Title: Improvements in or Relating to Disease-Resistance of Plants

Field of the Invention

This invention relates to disease-resistance of plants, particularly resistance to nematodes.

Background of the Invention

Nematodes are small wormlike animals, many of which are plant, animal or human parasites which cause a variety of diseases.

All nematodes are bounded by a tough elastic cuticle synthesised by the underlying hypodermis. The major structural component of the nematode cuticle is collagen, cross-linked by disulphide bonds and located primarily in the basal and inner cortical layers. The external cortex, containing the epicuticle, is composed of a cross-linked protein called cuticulin.

Studies on the nematodes *C. elegans* and *Ascaris* spp. indicate that nematode cuticular collagens differ markedly from vertebrate interstitial collagens in molecular weight, assembly and mode of cross-linking. A significant difference between the derived amino acid sequence of cloned *C. elegans* and vertebrate collagen genes is that those of nematodes have relatively short (24-66) Gly-X-Y repeats (as opposed to 300 or more in vertebrates), and these repeats are interrupted by stretches where Gly is not present in every third position; cysteine residues provide the potential for intermolecular disulphide bridges. However, only a few of the many genes for collagen in *C. elegans* have been looked at in detail, and those from other nematodes hardly at all (Selkirk et al. 1989, Molec. Biochem. Parasitol. 32, 229-246; Cox et al. 1990 Exp. Parasitol. 70, 175-185).

Plant pathogenic nematodes are a major agricultural problem causing significant crop and

yield losses. Plant tissue, particularly root tissue, is damaged by nematode feeding. Such feeding can cause mechanical tissue damage and the accompanying injection of nematode enzymes can cause further tissue disintegration. Nematode infections of roots result in root galls, and distortions in root growth. Similar symptoms accpmpany nematode infections of other parts of the plants.

Important plant pathogenic nematodes include: root knot nematodes, such as Meloidogyne (e.g. *M. incognita*); cyst nematodes, including Heterodera (e.g. *H. glycines*, *H. schachtii*) and Globodera (e.g. *G. rostochiensis*, *G. pallida*) particularly on soyabean, wheat, sugar beet and potatoes; lesion nematodes such as Pratylenchus; burrowing nematodes exemplified by *Radopholus similis*; stem and bulb nematodes, including Ditylenchus, (e.g. *D. dipsaci*); citrus nematodes, including Tylenchulus; seed gall nematodes, such as Anguina; and foliar nematodes typified by Aphelenchoides.

Nematode infection can be accompanied by bacterial or fungal infection. In such plant-disease complexes, damage caused by nematodes can lead to enhanced severity of bacterial or fungal infection. For example, Fusarium and Verticillium wilts, Pythium damping off, Rhizoctonia and Phytophthora root rots as well as *Pseudomonas solanacearum* and *Corynebacterium insidiosum* bacterial wilts are enhanced by nematode infection. In addition, several nematodes (Xiphinema, Longidorus, Trichodorus and Paratrichodorus) are vectors for plant pathogenic viruses.

Attempts to control nematode infection have led to the development (by conventional breeding techniques) of nematode resistant cultivars of a number of crop species. In the case of cyst nematode resistant cultivars, the resistance is actually to nematode multiplication rather than invasion, so plant damage can still occur. It is therefore also necessary to select for tolerance in such plants.

Plant pathogenic nematodes have also been controlled by use of crop rotation or by the use of chemical nematicides.

Nematicides used commercially are generally either fumigants (e.g. halogenated aliphatic

hydrocarbons and methyl isothiocyanate precursor compounds) or non-fumigants (e.g. organophosphates and oximecarbamates). However, the use of chemical nematicides poses an increasing number of difficulties. In particular, nematicides are highly toxic to other animals, including man. They are therefore hazardous not only to the user but also to the environment. In the USA, several nematicides (e.g., DBCP, EDB, D-D, aldicarb and carbofuran) have already been found in the groundwater. Due to their harmful effects on humans and other non-target organisms, their persistence in the soil, and their concentration in ground water, nematicides are being withdrawn from the market worldwide. As a result, there is today a pressing need for more effective, and safer, means to control plant-parasitic nematodes.

Some interest has attached to the discovery that microorganisms isolated from collagen or chitin amended soils were effective in controlling soil nematodes, as was the culture medium in which these microorganisms had been grown. An example of such a microorganism from collagen amended soil is *Cunnighamella elegans* whose use as a nematicidal organism is described in EP O 354 491. The fungus is not parasitic on nematodes so its action appears to be indirect. Culture filtrates from this organism were effective in suppressing nematodes, and were found to contain both protease and collagenase activity (Galper et al. 1991 J. Nematology 23, 269-274). These authors state "We suspect that the suppression of *M. javanica* by collagen amendments of soil results from the production of collagenase and the subsequent damage of the nematode cuticle".

Pure proteases and collagenases have effects on a number of plant parasitic nematodes *in vitro* (Miller & Sands 1977, J. Nematol: 9, 192-197). However many collagenases have proteolytic activity and several proteases can hydrolyse denatured collagen (gelatin) so it is by no means certain that the effect on nematodes is due solely to a collagenolytic activity.

A large number of enzymes from a wide variety of sources have been identified as having collagenolytic activity. For instance, in bacteria the enzyme from *Clostridium histolyticum* has been particularly intensively studied, and two recent reports describe the characterisation of other bacterial collagenases with different molecular structures

(Takeuchi et al., 1992 Biochem. J. 281, 703-708, Kato et al., 1992, J. Bact. 174, 3889-3895). As mentioned above. enzymes with collagenase activity have been identified in fungi which are nematicidal. Other collagenases have been found in sea urchins (Lepage & Gache, 1990 EMBO J. 9, 3003-3012), crabs (Arnoux et al., 1990, J. Computer Aided Design 4, 107-116), crayfish (Bode et al. 1992, Nature 358, 164-167) and insects (Lecroisey et al., 1987, J. Biol. Chem. 262, 7546-7551). Collagenases are also present in higher organisms.

Amongst the best studied group of enzymes with collagenolytic activity are the matrix metalloproteases (Matrisian, 1990 Trends in Genetics 6, 121-125). Collagenolytic matrix metalloproteases have the following characteristics: (1) they are proteinases that degrade at least one component of the extracellular matrix of higher organisms; (2) they contain a zinc ion (hence the name metalloprotease) and are inhibited by chelating agents; (3) they are secreted in a latent pro-enzymic form and require activation for proteolytic activity; (4) they are inhibited by specific inhibitors of metalloproteases (TIMPs); and (5) they share sequence similarities. Three subclasses have been described, each subclass containing several members:

(a) Interstitial collagenases: (commonly called "collagenasen", "collagenase type 1" or MMP1) degrade native collagens types I, II & III. These collagens (types I-III) in native form are not susceptible to stromelysin or to gelatinase. The enzyme cleaves a single bond between Gly-Ile or Gly-Leu three quarters of the way along the collagen triple helix. The collagen molecule unwinds and is then degraded by other proteases or metalloproteases.

(b) Gelatinases: (or MMP2) preferentially degrades denatured collagen types IV (this is basement membrane collagen), V & VII.

(c) Stromelysin: (or MMP3) has very broad activity but does not degrade interstitial collagen. MMP3 has approximately 54% sequence identity at amino acid level and 71% nucleotide similarity to MMP 1. Gelatinase is evolutionarily more distant.

Thus there are many different types of collagenase. each with different properties and

characteristics.

One attempt at biocontrol of nematodes involved the expression of human collagenase genes in transgenic plants, as briefly described by Havstad et al., (1991, Abstract 345 of the Third International Congress of Plant Molecular Biology: Molcular Biology of Plant Growth and Development, Tucson Arizona, USA).

Three different collagenase cDNAs were used in two types of construct and expressed in plants. The collagenases were (i) collagenase (expressed by construct p Coll85.2, Goldberg et al. 1986), an MMP-1 type enzyme, (ii) a gelatinase, (expressed by construct pGel 186.2 Collier et al. 1988), an MMP-2 enzyme, and (iii) a stromelysin, expressed by construct p. Strom, (Wilhelm et al. 1987) an MMP-3 enzyme. The enzymes encoded by these DNA constructs were either:

(a) The prepro enzymes, which thus had (in sequence) the signal peptide, the procollagenase peptide, the mature collagenase enzyme. The coding region of the construct was followed by the untranslated 3' region which contains the poly(A) addition sequences from a non plant source.

(b) The mature collagenase sequence "with or without" the poly(A) sequence from a non plant source.

The highest levels of expression reported were for a mature pStrom with a plant poly(A).

The abstract disclosed that "We are currently analysing plants transformed with the complete constructs of pStrom and pCol 185.2 and tailoring pGel 186.2 for expression in plants. Nematode-effect assays with Meloidogyne (rootknot) and Pratylenchus are currently underway". As far as the present inventors can ascertain, no results were published. In view of the time which has now elapsed, it would therefore seem that Havstad et al. were unable to obtain transgenic plants with increased resistance to nematodes. Accordingly, by definition, the disclosure of Havstad et al. cannot be considered enabling.

Summary of the Invention

In one aspect the invention provides a nucleic acid molecule capable of increasing the resistance to nematode infection of a plant, comprising a nucleotide sequence encoding a polypeptide with collagenase activity or a precursor thereof, the nucleotide sequence being in operable linkage to a promoter active in plants.

For present purposes, the term collagenase refers to any enzyme capable of degrading collagen. Preferably the collagenase or precursor thereof is a metalloprotease, more preferably a matrix metalloprotease, and most preferably a type I matrix metalloprotease.

An example of a suitable starting material is the sequence of a type 1 matrix metalloprotease (MMP), shown in Figure 1. The particular sequence identified in Figure 1 encodes the active matrix metalloprotease (i.e. without pre-pro-sequence). Such a sequence may be modified such that it directly encodes an active form of the enzyme expressible in plants. This is conveniently achieved by modifying the nucleotide sequence to include an ATG start codon (encoding methionine) substantially immediately adjacent 5' to, and in frame with, the sequence encoding the mature MMP enzyme.

Advantageously therefore, the nucleotide sequence encodes a polypeptide which lacks the "pre-pro" portions of the collagenase enzyme, the polypeptide thus not requiring any processing before displaying collagenolytic activity.

Desirably the nucleic acid molecule is adapted in various ways to improve expression in plants of the sequence encoding the collagenolytic polypeptide or precursor.

Examples of alterations to improve expression in host plants include changes to the sequence around the ATG codon, especially immediately adjacent 5' of the ATG codon, so as to render the sequence more closely consistent with the consensus sequence for a plant initiation codon. Desirably the sequence will also comprise a polyadenylation signal derived from a plant source.

In addition to improving the level of expression of the mature collagenase in host plants, other alterations to the sequence are possible which can introduce optional desired characteristics into the protein. For instance, the nucleotide sequence can be altered to more closely resemble plant codon usage, and leader (or signal) sequences can be inserted between the ATG codon and the sequence encoding the mature collagenase.
For the purposes of the present application, the presence of a leader sequence is not considered to prevent a polypeptide being thought of as "mature", if removal of the leader sequence alone is sufficient to leave a fully active collagenase.

Conveniently such a leader or signal sequence comprises the patatin signal sequence, but other signal sequences which target the protein out of the plant cell are known to those skilled in the art and should also be suitable, such as that from a pathogenesis related ("PR") plant gene.

The nucleic acid molecule of the invention may conveniently be a vector. Such a vector will comprise a nucleotide sequence encoding a polypeptide with collagenase activity or precursor thereof (preferably a collagenolytic metalloprotease), in operable linkage to a promoter active in plants. Conveniently, the vector will further comprise an origin of replication effective in bacteria, and/or regulatory signals recognised in plants.

A number of suitable promoters, active in plants, are available to those skilled in the art and are known from the literature. These include constitutive and, more preferably, inducible promoters. Desirably the promoter should be induced in response to nematode attack.

In one embodiment, the molecule also comprises means for preventing the expression in bacteria of the polypeptide having collagenase activity. Typically this comprises a 'spacer' fragment located between the collagenase coding sequence and an adjacent bacterial promoter. This facilitates the manipulation in bacteria of nucleotide sequences encoding polypeptides having collagenolytic activity, which the inventors have typically found to be toxic for commonly used laboratory bacterial strains. A suitable spacer fragment may be derived from the barley beta-glucanase gene.

A molecule in accordance with the invention may be introduced into a plant or part thereof, using techniques well known to those skilled in the art (e.g. transformation or electroporation). Thus in another aspect the invention provides a method of increasing the resistance of a plant to a nematode, comprising introducing into the plant a nucleic acid molecule in accordance with the invention, so as to cause the plant to express, or to increase the expression of, a polypeptide possessing collagenase activity, or a precursor thereof.

In yet another aspect the invention provides a transgenic plant or the progeny thereof, having increased resistance to nematodes, comprising a nucleic acid molecule in accordance with the invention.

In a further aspect, the invention provides a method of reducing or eliminating plant-infecting nematodes from an area of land, comprising planting the area with nematode-resistant plants comprising a nucleic acid molecule in accordance with the invention.

Nematodes are unable to replicate in such plants, thus the number of nematodes in the area will be reduced. In principle, if there are very few nematode-susceptible plants in the area of land, it should be possible to eliminate all plant-infecting nematodes from the area.

The invention may be better understood by reference to the following illustrative example and drawings, wherein:

Figure 1 shows the nucleotide sequence encoding a mature MMP type I enzyme, together with the amino acid sequence of the mature polypeptide;

Figure 2 shows the sequence of a spacer fragment used in constructing a vector which did not express in bacteria;

Figure 3 summarises the method for construction of the MET COL and PAT COL plasmids;

Figure 4 shows *in vitro* processing of the polypeptide expressed by the PAT COL construct; and

Figures 5 to 7 show bar charts of the number of nematode cysts in resistant transgenic plants comprising a nucleotide sequence in accordance with the invention, compared with control plants.

Example

Alteration of Type I MMP gene to Encode for Active Enzyme

The DNA encoding the pre-pro-form of an MMP type I enzyme was isolated, the mature form of which had the sequence shown in Figure 1 (nucleotide sequence Seq. ID No. 1, amino acid sequence Seq. ID No. 2). The DNA sequence was adapted for expression in plants as described below.

The DNA fragment coding for the active MMP described above lacks a protein synthesis initiation codon (as the protein is synthesised as a pre-pro-protein *in vivo*), so an ATG (Met) codon was placed immediately upstream of the mature enzyme sequence with the aim of expressing an active MMP polypeptide in the plant. This clone was designated MET COL. The procedure was designed to minimise the number of extra amino acids that are placed in front of the mature enzyme, so as to minimise the extraneous amino acid content of the enzyme.

The polymerase chain reaction (PCR) was performed on a nearly full-length clone of the MMP type I gene. The forward oligonucleotide (RS-5, Seq. ID No. 3) was designed to contain a *Bam*HI site, plant initiation codon context (Lutcke et al., 1987 EMBO J. 6, 43-48), ATG codon and sequences homologous to the 5' end of the mature MMP sequence:

					MMP sequence
RS-5	5'-CGC	GGATCC	AACA	ATG	TTTGTTCTCACTCCAGGG-3'
		*Bam*HI	Context	Met	F V L T P G

The reverse primer (MOH-32, Seq. ID No. 4) was designed to contain a *Sal*I site, a termination codon and sequences homologous to the 3' end of the MMP gene:

MOH-32	5'-G	GTCGAC	TAA	TTTTTCCTGCAGTTGAAC-3'
		*Sal*I	stop codon	N K R C N F MMP Sequence

30 cycles of PCR were performed, each cycle consisting of 30 seconds at 94°C, 1 minute at 45°C and 2 minutes at 72°C.

The resulting 1.1kb fragment was cloned into the *Bam*HI-*Sal*I sites of BS (Bluescript Stratagene) and was sequenced in full to ensure that the predicted amino acid sequence of the MET COL clone was identical to that of Figure 1.

MET COL was then transferred as a *Bam*HI-*Kpn*I fragment into the 35S promoter - Nos terminator cassette PBI 220.5 and subsequently transferred into Bin 19 (Bevan, M.W. 1984 Nucleic Acid Res. 12, 8711-8721) as a *Hin*dIII-*Eco*RI fragment.

Fusion of the Patatin Signal Sequence

The sequence encoding MMP was further adapted to expression in plants by including a stretch of nucleotides which encoded a plant signal sequence (to make a construct termed "PAT COL").

The purpose of making these constructs is to direct the MMP enzyme to the extracellular compartment of the plant cell to attack the nematode cuticle.

(a) A construct was made that placed the patatin signal sequence (Iturriaga et al., 1989 Plant Cell 1, 381-390) immediately upstream of the mature MMP sequence. The fusion ("+1") contains the patatin signal sequence and the first amino acid of the mature patatin protein fused to the N terminus of the mature MMP. The construct is shown below. The usual convention for single letter amino acid codes is used.

signal sequence (Seq. ID No. 5)

+ 1 MATT RSF LIL FFMILATTSSTCA - T - FVL ...
Patatin MMP Sequence

The patatin leader sequence was cloned by PCR from lambda pat 18 (Bevan et al., 1986 Nucl. Acids Res. 14, 4625-4638 and Goldsbrough, 1989 Ph.D. thesis "A molecular analysis of Patatin gene expression", Council for National Academic Awards, London) which contains 5kb of the 5' end of the patatin gene in pUC.

The forward oligonucleotide (RS-1, Seq. ID No. 6) contained a *Bam*HI site, plant context and sequences homologous to the 5' end of the patatin coding region:

RS-1 5'-CGC GGATCC AACA ATG GCA ACT ACT AAA TCT-3'
*Bam*HI Context M A T T K S
Patatin

The reverse oligo (RS-3) was used in conjunction with RSl to clone the signal sequence plus the first amino acid of the mature patatin respectively (Seq. ID No. 7):

*Dra*I
+ 1 RS-3 5'-AGT TCA ACA TGT GCT ACG TTT AAA GCG-3'
S S T C A T F
signal sequence

The oligonucleotide was designed to contain a *Dra*I site. Cleavage with *Dra*I leaves a TTT codon which encodes phenylalanine (i.e. the first residue of the mature MMP).

(b) The PCR products were purified and digested with *Bam*HI and *Dra*I.

(c) The 5' sequence of mature MMP, lacking the first Phe codon, was also cloned by PCR using a forward primer with a *Sna*BI site (RS-4, Seq. ID No. 8) and sequences homologous to MMP and reverse primer MOH-32.

*Sna*BI
RS-4 5'- GCG TACGTA CTC ACT CCA GGG AAC CCT-3'
V L T P G N P

The sequence of RS-4 differs slightly from the cDNA sequence in that the valine residue is encoded by the codon GTA in the primer rather than by GTT. This alteration was made in order to introduce a *Sna*BI site, to facilitate cloning. Cleavage with *Sna*BI leaves a GTA codon which encodes valine (ie the second amino acid of the mature MMP), so the encoded amino acid sequence is not changed by the alteration in nucleotide sequence.

(d) The PCR product was cleaved with *Sna*BI and *Sal*I and cloned into the *Sna*BI-*Sal*I sites of a BS vector modified to contain a single *Sna*BI site in place of *Kpn*I.

The clone was sequenced in full to ensure that the predicted amino acid sequence was identical to that of Figure 1.

The insert was removed by cleavage with *Sna*BI and *Sal*I and ligated with the patatin signal sequence PCR products from (b) (see Figure 3) and inserted into BS cut with *Bam*HI and *Sal*I. Fusion products were sequenced to ensure that the constructs were correct.

Figure 3 summarises the construction of MET COL and PAT COL. The signal sequence and 'pro' sequence were removed from the sequence encoding a pre-pro-collagenase, to leave a sequence encoding the mature polypeptide with an ATG start codon added at the 5' end to make MET COL. To make PAT COL, the patatin leader sequence was fused to the 5' end of MET COL.

When the fusion construct was cloned into the pBI220.5 vector (as a *Bam*HI-*Kpn*I fragment) transformants were recovered but they grew poorly in liquid culture and on stock plates. Transformants yielded very low amounts of plasmid DNA. The 35S-Fusion-Nos construct was then cloned into Bin 19 as a *Hin*dIII-*Eco*RI fragment. This vector was used to transform agrobacteria strains (LB4404, GV 2260 and 3850) but transformants were recovered only at very low frequency and often contained rearranged DNA. These data suggested that the fusion construct and/or fusion product had a deleterious effect on *E. coli* and Agrobacterium. The fusion construct was, therefore, cloned into a modified PBI 220.5 vector. The vector was modified by insertion of a 540bp

*Sph*I fragment into the *Sph*I polylinker site between the lacZ promoter and the CaMV 35S promoter. The 540bp *Sph*I "spacer" fragment was derived from the upstream promoter region of a barley (1-3, 1-4) Beta-glucanase gene and includes 12 bp of PUC vector DNA at the 5' end. The sequence (Seq. ID No. 9) is presented in Figure 2. It was hoped that the pBI 220.5 vector modified in this way would prevent transcriptional readthrough of the fusion protein from the lacZ promoter, which might have been causing accumulation of a potentially deleterious fusion polypeptide in the bacterial cells. Transformants containing the fusion construct were obtained and the construct was cloned into Bin 19 as a *Hin*dIII-*Eco*RI fragment. This vector was then used to transform Agrobacteria and transformants were obtained having the expected plasmid structure.

All agrobacteria strains were grown on YEP with appropriate antibiotics. The MMP constructs in the Bin 19 vector were introduced into agrobacterium strain GV 2260, by electroporation and transformants selected on kanamycin.

In vitro Processing

The MET COL and PAT COL fusion constructs in the BS vector were used as templates for *in vitro* transcription of RNA using standard procedures. *In vitro* transcripts were translated in a rabbit reticulocyte *in vitro* translation system in the presence (4b, 4c) or absence (4a) of dog pancreatic microsomes. The products were separated on standard 10% acrylamide gels (SDS PAGE) and detected by autoradiography (Figure 4). MET-COL products ("Met") are shown on the right of each gel, PAT-COL products ("Pat") on the left.

Figure 4a-c shows that the predominant translation product from the MET COL construct has an apparent molecular weight (Mwt) of 41kD (large arrow) which corresponds closely to the expected size. Minor bands are detected at 38kD and 32kD (small arrows) and may correspond to degradation products. The predominant translation product from the PAT COL construct has an apparent Mwt of 42kD (large arrow 4a), corresponding to a patatin signal sequence-MMP I fusion. On addition of 0.7μl (4b) and 1.4μl (4c) of microsomes, the band size is decreased to that of the collagenase (mature) peptide indicating that correct

processing of the patatin signal sequence has occurred. These results show that the mature collagenase protein will be present in the extracellular space of plant cells.

Transformation of Plants

The MMP expression vectors described above were introduced into plants.

The plants were originally derived from tuber sprouts and are the widely available varieties Désirée and Maris Piper (or 83 N28-47 control plants). The plants were grown as *in vitro* shoot cultures at 24°C under continuous light (70μE m sec) in 150 x 25mm glass tubes loosely capped with Magenta lids (Magenta Corp., Chicago, Ill. USA). Nodal stem cuttings with one leaf were taken from 5-6 week old plants and planted in MS (Marashige and Skoog) medium containing 1% sucrose solidified with 0.9% agar (Sigma) and supplemented (after autoclaving) with STS which promoted plant growth and produced larger leaves. The STS (silver thiosulphate) stock solution (6mM or 1.5 mg/ml.) was prepared by adding 12mM $AgNO_3$ to an equal volume of 96mM $Na_2S_2O_3$ and used at a concentration of 1μg/ml.

Leaf explants were taken from plantlets at 5-6 weeks after subculturing; cut across the base (discarding the petiole and the lower 1-2mm of leaf base) and the remaining leaf used in transformation experiments. These were carried out essentially as published by Visser et al. (1989 Plant Mol. Biol. 12, 329-337) but slightly altered from the protocol published. Briefly, explants were floated overnight on liquid M387. The medium (M387) contained MS salts and vitamins supplemented with: 10g/l sucrose; 80mg/l NH_4NO_3; 147mg/l $CaCl_2$; 10mg/l NAA; and 10mg/l BAP. The next day the explants were soaked in an overnight culture of *A. tumefaciens* for 15 minutes, blotted dry and placed on solidified M379 callus induction media (MS salts) and vitamins supplemented with: 1g/l sucrose; 4g/l mannitol; 0.0175mg/l IAA; 2.25mg/l BA and solidified with 0.8% agar). After two days of co-cultivation the explants were placed on plates with 500μg/ml cefotaxime (Claforan. Roussel) and five to seven days later the explants were transferred to M384 (shoot induction medium: MS supplemented with 15g/l sucrose; 2.25mg/l BAP; 5mg/l giberellin GA3 and 200mg/l cefotaxime and 50μg/ml kanamycin).

Every three to four weeks the explants were transferred to fresh M384 medium with 200μg/ml cefotaxime and 50μg/l kanamycin. Transformed shoots were removed from the explants and grown *in vitro* as described above before transfer to soil for further growth. Further details of the method are described by Higgins et al. (1992 Plant Sci. 82, 109-118) and Hulme et al. (1992 Plant Cell, Tiss. and Org. Cult. 31, 161-167).

Nematode Resistance Tests

Two types of test for nematode resistance were performed using Potato cyst nematode *G. pallida* Pa2/3: pot tests on whole plants in the greenhouse; and canister tests on roots growing on tuber explants. Plants that showed resistance in the greenhouse pot test were re-tested in canister tests.

Nematode Pot Tests

Plants were grown in infested soil, obtained from a nematode bed maintained especially for the purpose. As the concentration of cysts in the bed was high, the soil was mixed with peat to give a concentration of approximately 20 eggs and larvae per gram of soil. Approximately 2.5cm of the soil/compost mixture was put in the base of a 9cm pot and the pot then filled with sterilised potting compost. Tissue culture plants were planted in the pots and grown under lights (14h photoperiod) at about 20°C for 8-10 weeks. The plants were then tipped out of the pots and the rootballs assessed for the presence of cysts. Only clones with a mean score of less than one cyst per rootball were retained.

Nematode Canister Tests

Tubers from greenhouse grown transgenic plants were used to assess the resistance to nematodes in a closed container test. (Phillips et al., 1990 Ann. Appl. Biol. 96, 317-322). Four replicates (REP1-4) were taken from each transgenic plant. After six weeks the number of visible females was counted. The results are shown in table 1 and summarised below.

Results of Nematode Tests

The results from the two constructs are presented in table 1, where either the patatin signal sequence is fused to the mature collagenase gene (PAT COL), or where the mature collagenase construct (MET COL) is used without a signal sequence.

PAT COL constructs: a total of 61 Maris Piper transformants were tested, and eight clones were found resistant in canister tests. Seventy-seven Désirée transformants were tested and eighteen showed resistance.

MET COL constructs: a total of 86 Maris Piper transformants were tested for resistance in glass house tests. Of these, seven were selected for re-test in canisters, of which four (#2, #25, #31 & #89) displayed moderate levels of resistance.

Nine Désirée plants were also tested (2 in pot tests & seven in canisters), but none were resistant.

In summary: Resistant lines (to *G. pallida* Pa 2/3)

Construct	Maris piper	Désirée
MET COL	4/86	0/9
PAT COL	8/61	18/77

It is clear from these results (and those shown in table 1) that PAT COL provides superior levels of resistance than MET COL in both potato varieties, and that very high levels of resistance are only seen with PAT COL constructs. In all cases the levels of resistance were higher than for a conventionally bred partially resistant variety (83 N 28-47). The variation seen from transformant to transformant using the same construct is commonly noted with the expression of transgenes and is ascribed to "position effects". The reason why more resistant Désirée than Maris Piper plants were obtained is not clear but may be due to the relative ease of transformation of Désirée, which could result in higher numbers

of transgenes per plant.

The results of the cannister tests are displayed graphically in Figures 5 to 7. Figure 5 is a bar chart showing the number of *G. pallida* cysts found in the resistant Désirée PAT COL transgenic lines compared with control varieties of Désirée, Maris piper and the partially resistant 83N plants. Similarly, Figure 6 shows the number of cysts in resistant transgenic PAT COL Maris piper plants, and Figure 7 shows the number of cysts in resistant transgenic MET COL Maris piper plants.

It is extremely encouraging that, where transgenic plants displayed resistance to *G. pallida*, the level of resistance could be exceptionally high.

Table 1

1. Construct : PAT-COL

a. Cultivar: Désirée

77 transformants were tested in canisters

CLONE	REP 1	REP 2	REP 3	REP 4	MEAN
44i	-	0	-	0	0
29ii	0	0	-	0	0
48	0	-	0	0	0
61ii	0	-	0	0	0
55i	0	-	-	0	0
70i	0	1	5	1	1.75
64i	0	0	2	4	1.5
67i	-	7	0	1	2.67
9iii	-	6	2	1	3.0
8Bi	3	2	-	1	2.0
5i	5	1	-	0	2.0
59i	0	0	1	0	0.25
15i	-	-	8	0	4
28i	17	0	-	1	6.0
76i	12	11	11	0	8.5
17i	13	0	2	0	3.75
66i	12	0	0	0	3.0
54i	-	-	22	9	15.5
59 other clones					>20
Desiree (susceptible)	>100	>100	>100	>100	>100

Table 1 (cont'd)

b. Cultivar: Maris Piper

61 transformants were tested in canisters

CLONE	REP 1	REP 2	REP 3	REP 4	MEAN
32ii	-	-	0	3	1.5
9i	1	2	1	1	1.25
9ii	23	22	6	-	17.0
6i	4	13	20	32	17.25
4i	30	13	3	6	13.5
1Bi	2	18	6	4	7.5
17i	18	31	29	20	24.5
18i	0	3	7	21	7.75
53 other clones					>20
Maris Piper (susceptible)	>100	>100	>100	>100	>100

Table 1 (cont'd)

2. Construct: MET-COL

a. Cultivar: Désirée

9 transformants were tested (2 in pots, 7 in canisters); none were resistant.

b. Cultivar: Maris Piper

86 transformants were tested in pots; 5 lines were selected as possibly resistant and used in canister tests.

CLONE	REP 1	REP 2	REP 3	REP 4	MEAN
#2	27	14	-	-	20.5
#25	5	14	23	15	14.25
#31	0	8	21	27	14.0
#89	16	19	4	5	11.0
1 other clone					>20
Maris Piper (susceptible	>100	>100	>100	>100	>100
83N28-47 (partially resistant)	76	80	70	84	77.5

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Unilever PLC
(B) STREET: Unilever House, Blackfriars
(C) CITY: London
(E) COUNTRY: United Kingdom
(F) POSTAL CODE (ZIP): EC4P 4BQ

(A) NAME: Unilever NV
(B) STREET: Weena 455
(C) CITY: Rotterdam
(E) COUNTRY: Netherlands
(F) POSTAL CODE (ZIP): 3013 AL

(ii) TITLE OF INVENTION: Improvements in or Relating to Disease-Resistance of Plants

(iii) NUMBER OF SEQUENCES: 9

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1110 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..1110

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
TTT GTT CTC ACT CCA GGG AAC CCT CGG TGG GAG AAC ACA CAC CTG ACC      48
Phe Val Leu Thr Pro Gly Asn Pro Arg Trp Glu Asn Thr His Leu Thr
 1               5                  10                  15

TAC AGG ATT GAA AAT TAC ACA CCA GAT CTG TCA AGA GAA GAT GTG GAC      96
Tyr Arg Ile Glu Asn Tyr Thr Pro Asp Leu Ser Arg Glu Asp Val Asp
            20                  25                  30

CGT GCC ATT GAG AAA GCC TTT CAA CTC TGG AGC AAT GTC TCA CCC TTG     144
Arg Ala Ile Glu Lys Ala Phe Gln Leu Trp Ser Asn Val Ser Pro Leu
        35                  40                  45
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ACC TTC ACC AAG GTC TCC GAG GGT CAA GCA GAC ATA ATG ATA TCC TTT      192
Thr Phe Thr Lys Val Ser Glu Gly Gln Ala Asp Ile Met Ile Ser Phe
     50                  55                  60

GTC AGG GGA GAT CAT CGT GAC AAT TCT CCC TTT GAT GGA CCT GGA GGA      240
Val Arg Gly Asp His Arg Asp Asn Ser Pro Phe Asp Gly Pro Gly Gly
 65                  70                  75                  80

AAC CTT GCT CAT GCT TTT CAG CCA GGC CCA GGT ATT GGA GGG GAT GCT      288
Asn Leu Ala His Ala Phe Gln Pro Gly Pro Gly Ile Gly Gly Asp Ala
                 85                  90                  95

CAT TTT GAT GAA GAT GAA AGG TGG ACC AAA AAT TTC AGA GAT TAT AAC      336
His Phe Asp Glu Asp Glu Arg Trp Thr Lys Asn Phe Arg Asp Tyr Asn
            100                 105                 110

TTG TAT CGT GTG GCT GCT CAT GAA CTG GGC CAT TCC CTT GGC CTT TCT      384
Leu Tyr Arg Val Ala Ala His Glu Leu Gly His Ser Leu Gly Leu Ser
        115                 120                 125

CAT TCC ACT GAC ATT GGG GCT TTG ATG TAC CCC AAC TAC ATC TAC ACT      432
His Ser Thr Asp Ile Gly Ala Leu Met Tyr Pro Asn Tyr Ile Tyr Thr
    130                 135                 140

GGT GAT GTT CAG CTG TCC CAA GAT GAC ATT GAT GGC ATC CAG GCC ATC      480
Gly Asp Val Gln Leu Ser Gln Asp Asp Ile Asp Gly Ile Gln Ala Ile
145                 150                 155                 160

TAT GGA CCT TCC GAA AAC CCC GTT CAG CCA AGT GGC CCA CAA ACC CCA      528
Tyr Gly Pro Ser Glu Asn Pro Val Gln Pro Ser Gly Pro Gln Thr Pro
                165                 170                 175

CAA GTG TGT GAT AGT AAG CTA ACT TTT GAT GCT ATA ACT ACA CTT CGG      576
Gln Val Cys Asp Ser Lys Leu Thr Phe Asp Ala Ile Thr Thr Leu Arg
            180                 185                 190

GGA GAA CTA ATG TTC TTT AAA GAC AGG TTC TAC ATG CGC ACA AAT TCC      624
Gly Glu Leu Met Phe Phe Lys Asp Arg Phe Tyr Met Arg Thr Asn Ser
        195                 200                 205

TTC TAC CCT GAA GTG GAG CTC AAT TTC ATT TCT GTG TTC TGG CCA CAA      672
Phe Tyr Pro Glu Val Glu Leu Asn Phe Ile Ser Val Phe Trp Pro Gln
    210                 215                 220

GTG CCA AAT GGA CTT CAA GCT GCT TAT GAG ATT GCC GAT AGA GAT GAA      720
Val Pro Asn Gly Leu Gln Ala Ala Tyr Glu Ile Ala Asp Arg Asp Glu
225                 230                 235                 240

GTC CGG TTT TTC AAA GGT AAC AAG TAC TGG GCT GTT AGG GGG CAG GAT      768
Val Arg Phe Phe Lys Gly Asn Lys Tyr Trp Ala Val Arg Gly Gln Asp
                245                 250                 255

GTG CTA TAC GGA TAC CCC AAG GAC ATC CAC AGA TCC TTT GGC TTC CCT      816
Val Leu Tyr Gly Tyr Pro Lys Asp Ile His Arg Ser Phe Gly Phe Pro
            260                 265                 270
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AGT ACT GTG AAG AAT ATC GAT GCT GCT GTT TTT GAG GAA GAT ACT GGG      864
Ser Thr Val Lys Asn Ile Asp Ala Ala Val Phe Glu Glu Asp Thr Gly
        275                 280                 285

AAA ACA TAC TTC TTT GTT GCT CAC GAA TGC TGG AGG TAT GAT GAA TAT      912
Lys Thr Tyr Phe Phe Val Ala His Glu Cys Trp Arg Tyr Asp Glu Tyr
    290                 295                 300

AAA CAA TCC ATG GAT ACA GGT TAT CCC AAA ATG ATA GCA GAA GAA TTT      960
Lys Gln Ser Met Asp Thr Gly Tyr Pro Lys Met Ile Ala Glu Glu Phe
305                 310                 315                 320

CCT GGG ATT GGC AAC AAA GTT GAT GCT GTT TTC CAG AAA GAC GGA TTT     1008
Pro Gly Ile Gly Asn Lys Val Asp Ala Val Phe Gln Lys Asp Gly Phe
                325                 330                 335

CTA TAT TTC TTC CAT GGA ACA AGG CAG TAC CAA TTT GAT TTC AAA ACG     1056
Leu Tyr Phe Phe His Gly Thr Arg Gln Tyr Gln Phe Asp Phe Lys Thr
            340                 345                 350

AAG AGA ATT TTG ACT CTC CAA AAA GCT AAT AGC TGG TTC AAC TGC AGG     1104
Lys Arg Ile Leu Thr Leu Gln Lys Ala Asn Ser Trp Phe Asn Cys Arg
        355                 360                 365

AAA AAT                                                            1110
Lys Asn
    370
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 370 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Phe Val Leu Thr Pro Gly Asn Pro Arg Trp Glu Asn Thr His Leu Thr
 1               5                  10                  15

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

Arg Ala Ile Glu Lys Ala Phe Gln Leu Trp Ser Asn Val Ser Pro Leu
        35                  40                  45

Thr Phe Thr Lys Val Ser Glu Gly Gln Ala Asp Ile Met Ile Ser Phe
    50                  55                  60

Val Arg Gly Asp His Arg Asp Asn Ser Pro Phe Asp Gly Pro Gly Gly
65                  70                  75                  80
```

Asn Leu Ala His Ala Phe Gln Pro Gly Pro Gly Ile Gly Gly Asp Ala
85 90 95

His Phe Asp Glu Asp Glu Arg Trp Thr Lys Asn Phe Arg Asp Tyr Asn
100 105 110

Leu Tyr Arg Val Ala Ala His Glu Leu Gly His Ser Leu Gly Leu Ser
115 120 125

His Ser Thr Asp Ile Gly Ala Leu Met Tyr Pro Asn Tyr Ile Tyr Thr
130 135 140

Gly Asp Val Gln Leu Ser Gln Asp Asp Ile Asp Gly Ile Gln Ala Ile
145 150 155 160

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

Gln Val Cys Asp Ser Lys Leu Thr Phe Asp Ala Ile Thr Thr Leu Arg
180 185 190

Gly Glu Leu Met Phe Phe Lys Asp Arg Phe Tyr Met Arg Thr Asn Ser
195 200 205

Phe Tyr Pro Glu Val Glu Leu Asn Phe Ile Ser Val Phe Trp Pro Gln
210 215 220

Val Pro Asn Gly Leu Gln Ala Ala Tyr Glu Ile Ala Asp Arg Asp Glu
225 230 235 240

Val Arg Phe Phe Lys Gly Asn Lys Tyr Trp Ala Val Arg Gly Gln Asp
245 250 255

Val Leu Tyr Gly Tyr Pro Lys Asp Ile His Arg Ser Phe Gly Phe Pro
260 265 270

Ser Thr Val Lys Asn Ile Asp Ala Ala Val Phe Glu Glu Asp Thr Gly
275 280 285

Lys Thr Tyr Phe Phe Val Ala His Glu Cys Trp Arg Tyr Asp Glu Tyr
290 295 300

Lys Gln Ser Met Asp Thr Gly Tyr Pro Lys Met Ile Ala Glu Glu Phe
305 310 315 320

Pro Gly Ile Gly Asn Lys Val Asp Ala Val Phe Gln Lys Asp Gly Phe
325 330 335

Leu Tyr Phe Phe His Gly Thr Arg Gln Tyr Gln Phe Asp Phe Lys Thr
340 345 350

Lys Arg Ile Leu Thr Leu Gln Lys Ala Asn Ser Trp Phe Asn Cys Arg
355 360 365

Lys Asn
370

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CGCGGATCCA ACAATGTTTG TTCTCACTCC AGGG 34

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GGTCGACTAA TTTTTCCTGC AGTTGAAC 28

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Met Ala Thr Thr Lys Ser Phe Leu Ile Leu Phe Phe Met Ile Leu Ala
1 5 10 15

Thr Thr Ser Ser Thr Cys Ala Thr Phe Val Leu
20 25

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CGCGGATCCA ACAATGGCAA CTACTAAATC T 31

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AGTTCAACAT GTGCTACGTT TAAAGCG 27

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GCGTACGTAC TCACTCCAGG GAACCCT 27

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 540 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GCATGCCTGC AGGTCGACCT CCATTCTGCC CCTACGATAT TCCTGGCCCG TTTTGTTTAA 60

TAAATCCAGC TAGCTAGTGC CGTGCAAGTA CTACACAATT TATGCCATAC CATTGATTGG 120

CGATGGCACT CTTAAGTGTA CACGTACTGT AAAAATACAT GTATTCTTTC TAGCATAATT 180

GATGTACCAA CTATCAAGTG TAACACTTTA ATAGAGTAGA TTGGTGTAGA GTTAGTTGGT 240

AAACCAAGCA AATCAGGGAG GGTAAAAGAT TCACTGCACT GGAAGCTAGT CAAAGTTCAT 300

TCCTTCCTTT AAGGTTATCT AGTTCCTTTT TCTCCTTGCT CGTGGTAGAG TAGCTAGTAG 360

CTAGTGACAA GTCGGTCAAG GCGCCGGCCG TGAAAATAGC AATGTTCCTC GGCCGTGTGC 420

GTGCATCTGA CACCAACTCG TGACTGTAAC AAAAACAATA TATAAGTGCT GCATCAGCCA 480

CCAAAGTCTA GAGAGAAAGA GATAAAAAAA ATGCGCAAAG CTAGAGGCTT ACACGCATGC 540



Claims

1. A nucleic acid molecule capable of increasing the resistance to nematode infection of a plant, comprising a nucleotide sequence encoding a polypeptide with collagenase activity or a precursor thereof, said nucleotide sequence being in operable linkage to a promoter active in plants.

2. A nucleic acid molecule according to claim 1, wherein the nucleotide sequence encodes a metalloprotease or a precursor thereof.

3. A nucleic acid molecule according to claim 1 or 2, wherein the nucleotide sequence encodes a matrix metalloprotease (MMP), or a precursor thereof.

4. A nucleic acid molecule according to any one of claims 1, 2 or 3, wherein the nucleic acid sequence encodes a type I matrix metalloprotease (MMP-I), or a precursor thereof.

5. A nucleic acid molecule according to any one of the preceding claims, wherein the nucleic acid sequence encodes a mature polypeptide having collagenase activity.

6. A nucleic acid molecule according to any one of the preceding claims, wherein the nucleic acid sequence encodes a polypeptide having a signal sequence.

7. A nucleic acid molecule according to any one of the preceding claims, wherein the nucleic acid sequence encodes a polypeptide having a signal sequence from the patatin gene or from a plant pathogenesis related (PR) gene.

8. A nucleic acid molecule according to any one of the preceding claims, comprising a nucleotide sequence encoding substantially the amino acid sequence shown in Figure 1.

9. A nucleic acid molecule according to any one of the preceding claims, comprising

substantially the nucleic acid sequence shown in Figure 1.

10. A nucleic acid molecule according to any one of the preceding claims, further comprising a spacer fragment.

11. A nucleic acid molecule according to any one of the preceding claims, comprising a spacer fragment comprising a promoter active in plants.

12. A nucleic acid molecule according to any one of the preceding claims, comprising substantially the sequence shown in Figure 2.

13. A vector comprising a nucleic acid molecule in accordance with any one of the preceding claims.

14. A method of increasing the resistance of a plant to a nematode, comprising introducing into the plant a nucleic acid molecule in accordance with any one of the preceding claims, so as to cause the plant to express, or to increase the expression of, a polypeptide possessing collagenase activity or a precursor thereof.

15. A transgenic plant or the progeny thereof having increased resistance to nematodes, comprising a nucleic acid molecule in accordance with any one of claims 1 to 13.

16. A method of reducing or eliminating plant-infecting nematodes from an area of land, comprising planting the area with nematode-resistant plants comprising a nucleic acid molecule in accordance with any one of claims 1 to 13.

17. A method according to claim 16, further comprising the reduction or eradication of nematode-susceptible plants, if any, in the area of land.