagctttac 600
 agaacaccag cttcggaag gccaaactgc ct 632

<210> 69

<211> 640

<212> DNA

<213> unknown

<220>

<223> B. croci NEP1 cDNA

<400> 69

caagggtgct ccattgagg agaacacat ccaagctcgt gccgtcgtc ctcatgactc 60
 cattaacca tggggagaaa acgtacctgg taacgccttg ggtaacacct tgaagagatt 120
 cgagccattc cttcacattg ctcatggtg tcaaccatac agtcagtcg atggtaatgg 180

P6000092.ST251

taacactagt ggtggactcc aagatactgg aaatgtctca gccggctgtc gtgatcagag 240
 caaagggtcaa acctacgtcc gaggtggctg gtctggaggt cgctatggta tcatgtacgc 300
 ctggtatttc cccaaggatc aaccagcagc cggaaacgtt gttggaggcc atcgtcacga 360
 ctgggagcac atcgtcgtct gggtaacaa ccctgaagtc ggcaaccca ccttgatcgg 420
 tgccgccgca tctggtcacg gaagtgtgaa gaagacgacc aacccccaac gccagggtga 480
 tagattgaag gtcgaatact atgtctcttt ccctaccaac cacgagttgc agttcaccaa 540
 cacattgggc agagatttgc caatgatgtg gtatgacttc ttgccagctg ttagcaagac 600
 agctttacag aacaccagct tcggaagggc caactgtcct 640

<210> 70

<211> 640

<212> DNA

<213> unknown

<220>

<223> B. hyacinthi NEP1 cDNA

<400> 70

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 cattaacca tggggagaaa acgtacctgg taacgccttg ggtaacacct tgaagagatt 120
 cgagccattc cttcacattg ctcattggtg tcaaccatac agtgcagtcg atggtaatgg 180
 taacactagt ggtggactcc aagatactgg aaatgtctca gccggctgtc gtgatcagag 240
 caaagggtcaa acctacgtcc gaggtggctg gtctggaggt cgctatggta tcatgtacgc 300
 ctggtatttc cccaaggatc aaccagcagc cggaaacgtt gttggaggcc atcgtcacga 360
 ctgggagcac atcgtcgtct gggtaacaa ccctgaagtc ggcaaccca ccttgatcgg 420
 tgccgccgca tctggtcacg gaagtgtgaa gaagacgacc aacccccaac gccagggtga 480
 tagattgaag gtcgaatact atgtctcttt ccctaccaac cacgagttgc agttcaccaa 540
 cacattgggc agagatttgc caatgatgtg gtatgacttc ttgccagctg ttagcaagac 600
 agctttacag aacaccagct tcggaagggc caactgtcct 640

<210> 71

<211> 741

<212> DNA

<213> unknown

P6000092.ST251

<220>

<223> B. elliptica NEP1 cDNA

<400> 71

atgtacttct ccaacgcaaa attcctttct atcctcgtc cgcgtgcagc agtcaagggt 60
 gtcgccattg aggagaacac tattcaagct cgtgccgtcg ttaatcatga ctctatcaac 120
 ccatggggag aaaacgtacc tggtaacgcc ttgggtaaca cttgaagag attcgagcca 180
 ttcttcaca ttgctcatgg ttgtcaacca tacagtgcgg ttgatggta tggaataacc 240
 agtggtggac tccaagatac tggcaatgtc tcagccggtt gccgtgatca gagcaagggt 300
 caaacctatg tcagagggtg ctggtctgga ggtcgctatg gtatcatgta cgcctggat 360
 ttcccaagg atcaaccagc ggctggaaac gttgttgag gccatcgta cgactgggag 420
 cacatcgctg tctgggtcaa caaccctcc gtcgccaacc ccacctgat cggtgccgcc 480
 gcatctggtc acggaagtgt gaagaagacg accaatcccc aacgccaggg ttagatattg 540
 aaggtcgaat actatgtctc ttccaacc aaccacgagt tgcaattcac caacacattg 600
 ggagagatt tgcaatggt gtgtacgac ttctgccag ctgttagca gacagctta 660
 cagaacacca gcttcgaaa agccaactgt ctttcaatg accacaactt cgccaacaac 720
 ctcgccaagg ctgcaatcta g 741

<210> 72

<211> 640

<212> DNA

<213> unknown

<220>

<223> B. squamosa NEP1 cDNA

<400> 72

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 tatcaacca tggggagaaa acgtacctgg taacgccttg ggtaaacctt tgaagagatt 120
 cgagccattc cttcacattg ctcatggtg tcaaccatac agtcggttg atggttatgg 180
 aaataccagt ggtggtctcc aagatactgg caatgtctca gccggctgcc gtgatcagag 240
 caagggtcaa acctatgtca gaggtggctg gtctggaggt cgctatgta tcatgtacgc 300
 ctggtatttc cccaaggatc aaccagcggc tggaaacgtc gttggaggcc atcgtcacga 360
 ctgggagcac atcgtcgtct gggtaacaa ccttccgtc gccaaacca cttgatcgg 420

P6000092.ST251

tgccgccga tctgtcacg gaagtgtgaa gaagacgacc aatccccaac gacagggtga 480
tagattgaag gtogaatact atgtctctt cccaaccaac cagagttgc agttcaccaa 540
cacattgggc agagatttgc caatgatgtg gtacgacttc ttgccagctg ttagcaagac 600
agctttacag aacaccagct tcggaaaggc caactgtcct 640

<210> 73
<211> 635
<212> DNA
<213> unknown

<220>
<223> B. ranunculi NEP1 cDNA

<400> 73
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acctatgggg agaaaacgta cctggtaacg ccttgggtaa cactttgaag aaattcgagc 120
cattccttca cattgctcat ggttgtaac catacagtgc agttgatggt aatggaaata 180
ccagtgttgg actccaagat actggcaatg tctcagccgg ttgccgtgat cagagcaagg 240
gtcaaaccta tgcagaggt ggctggctg gaggtcgcta tggatcatg tacgcctggt 300
atttcccaa ggaatcaaca gcggctggaa acgtcgttgg aggccatgt cagactggg 360
agcacatcgt agtttgggtc aacaacccta acgtcggcaa cccaccttg atcggtgccg 420
ccgcctctgg tcacggaagt gtgaagaaga cgaccaatcc ccaacgccag ggtgatagat 480
tgaaggtcga atactatgtc tcttcccaa caaacacga gttgcagttc accaacacat 540
tgggcagaga ttgccaatg atgtggtacg acttcttggc agctgttagc aagacagctt 600
tacagaacac cagcttcgga aaggccaact gtcct 635

<210> 74
<211> 640
<212> DNA
<213> unknown

<220>
<223> B. ficariarum NEP1 cDNA

<400> 74
caagggtgct ccattgagg agaaccat ccaagctcgt gccgtcgta atcatgactc 60
tatcaacca tggggagaaa acgtacctg taacgccttg ggtaaacctt tgaagagatt 120

P6000092.ST251

cgagccattc cttcacattg ctcatggtg tcaaccatac agtgcagttg atggtaatgg 180
 aaataccagt ggtggactcc aagatactgg caatgtctca gccggctgcc gtgatcagag 240
 caagggtcaa acctatgtcc gaggtggctg gtctggaggt cgctatggtg tcatgtacgc 300
 ctggtatttc cccaaggatc aaccagcggc tggaaacgtc gttggaggcc atcgtcacga 360
 ctgggagcac atcgtgtttt gggtaacaa ccctaactg ggcaacccca cctgatcgg 420
 tgccgccgca tctggtcacg gaagtgtgaa gaagacgacc aatccccaac gccagggtga 480
 tagattgaag gtcgaatact atgtctctt cccaaccaac cacgagttgc agttcacaa 540
 cacattgggc agagatttgc caatgatgtg gtacgacttc ttgccagctg ttagcaagac 600
 agctttacag aacaccagct tcggaaaggc caactgtcct 640

<210> 75

<211> 640

<212> DNA

<213> unknown

<220>

<223> B. galanthina NEP1 cDNA

<400> 75

caagggtgct ccattgagg agaacacat tcaagctcgt gccgacattg atcatgactc 60
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 cgagccattc cttcacattg ctcatggtg tcaaccatac agtgcagttg atggttatgg 180
 taacaccagt ggtggtctcc aagatactgg aaatgtctca gccagctgcc gtgatcagag 240
 caagggtcaa acctatgtcc gaggtggctg gtccggaggt cgctatggtg tcatgtacgc 300
 ctggtatttc cccaaggatc agccaacagc cggaaatgt atcgagggcc atcgtcacga 360
 ctgggaatac atcgtcgtct gggtaacaa ccctgaagtc ggcaacccca cctgatcgg 420
 tgccgccgca tctggtcacg gaagtgtgaa gaagacgacc aatccccaac gccagggtga 480
 tagattgaag gtcgaatact atgtctctt cccaaccaac cacgagttgc agttcacga 540
 cacattgggc agagatttgc caatgatgtg gtacgacttc ttgccagctg ttagcaagac 600
 agctttacag aacaccagct tcggaaaggc caactgtcct 640

<210> 76

<211> 659

<212> DNA

P6000092.ST251

<213> unknown

<220>

<223> B. aclada NEP1 cDNA

<400> 76

tcgctgccgc tgcagcagtc aagggtgctc caattgagga gaacaccatc caagcacgcg 60

ccgctcgttc tcattgctg atcaacccat ggggagagaa cgtacctggc aacgccttgg 120

gtaacacttt gaagaaattc gagccattcc ttcacattgc ccatggttgt caaccataca 180

gtgcagttga tggtaattgt aacaccagtg gtggactcca agatactgga aatgtctcag 240

ccggctgccg tgatcaggcc aagggtcaaa cctatgtcag aggtggctgg tctggaggtc 300

gctatggtat catgtacgcc tggatttccc ccaaggatca accagcagcc ggaaacgtcg 360

ttggaggcca cgtcacgac tgggagcaca tcgtcgtctg ggtcaacaac ccctctatcg 420

ctaaccacac ttaatcggg gccgccgcat ctggtcacgg aagtgtcaag aagacaacaa 480

acccccaacg ccaggcgac agattgaagg tcgaatacta tgtctcttc ccaaccaacc 540

acgagttgca gttaccaat accctgggca gagattgcc aatgatgtgg tatgactct 600

tgccagctgt tagcaagaca gctttacaga acaccagctt tggaaggcc aattgtcca 659

<210> 77

<211> 671

<212> DNA

<213> unknown

<220>

<223> B. porri NEP1 cDNA

<400> 77

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cggctcgttc tcacgattca atcaacccat gccagaaaa cgttcctggt aacgccttgg 120

gtaacacttt gaagaaattc gagccattcc tccacattgc tcattggtgt caaccataca 180

gtgcagttga tggatacggg aacaccagtg gtggactcca agactcaggt agcccttcag 240

gcggttgccg tgatcagagc aagggccaaa cctatgtccg aggtggctgg tctggaggtc 300

gctatggtat catgtacgcc tggacttccc cttaaagatca gccatcccc ggaaacgtcg 360

ttggaggcca tcgtcacgac tgggagcacg ttgtcgtctg ggttaacaac cctccgtcg 420

ccaaccctgt cttaatcggc gccggtgcat ccggtcacgg aaagatgaag aaaaccacaa 480

P6000092.ST251

accccaacg ccagggcgat agagtcaagg tggaatactt caccacttc cctaccaacc 540
 acgagttgca gttcacgat actttgggca gagatttacc aatgatgtgg tatgacttct 600
 tgccagctgc tagcaagact gctttacaga acaccgatti tggaaaggct gtctgtccat 660
 tgaatgatgc a 671

<210> 78
 <211> 723
 <212> DNA
 <213> unknown

<220>
 <223> B. tulipae NEP1 cDNA

<400> 78
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 accatccaag ctcgtgccgt cgttgctcat gactccatca acccatggcc agaaaacgtc 120
 cctggtaacg ccataggtaa cactttgagg aaattcgagc cgttcattca cattgctcat 180
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 aacaaccctg ccgtccctga ccccgcttg atcgggtgcc cgcacatctg tcacggaagt 480
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 tcggccaact gtccttcaa tgaccacaac ttcgccaaca acctcgccaa ggctcaaata 720
 tag 723

<210> 79
 <211> 687
 <212> DNA
 <213> unknown

<220>
 <223> B. convoluta NEP2 cDNA

<400> 79

P6000092.ST251

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 agcttgagtc tcgggccgtt atcaattccg atgccgttgt aggatttccc gaaactgttc 120
 ctacggggac agtaggaaca gtttatgaga catatcaacc atatctttat gtagtcaatg 180
 gatgtgtacc attccctgca gtcgatgcat cgggtaatac aggtggtggc ttggcaccaa 240
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 ccacaaccgc cgacaatc gttgccgtt gtccttcgc ccatggaggc tgggattgtt 480
 ccaccgatgg ttatccctt tctggtacca gcccttcat caagtacgaa agtatctggc 540
 ctatcgatca ttcaatgggt ctactagta ctgttggtgg acaacaacct atgattgctt 600
 gggaatcttt acctaccgct gctcaaactg ctctgagac gactgattc ggctctgca 660
 atgttcctt cattccatct gccttcg 687

<210> 80

<211> 687

<212> DNA

<213> unknown

<220>

<223> B. porri NEP2 cDNA

<400> 80

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 ctacggggac agtaggaaca gtttatgaga catatcaacc atatcttaag atagtcaatg 180
 gatgtgtacc attccctgca gtcgatgcat cgggtaaacac aggtggtggt ttggcaccaa 240
 ctggcagtag caacgggtgat tgcagcagta gtaccggaca agtatacgtc cgaggagcac 300
 aaagcggatc atactacggt atcatgtact cctggtacat gccaaaagac gacccctcaa 360
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 gcacaaccgc cgacaatc gtcgccgtgt gtccttcgc ccacggagac tgggattgtt 480
 ccaccgatga atttccctt tctggtacca gtccttcat ccaatacgaa agtatctggc 540
 cgatcgatca ttcaatgggt ctactagta ctgttggtggg acaacaacct atgattgcgt 600

P6000092.ST251

gggaatcgtt acctactgct gctcaaactg ctcttgagac gacggatttt gggtcggcga 660

atgttccttt cattccatcg gcttttg

687

<210> 81

<211> 687

<212> DNA

<213> unknown

<220>

<223> B. paeoniae NEP2 cDNA

<400> 81

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agcttgagtc tcgggccgtt atcagttccg atgccgttgt aggattccc gaaactgttc 120

ctagcgggac agtaggaaca gtctatgaga catatcaacc atatcttgat gtagtcaatg 180

gatgcgtacc attccctgcc gtcgatgcat cgggtaacac ggggtggtgt ttggcaccaa 240

ctggcagtag caacgggtgt tgcagcagta gtactggta agtatactgc cgaggagcac 300

aaagcggatc atactacggt atcatgtact cctggtacat gccaaaagac gagccctcaa 360

ccggtattgg tcaccgtcac gactgggaag gtgtaatcgt ctggtcttcc agctcaaccg 420

ccacaaccgc cgacaacatc gtagccgtgt gtccttcgc ccatggaggc tgggattgtt 480

ccaccgatgg ttattccctt tctggtacca gtccttcat caagtacgaa agtatctggc 540

ctatcgatca ctcaatgggt cttactagta ctgttggtgg acaacaacct atgattgctt 600

gggaatcttt acctactgct gctcaaactg ctcttgagac gactgatttt gggtctgcga 660

atgttcatt cattccatct gttttcg

687

<210> 82

<211> 687

<212> DNA

<213> unknown

<220>

<223> B. croci NEP2 cDNA

<400> 82

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ccagcgggac agtgggaaca gtttatgaga catatcaacc atatcttaag atagtcaatg 180

P6000092.ST251

gttgcgtacc attccccgca gtcgatgcat cgggtaacac aggtggtggc ttggcaccaa 240
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 ctatcaatca ttcaatgggt ctactagca ctgttggtgg acaacaaccc atgattgctt 600
 gggaatcttt acctgctgct gctcaaactg ctcttgagac gaccgatttc ggctctgcga 660
 atgttccttt cattccatct gctttcg 687

<210> 83

<211> 687

<212> DNA

<213> unknown

<220>

<223> B. hyacinthi NEP2 cDNA

<400> 83

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 ccagcgggac agtgggaaca gtttatgaga catatcaacc atatcttaag atagtcaatg 180
 gttgcgtacc attccccgca gtcgatgcat cgggtaacac aggtggtggc ttggcaccaa 240
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 ctatcaatca ttcaatgggt ctactagca ctgttggtgg acaacaaccc atgattgctt 600
 gggaatcttt acctgctgct gctcaaactg ctcttgagac gaccgatttc ggctctgcga 660
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<210> 84

P6000092.ST251

<211> 687

<212> DNA

<213> unknown

<220>

<223> B. galanthina NEP2 cDNA

<400> 84

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ccagcgggaa agtaggaaca gtgtatgaga catatcaacc atatcttaag atagtcaatg 180
gttgcgtacc attccctgca gtcgatgcat cgggtaacac aggtgggtgt ttggcaccaa 240
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ccacaaccgc cagcaacatc gtagccgtct gtcctccgc ccatggaggc tgggattgtt 480
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ctatcgatca ttaaatgggt ctactagta ctgttggtgg acaacaacct atgattgctt 600
gggaatcttt acctctgct gctcaaactg ctcttgagac gaccgatttc ggcgctgcga 660
atgttccttt cattccatct gctttcg 687

<210> 85

<211> 687

<212> DNA

<213> unknown

<220>

<223> B. sphaerosperma NEP2 cDNA

<400> 85

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ccagcgggaa agtaggaaca gtttatgaga catatcaacc atatcttaag atagtcaatg 180
gatgtgtacc attccctgca gttgacgcag cgggcaacac aggtgggtggc ttggcaccaa 240
ctggcagtag caatggtag tgcagcagta gtaccggtca agtatacgtc cgaggagcac 300
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P6000092.ST251

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 gggaatcttt acctgctgct gctcaaactg ctcttgagac gaccgatttc ggcgctgcga 660
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<210> 86

<211> 687

<212> DNA

<213> unknown

<220>

<223> B. globosa NEP2 cDNA

<400> 86

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 ccagcggaac agtaggaaca gtttatgaga catatcaacc atatcttaag atagtcaatg 180
 gatgtgtacc attccctgca gttgacgcag cgggcaacac aggtggtggc ttggcaccaa 240
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 aaagcggatc atactacggt atcatgtact cctggtacat gccaaaagat gagccctcga 360
 ccggtattgg tcaccgtcac gactgggaag gtgtaatcgt ctggctctcc agctcagccg 420
 ccacaactgc cagcaacatt gtagccgtct gtccttccgc ccatggaggc tgggattgtt 480
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 ctatcgatca ttcaatgggg ctactagt ttccgttggtg acaacaacct atgattgctt 600
 gggaatcttt acccgctgct gctcaaactg ctcttgagac gaccgatttc ggcgctgcga 660
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<210> 87

<211> 687

<212> DNA

<213> unknown

<220>

<223> B. gladiolorum NEP2 cDNA

P6000092.ST251

<400> 87

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 ccagcgggaa agtaggaaca gtttatgaga catatcaacc atatcttaag atagtcaatg 180
 gatgtgtacc attccctgca gttgacgcag cgggcaacac aggtggcggt ttggcaccaa 240
 ctggcagtag caatgggatg tgcagtagta gtaccgggtca agtatacgtc cgaggagcac 300
 aaagcggatc atactacggt atcatgtact cctggtacat gccaaaagat gagccctcga 360
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 ccacaaccgc cagcaacatc gtagccgtct gtcctccgc ccatggaggc tgggattgtt 480
 ccaccgatgg ttattccctt tctggtacca gccccttat caagtacgaa agcatctggc 540
 ctatcgatca ttccatgggt ctactagtgt ctgttggtgg acaacaacct atgattgctt 600
 gggaatcttt acctgtgtgt gctcaaactg ctcttgagac gaccgatttt ggcgtgcga 660
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<210> 88

<211> 687

<212> DNA

<213> unknown

<220>

<223> B. polyblastis NEP2 cDNA

<400> 88

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 ccagcgggaa agtaggaaca gtttatgaga catatcaacc atttcttaag atagtcaatg 180
 gatgtgtacc attccctgca gttgacgcag cgggcaacac aggtgggtgt ttggcaccaa 240
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 aaagcggatc acactacggt atcatgtact cctggtacat gccaaaagat gagccctcaa 360
 ccgaaattgg tcaccgtcac gactgggaag gtgtaacgt ctggctctcc agctcaaccg 420
 ccacaaccgc cagcaacatt ttaccgtct gtcctccgc ccatggaggc tgggattgtt 480
 ccaccgatgg ttattccctt tctggacca gccccttat caagtacgaa agtatctggc 540

P6000092.ST251

ctatcgatca ttccatgggt cttactagcg ctattggtgt acaacaacct atgattgctt 600
 gggaatcttt acctgctgct gctcaaaagg ctcttgagac gaccgacttc ggctccgcga 660
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<210> 89

<211> 687

<212> DNA

<213> unknown

<220>

<223> B. narcissicola NEP2 cDNA

<400> 89

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 ccagcgggaa agtaggaaca gtttatgaga catatcaacc atatcttaag atcgtaatg 180
 gttgcgtacc attccctgct gtcgatgcat cgggtaacac agtggtgtgt ttggcaccaa 240
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 aaagcggatc atactacggt atcatgtact cctggtacat gccaaaagat gagccctcga 360
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 ccacaaccgc cagcaacatt gtagccgtct gtcctccgc ccatggaggc tgggattgtt 480
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 ctatcgatca ttccatgggt cttactagta gtgttggtgg acaacaacct atgattgctt 600
 gggaatcttt acctgctgct gcccaaactg ctcttgagac gaccgatttc ggctccgcga 660
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<210> 90

<211> 687

<212> DNA

<213> unknown

<220>

<223> B. byssoidae NEP2 cDNA

<400> 90

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P6000092.ST251

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 ccacaaccgc cagcaacatc gtagccgtct gtcctccgc ccatggaggc tgggattgtt 480
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 gggaatcttt acctgctgct gcacaaactg ctcttgagac gaccgatttc ggcgctgcga 660
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<210> 91

<211> 687

<212> DNA

<213> unknown

<220>

<223> B. squamosa NEP2 cDNA

<400> 91

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 ccacaaccgc cagcaacatc gtagccgtct gtcctccgc ccatggaggt tgggattgtt 480
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 ctgtcgatca ttaatgggt ctactagta ctgttgccgg acagcaacct atgattgcct 600
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P6000092.ST251

<210> 92
 <211> 687
 <212> DNA
 <213> unknown

<220>
 <223> B. ficariarum NEP2 cDNA

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 ccagcgggac ttaggaaca gtttatgaga catatcaacc atatctcaag atagtcaatg 180
 gctgtgtacc attccctgca gtcgatgcat cgggtaacac aggtggtggt ttgtcacaa 240
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<210> 93
 <211> 687
 <212> DNA
 <213> unknown

<220>
 <223> B. ranunculi NEP2 cDNA

<400> 93
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 ctggcagtag cagtgggtgga tgcagcagca gtaccggtca agtatacgtc cgaggagcac 300

P6000092.ST251

aaagtggatc atactacggt atcatgtact cctggtacat gccaaaagat gagccctcaa 360
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 atgtccatt cattccatct gttttcg 687

<210> 94

<211> 687

<212> DNA

<213> unknown

<220>

<223> B. aclada NEP2 cDNA

<400> 94

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 gggaatcttt acctgctgct gctcaaactg ctcttgagac gactgatttc ggctctgcga 660
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<210> 95

<211> 735

<212> DNA

<213> unknown

P6000092.ST251

<220>

<223> B. fabae NEP2 cDNA

<400> 95

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<210> 96

<211> 735

<212> DNA

<213> unknown

<220>

<223> B. pelargonii NEP2 cDNA

<400> 96

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P6000092.ST251

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<210> 97

<211> 653

<212> DNA

<213> unknown

<220>

<223> B. calthae NEP2 cDNA

<400> 97

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<210> 98

<211> 735

<212> DNA

<213> unknown

<220>

<223> B. tulipae NEP2 cDNA

<400> 98

P6000092.ST251

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 caacaaccta tgattgcttg ggaatctcta cctgctgctg ctcaaactgc tcttgagacg 660
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<211> 735

<212> DNA

<213> unknown

<220>

<223> B. elliptica NEP2 cDNA

<400> 99

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P6000092.ST251

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