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(54) **Title**: A PRIMER SEQUENCE FOR THE PREPARATION OF INSECTICIDAL PROTEIN FROM MICROSORIUM SCOLOPENDRIUM

(57) **Abstract**: A primer sequence for the preparation of insecticidal protein from Microsorium scolopendrium The present invention relates to primer sequences for the amplification of novel insecticidal gene, encoding a polypeptide which is toxic to whiteflies and lepidopteran caterpillars. Further, the present invention relates to a cDNA fragment having nucleotide sequence as set forth in SEQ ID NO: 1, coding for an insecticidal protein having an amino acid sequence as set forth in SEQ ID NO: 2. The polypeptide sequence as set forth in SEQ ID No: 2, which is toxic to whitefly (*B. tabaci*) and cotton bollworm (*H. armigera*). The instant invention provides a process for the preparation of insecticidal protein as set forth in SEQ ID No:2 and its application for insect control.

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A Primer Sequence for the preparation of insecticidal protein from Microsorium scolopendrium.

FIELD OF INVENTION

[001] The present invention relates to a polynucleotide fragment encoding an insecticidal protein which is toxic to whiteflies and lepidopteran caterpillars. Further, the instant invention provides DNA constructs, vectors, recombinant host cells, and methods of producing said protein.

BACKGROUND OF THE INVENTION

[002] Loss in crop yields due to the attack of various insects is a worldwide problem. Insect pests are primarily of two types- biting and chewing type (e.g. insects of orders *lepidoptera* and *coleoptera*) and piercing and sucking type (insects of order *hemiptera*). δ-endotoxins or Cry proteins of *Bacillus thuringiensis* cause mortality to larvae of some lepidopteran and coleopteran crop insects. Transgenic crops expressing Cry proteins have been developed and they offer high resistance against the target insects. However, the transgenic plants are not effective against sap sucking pests. Hence, an alternative insecticidal protein is required for the control of sap sucking pests. To achieve the target, the plant biodiversity can be screened. The approach may help in identification of a new insecticidal protein from a new and uncommon source.

[003] Ferns are the most ancient vascular plants. They differ from the more primitive lycophytes in having true leaves, and from seed plants (gymnosperms and angiosperms) in mode of reproduction, absence of flowers and seeds. Ferns show great degree of diversity than any other plant phyla except angiosperms. Success of ferns is often attributed to their less susceptibility to insect attack, although 9300 species of insects are reported to use them as a food source(Crooper-Drive 1978, Entomol. Exp. Appl. 24: 110-116). Ferns have not been reported to suffer severe insect attacks; it is mainly due to the high concentration of secondary metabolites and possible presence of insect resistant macromolecules. They are known to produce insect resistant

secondary metabolites such as ferulic acid, hydrolysable tannins, terpenes, alkaloids (Schaufelberger and Hostettmann 1983, Planta Med. 48:105-107; Asakawa 1990, Biologically active substances from bryophytes. Pages 259-287. In: R chopra, B Satish (eds). Bryophyte development: Physiology and Biochemisrty. CRC, Boston) and ecdysones mimics like insect hormone (Jones and Firn 1978, J Chem. Ecol. 4: 117-138; Lafont and Horn 1989; Phytoecdysteroids: structure and occurrence. Pages 39-64. In: J. Koolman (ed). Ecdysone: from chemistry to mode of action. Thieme, Stuttgart). There is one report where crude protein extract caused 70-100% mortality of *Spodoptera frugiperda* and *Helicoverpa zea* (Markham et al, 2006, Int. J. Plant Sci. 167: 111-117); however insecticidal feature could not be attributed to any protein.

[004] Many insecticidal lectins have been isolated from ferns. Enzyme thiaminase derived from ferns and moss has been demonstrated for insect resistance activity. Thiaminase in the fern *Nephrolepis exaltata* deterred feeding by southern armyworm (Hendrix 1977, Oecologia 26: 347-361).

[005] Plants have evolved sophisticated defense mechanisms by producing a wide array of defensive compounds that confer resistance against phytophagous predators and infection of viruses, bacteria, fungi and nematodes. The best known plant proteins supposedly involved in defense mechanisms are lectins, ribosome-inactivating proteins (RIPs) of types 1and 2, inhibitors of proteolytic enzymes and glycohydrolases (Ryan 1990, Annu. Rev. Phytopathol. 28: 425–449; Bowles 1990, Ann. Rev. Biochem. 59; 873–907; Chrispeels and Raikhel 1991, Plant Cell 3: 1–9; Barbieri et al. 1993, Biochem. J. 185: 203–210; Peumans and Van Damme 1995, Plant Physiol. 109: 347–352). Other plant proteins involved in the complex mechanisms of defense are the arcelins (Osborn et al, 1988, Science 240: 207–210), chitinases (Herget et al. 1990, Mol. Gen. Genet. 224: 469–476), canatoxins (Carlini et al. 1997, J. Econ. Entomol. 90: 340–348) and modified forms of storage proteins (Macedo et al. 1993, Comp. Biochem. Physiol. 105C: 89–94).

[006] Limitation in prior art: Presently no protein has been identified which causes significant mortality to whiteflies. Hence there was need to isolate whitefly specific

insecticidal protein. In this present invention, we have isolated a protein from fern which is toxic to whiteflies. The protein also causes serious growth inhibition to *Helicoverpa armigera* (a lepidopteran caterpillar). The prior art lacks isolation of an insecticidal proteins from a fern which is toxic to whiteflies at PPM level and growth inhibitory to *H. armigera*. There is no protein (gene) available in protein and nucleotide database which show sequence similarity with the protein and nucleotide sequence disclosed in the present invention.

OBJECTIVES OF THE PRESENT INVENTION

[007] The main objective of the present invention is to provide a cDNA sequence encoding an insecticidal protein having sequence as set forth in SEQ ID NO: 2.

[008] Another objective of the present invention is to provide a process for preparation of an insecticidal protein having sequence as set forth in SEQ ID NO: 2.

[009] Yet another objective of the present invention is to provide reagents for preparation of an insecticidal protein having sequence as set forth in SEQ ID NO: 2, comprising DNA constructs, vectors, and host cells.

[0010] Another objective of the present invention is to develop methods for the application of the said protein in integrated pest management.

SUMMARY OF THE INVENTION

[0011] In an aspect of the present disclosure, there is provided a cDNA fragment having nucleotide sequence as set forth in SEQ ID NO: 1.

[0012] In an aspect of the present disclosure, there is provided a polypeptide having insecticidal properties having amino acid sequence as set forth in SEQ ID NO: 2.

[0013] In an aspect of the present disclosure, there is provided a nucleotide fragment encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 2.

[0014] In an aspect of the present disclosure, there is provided a DNA construct comprising a cDNA fragment having nucleotide sequence as set forth in SEQ ID NO:

1; or a nucleotide fragment encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 2, operably linked to a promoter.

[0015] In an aspect of the present disclosure, there is provided a DNA vector comprising a DNA construct, said DNA construct comprising a cDNA fragment having nucleotide sequence as set forth in SEQ ID NO: 1; or a nucleotide fragment encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 2, operably linked to a promoter.

[0016] In an aspect of the present disclosure, there is provided a recombinant host cell comprising a DNA construct, said DNA construct comprising a cDNA fragment having nucleotide sequence as set forth in SEQ ID NO: 1; or a nucleotide fragment encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 2, operably linked to a promoter.

[0017] In an aspect of the present disclosure, there is provided a recombinant host cell comprising a DNA vector, said DNA vector comprising a DNA construct, said DNA construct comprising a cDNA fragment having nucleotide sequence as set forth in SEQ ID NO: 1; or a nucleotide fragment encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 2, operably linked to a promoter.

[0018] In an aspect of the present disclosure, there is provided a method of production of a polypeptide having insecticidal properties having amino acid sequence as set forth in SEQ ID NO: 2, said method comprising: (a) obtaining a recombinant host cell comprising a DNA construct, said DNA construct comprising a cDNA fragment having nucleotide sequence as set forth in SEQ ID NO: 1; or a nucleotide fragment encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 2, operably linked to a promoter; or a recombinant host cell comprising a DNA vector, said DNA vector comprising said DNA construct; (b) subjecting said recombinant host cell to conditions which result in expression of said polypeptide in said recombinant host cell.

[0019] In an aspect of the present disclosure, there is provided a cDNA fragment having nucleotide sequence as set forth in SEQ ID NO: 1; or a polypeptide having

insecticidal properties having amino acid sequence as set forth in SEQ ID NO: 2; or a nucleotide fragment encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 2 for use in inhibiting whitefly, or *H.armigera*.

[0020] In an aspect of the present disclosure, there is provided a set of primers having sequence as set forth in SEQ ID NO: 3 and SEQ ID NO: 4.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS

[0021] Figure 1 depicts the chromatogram of the proteins separated on Q-Sepharose (fast flow) column. The arrow indicates the fractions with insecticidal activity.

[0022] Figure 1 depicts the steps of purification of insecticidal protein from rhizome (MSR) monitored through SDS-PAGE: lane 1, protein molecular weight marker; lane 2, total extracted protein; lane 3, salted out ammonium sulfate fraction with insecticidal activity (20-50%); lane 4, biologically active protein after hydrophobic interaction chromatography; lane 5, after ion-exchange chromatography; and lane 6, after size-exclusion chromatography. The protein was purified to near homogeneity.

[0023] Figure 3 depicts the separation of purified insecticidal protein (from *M.scolopendrium* rhizome) on 2-D gel. Bioactive protein (molecular weight approximately 34kDa and pI-5.06) with BSA as control (molecular weight 66.409kDa and pI-5.64).

[0024] Figure 4 depicts the glycoprotein staining of the purified insecticidal protein: lane 1, pre-stained marker; lane 2, purified insecticidal protein; lane 3, BSA (negative control); and lane 4, Ribonuclease B (positive control).

[0025] Figure 5 depicts PMF of trypsinized insecticidal protein purified from *M.scolopendrium* rhizome.

[0026] Figure 6 depicts the construct (in pET28a vector backbone) for heterologous expression in bacteria.

[0027] Figure 7A depicts the purification of insecticidal protein expressed in *E.coli*: lane 1, protein molecular weight markers; lane 2, total bacterial protein before induction; lane 3, total bacterial protein after 4h of induction; lane 4, Ni-NTA purified

protein; and lane 5, protein purified on ion-exchange column. Figure 7B depicts western blot analysis of purified protein isolated from different sources: lane 1, pre-stained ladder; lane 2, BSA as negative control; lane 3, insecticidal protein purified from rhizome; and lane 4, insecticidal protein purified from *E.coli*.

DETAILED DESCRIPTION OF THE INVENTION

[0028] Whitefly is an important insect pest of several agriculturally important crops. It is responsible for the huge damage to crop productivity globally. There is a lack of any sustainable method available for their efficient management till date. As a consequence, we explored plants for a protein having potential to target insect. Extensive screening of plants for whitefly toxic proteins identified *Microsorium* spps (ferns) and purified a new insecticidal protein from its rhizomes and leaves. The method of purification involved extraction of total soluble protein from tissues followed by salting out of protein with ammonium sulfate and then purification on chromatography columns (hydrophobic interaction, ion exchange, and size exclusion) as explained in Example 1. At each stage of purification, protein fractions were dialyzed against 20mM TrisCl (pH 8) and evaluated for insecticidal activity. The fractions with insecticidal activity were taken to the next step of purification. The purified protein was evaluated for efficacy against whitefly (*B.tabaci*), cotton bollworm (*H.armigera*), aphid (*A.gossypii*), and cotton leaf worm (*S.litura*) by incorporating the protein in the artificial diet (Example 5). The protein caused mortality of whiteflies (Table 2 and 3) and cotton bollworm (Table 4). The purity and pI of the purified insecticidal protein was examined on 2D PAGE (Figure 3). The purified protein was subjected to Mass Spectrometric analysis on MALDI-TOF-TOF platform (Figure 5). MS and MS/MS analysis and search in protein and nucleic acid database could not identify similar sequence, establishing the novelty of the insecticidal protein. *de novo* sequencing of the selected peptides were performed which helped in designing degenerate primers. Total RNA was isolated from rhizome and cDNA was synthesized. Toxin protein encoding gene was amplified from cDNA

using primers dGSP1, dGSP2, and dGSP3 and cloned. *de novo* sequencing of trypsin digested peptide was performed to find out possible amino acid sequence in purified protein. This was done to (a) identify the insecticidal protein if possible, and (b) design degenerate primers for cloning the gene. A total of 7 peptide sequences were obtained, none of which showed any significant similarity to any protein in protein database. The cloned cDNA was 1477bp long, of which protein encoding ORF was 1062bp long (SEQ ID NO: 1). The ORF encodes insecticidal protein of 353 amino acid residues (SEQ ID NO: 2). The protein sequence matched with the MS and MS/MS data generated with the proteins. This established that the purified insecticidal protein and *in silico* translated protein from the clone gene is the same. One domain of the insecticidal protein matched with a domain of Natterin-like protein of fish. However, primary structure of the protein of the present invention does not show homology with any plant derived protein in the available database.

[0029] The gene encoding the insecticidal protein was cloned in *E.coli* expression vector having 6xHis-tag at N-terminus. The tagged protein was purified (Example 6) and evaluated for insecticidal activity against whitefly and *H.armigra*. The insecticidal protein exhibited at-par insecticidal activity.

[0030] In an embodiment of the present disclosure, there is provided a cDNA fragment having nucleotide sequence as set forth in SEQ ID NO: 1.

[0031] In an embodiment of the present disclosure, there is provided a cDNA fragment as described herein, wherein said fragment encodes an insecticidal protein.

[0032] In an embodiment of the present disclosure, there is provided a cDNA fragment as described herein, wherein said fragment encodes a polypeptide having amino acid sequence as set forth in SEQ ID NO: 2.

[0033] In an embodiment of the present disclosure, there is provided a cDNA fragment having nucleotide sequence as set forth in SEQ ID NO: 1, said cDNA fragment encoding a polypeptide with insecticidal properties having amino acid sequence as set forth in SEQ ID NO: 2.

[0034] In an embodiment of the present disclosure, there is provided a polypeptide having insecticidal properties having amino acid sequence as set forth in SEQ ID NO: 2.

[0035] In an embodiment of the present disclosure, there is provided a polypeptide having insecticidal properties as described herein, wherein said polypeptide is encoded by a sequence as set forth in SEQ ID NO: 1.

[0036] In an embodiment of the present disclosure, there is provided a nucleotide fragment encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 2.

[0037] In an embodiment of the present disclosure, there is provided a nucleotide fragment as described herein, wherein said nucleotide fragment sequence is as set forth in SEQ ID NO: 1.

[0038] In an embodiment of the present disclosure, there is provided a DNA construct comprising a cDNA fragment having nucleotide sequence as set forth in SEQ ID NO: 1, operably linked to a promoter.

[0039] In an embodiment of the present disclosure, there is provided a DNA construct comprising a cDNA fragment as described herein, wherein said cDNA fragment encodes a polypeptide having amino acid sequence as set forth in SEQ ID NO: 2.

[0040] In an embodiment of the present disclosure, there is provided a DNA construct comprising a nucleotide fragment encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 2, operably linked to a promoter.

[0041] In an embodiment of the present disclosure, there is provided a DNA construct comprising a nucleotide fragment as described herein, wherein said nucleotide fragment sequence is as set forth in SEQ ID NO: 1.

[0042] In an embodiment of the present disclosure, there is provided a DNA vector comprising a DNA construct, said DNA construct comprising a cDNA fragment having nucleotide sequence as set forth in SEQ ID NO: 1, operably linked to a promoter.

[0043] In an embodiment of the present disclosure, there is provided a DNA vector comprising a DNA construct comprising a cDNA fragment as describe herein, wherein said cDNA fragment encodes an insecticidal protein having amino acid sequence as set forth in SEQ ID NO: 2.

[0044] In an embodiment of the present disclosure, there is provided a DNA vector comprising a DNA construct, said DNA construct comprising a nucleotide fragment encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 2, operably linked to a promoter.

[0045] In an embodiment of the present disclosure, there is provided a DNA vector comprising a DNA construct comprising a nucleotide fragment as described herein, wherein said nucleotide fragment sequence is as set forth in SEQ ID NO: 1.

[0046] In an embodiment of the present disclosure, there is provided a recombinant host cell comprising a DNA construct, said DNA construct comprising a cDNA fragment having nucleotide sequence as set forth in SEQ ID NO: 1, operably linked to a promoter.

[0047] In an embodiment of the present disclosure, there is provided a recombinant host cell comprising a DNA construct comprising a cDNA fragment as described herein, wherein said cDNA fragment encodes an insecticidal protein having amino acid sequence as set forth in SEQ ID NO: 2.

[0048] In an embodiment of the present disclosure, there is provided a recombinant host cell comprising a DNA construct, said DNA construct comprising a nucleotide fragment encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 2, operably linked to a promoter.

[0049] In an embodiment of the present disclosure, there is provided a recombinant host cell comprising a DNA construct comprising a nucleotide fragment as described herein, wherein said nucleotide fragment sequence is as set forth in SEQ ID NO: 1.

[0050] In an embodiment of the present disclosure, there is provided a recombinant host cell as described herein, wherein said recombinant host cell is of bacterial origin.

[0051] In an embodiment of the present disclosure, there is provided a recombinant host cell as described herein, wherein said recombinant host cell is *E.coli*.

[0052] In an embodiment of the present disclosure, there is provided a recombinant host cell as described herein, wherein said recombinant host cell is of fungal origin.

[0053] In an embodiment of the present disclosure, there is provided a recombinant host cell as described herein, wherein said recombinant host cell is of plant origin.

[0054] In an embodiment of the present disclosure, there is provided a recombinant host cell as described herein, wherein cDNA fragment is host genome encoded.

[0055] In an embodiment of the present disclosure, there is provided a recombinant host cell as described herein, wherein nucleotide fragment is host genome encoded.

[0056] In an embodiment of the present disclosure, there is provided a recombinant host cell as described herein, wherein cDNA fragment is encoded by extra-cellular host genome.

[0057] In an embodiment of the present disclosure, there is provided a recombinant host cell as described herein, wherein nucleotide fragment is encoded by extra-cellular host genome.

[0058] In an embodiment of the present disclosure, there is provided a method of production of a polypeptide having insecticidal properties having amino acid sequence as set forth in SEQ ID NO: 2, said method comprising: (a) obtaining a recombinant host cell comprising a DNA construct, said DNA construct comprising a cDNA fragment having nucleotide sequence as set forth in SEQ ID NO: 1, operably linked to a promoter; and (b) subjecting said recombinant host cell to conditions which result in expression of said polypeptide in said recombinant host cell.

[0059] In an embodiment of the present disclosure, there is provided a method of production of a polypeptide having insecticidal properties having amino acid sequence as set forth in SEQ ID NO: 2, said method comprising: (a) obtaining a recombinant host cell comprising a DNA construct, said DNA construct comprising a nucleotide fragment encoding said polypeptide, operably linked to a promoter; and (b) subjecting

said recombinant host cell to conditions which result in expression of said polypeptide in said recombinant host cell.

[0060] In an embodiment of the present disclosure, there is provided a method of production of a polypeptide having insecticidal properties having amino acid sequence as set forth in SEQ ID NO: 2 comprising obtaining a recombinant host cell comprising a DNA construct comprising a nucleotide fragment as described herein, wherein said nucleotide fragment sequence is as set forth in SEQ ID NO: 1.

[0061] In an embodiment of the present disclosure, there is provided a method of production of a polypeptide as described herein, wherein said method further comprises the step of isolation or purification of said polypeptide.

[0062] In an embodiment of the present disclosure, there is provided a cDNA fragment having nucleotide sequence as set forth in SEQ ID NO: 1 for use in inhibiting whitefly, or *H.armigera*.

[0063] In an embodiment of the present disclosure, there is provided a use of a cDNA fragment as described herein, wherein said cDNA fragment encodes an insecticidal protein having amino acid sequence as set forth in SEQ ID NO: 2.

[0064] In an embodiment of the present disclosure, there is provided a use of a polypeptide having insecticidal properties having amino acid sequence as set forth in SEQ ID NO: 2 for use in inhibiting whitefly, or *H.armigera*.

[0065] In an embodiment of the present disclosure, there is provided a use of a polypeptide as described herein, wherein said polypeptide is encoded by a sequence as set forth in SEQ ID NO: 1.

[0066] In an embodiment of the present disclosure, there is provided a set of primers having sequence as set forth in SEQ ID NO: 3 and SEQ ID NO: 4.

[0067] In an embodiment of the present disclosure, there is provided a set of primers as described herein, wherein said primers are useful in a PCR reaction to produce an amplicon encoding a protein having amino acid sequence as set forth in SEQ ID NO: 2.

[0068] In an embodiment of the present disclosure, there is provided a set of primers as described herein, wherein primer of SEQ ID NO: 3 is forward primer.

[0069] In an embodiment of the present disclosure, there is provided a set of primers as described herein, wherein primer of SEQ ID NO: 4 is reverse primer.

[0070] In an embodiment of the present disclosure, a fragment having sequence as set forth in SEQ ID NO: 1 is obtained by a PCR reaction using primers having sequence as set forth in SEQ ID NO: 3 and SEQ ID NO: 4.

[0071] In an embodiment of the present disclosure, there is provided an insecticidal protein having sequence as set forth in SEQ ID NO: 2 of size ~34kDa on SDS-PAGE.

[0072] In an embodiment of the present disclosure, there is provided an insecticidal protein which exhibits activity against *Bemisia tabaci*, *Helicoverpa armigera* or any other crop insects for example, insect from order of *lepidotera*, *homodoptera*, *coleopteran*, and *diptera*.

[0073] In an embodiment of the present disclosure, SEQ ID NO: 1 comprised of 1-1062 nucleotides or the sequence complementary thereto, which encodes full length *M.scolopendrium* insecticidal polypeptide , wherein nucleotide sequence 1059 to1062 is stop codon.

The novelty of the disclosed protein has been established on the basis of below mentioned points:

[0074] No prior art is available on the isolation of an insecticidal protein from fern *Microsorium scolopendrium* and other two species (*M.punctatum*, *M.alternifolium*), toxic to whitefly (*Bemisia tabaci*) and *H.armigera*, and cloning the respective gene.

[0075] Blast analysis of the insecticidal protein disclosed in the present invention does not show significant homology (greater than 40%) with any plant derived protein in the available database.

[0076] The above points establish that the insecticidal protein is new and novel.

Sequences:

[0077] SEQ ID NO: 1 depicts the cDNA sequence of insecticidal protein.

ATGGCGCTGTACCAGACACCCGTGTATGTCATCGGAGGGCAAGGTGGCAACTCGTTTACT
TATGACCAGAGCAGGAACGGGAAGGTGTTGAGGAAGATTGGGGTGTGGGCTGGCGAGTGGCAGCTGC
GCGGCATCCGGGTATGGATGTCTGGCTCCGATACGCCGGCTACCTTTGGCTCAGCCTCGGGCTCTTATT
CTGAGTATACATTTGCGGATGGCGAGCGCATCACCCGCTTGTCC
TTGTGGGGCAACGGTGCTGGTACGCGTTCCGGAGCTATTAGATTCTACACGACAACTGGA
GGCACATTTTTCCCAAAAATGACATCTTGGGGCTTACAGACTGAGTATCCAATTGATGTG
GCATCTGGTCTTTGTGTGGGAATCTTAGGACGAGCTAGTGCTGATGTTGATTCATTGGGG
TTCCTATTTCTCCGAACCATAGCATCGGCTCGTATGATCAATGTAAGCTACCCAACCTTG
GGCTTGGAGCAAGCTGGAATTGTCCCTGTCACGCTTGATTCGTACAACGACTCCAATAAT
TCAGGTTCTATCTCCAAGAATTGGACTTTCTCTGGTAGTCGAACAGTTACAATATCATCA
TCATGGACGCTTACTACAGGGATAGAGGCACATGCTACTTTGACTGTTCAAGCAGGGATC
CCTTCAGTTGCAGAAGTTACTGGAGAGTTTGGATGGGCAGTGAGTGTGACCGGAAGCCAC
ACAACCACCGAAGAGGAAAGCCGAACGCTCCGTTGGGAGCAATCCGGAAC
CTTAGAGCCAGGACAATGGATTTCTCTCCAAGCTACCACTCGAAGAGGAACCATCACCTTACCCTACC
AAGGGACTATGGAGATCACCCTACAGTCTGGCACCGTGTTTCAATATCCCATATCCTCTATGTATTCTG
GTGTTGATTATACTAGTGTTGACATAATCAACACTGGAACTAGAGCATTGGATCAAGCTAAGGTGGAA
GATATTGATCAAAAATCCCAAGAAAAGGATCACAATGCCCAACCTAATGAACAAGTCCAAGAAAGCA
AACTCCTCTTTGTTGAGAA

[0078] SEQ ID NO: 2 depicts the amino acid sequence of insecticidal protein

MALYQTPVYVIGGQGGNSFTYDQSRNGKVLRKIGVWAGEWQLRGIRVWMSGSDTPATFGS
ASGSYSEYTFADGERITRLSLWGNGAGTRSGAIRFYTTTGGTFFPKMTSWGLQTEYPIDV
ASGLCVGILGRASADVDSLGFLFLRTIASARMINVSYPTLGLEQAGIVPVTLDSYNDSNN
SGSISKNWTFSGSRTVTISSSWTLTTGIEAHATLTVQAGIPSVAEVTGEFGWAVSVTGSH
TTTEEESRTLRWEQSGTLEPGQWISLQATTRRGTITLPYQGTMEITLQSGTVFQYPISSM
YSGVDYTSVDIINTGTRALDQAKVEDIDQKSQEKDHNAQPNEQVQESKLLFVE

[0079] SEQ ID NO: 3 depicts forward primer
atggcgctgt accagacacc cgtgtatg

[0080] SEQ ID NO: 4 depicts reverse primer
ttactcaaca aagaggagtt tgcttcttgg

EXAMPLES

[0081] The following examples are given by way of illustration of the present invention and therefore should not be construed to limit the scope of the present invention

Example 1

Extraction of total soluble protein and insecticidal activity guided purification

[0082] Plant materials were collected from the fern house of National Botanical Research Institute, Lucknow, India and total soluble proteins were extracted. Rhizomes or leaves were chopped into small pieces and crushed into fine powder under liquid nitrogen. Powdered tissues were suspended in ice cold protein extraction buffer {20 mM HEPES pH 8.0, 0.5 mM DTT, 1mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonylfluoride (PMSF) and 1 mm benzamidine} in the ratio 1:4 (w/v). The suspension was homogenized at $4^{O}C$, incubated for 1 h and then filtered through 4 layers of cheesecloth. The homogenate was spun (3000×g, $4^{0}C$, 30 min) and total soluble protein was collected as supernatant. The total soluble proteins were broadly fractionated with ammonium sulfate precipitation at the interval of 10% saturation. Each fraction was dialyzed against 20 mM TrisCl (pH 8) and evaluated for insecticidal activity. Ammonium sulfate fraction ranging 20-50% gave the maximum mortality against whiteflies and *H. armigera.* Those effective fractions were pooled for HIC purification on (hydrophobic interaction chromatography). Ammonium sulphate concentration in the protein samples was made up to 1M and loaded on the HIC column (Phenyl Sepharose, High Performance matrix), pre-equilibrated with loading buffer (20 mM HEPES pH 8 + 1 M ammonium sulfate). The column was washed with the same buffer until OD_{280} reached to less than 0.02. The proteins were eluted by linear decreasing gradient of ammonium sulfate, 1.0-0.0 M. The eluted fractions were dialyzed against 20 mM TrisCl (pH 8.0) and examined for insect bioassay. SDS-PAGE analysis of bioactive fractions showed many proteins on the gel. Bioactive fractions were pooled and loaded on Q sepharose (FF) anion exchange column, pre-equilibrated with 20 mM TrisCl (pH 8.0). The column was washed until OD_{280} reached to less than 0.02. The column bound proteins were eluted with a linear gradient of NaCl (0 to 1 M). The eluted fractions were dialyzed against 20 mM TrisCl (pH 8.0) and examined for insect bioassay. Bioactive fractions still had some undesired proteins. All fractions were pooled, dialyzed against 20 mM Tris (pH 8.0) containing 200 mM NaCl and

subjected to separation on Superdex 200 column, equilibrated with protein sample buffer. All the fractions were dialyzed to remove salt and insect bioassay was performed. The fractions which had highest biological activity were analyzed on denaturing PAGE, it consisted of single protein.

[0083] The purified protein was subjected to 2-dimension polyacrylamide gel electrophoresis. Coomassie stained gel showed four spots of similar molecular weight. Peptide mass fingerprinting of all 4 spots were nearly identical. This indicated that insecticidal protein in source plant was present in 4 isoforms (fig. 3).

Example 2

Partial characterization of the insecticidal protein

[0084] To examine whether insecticidal protein purified from *M. scolopendrium* rhizome, is a glycoprotein, purified protein was resolved on the denaturing PAGE and transferred to a PVDF membrane. Membrane was first incubated with biotin conjugated Concanavalin A (Con A binds to the sugar moiety of glycoprotein containing α-D-mannose or α-D-glucosyl residues at the terminus) and then with alkaline phosphatase conjugated streptavidin. After incubation with substrate, blue colored insoluble reaction product was visualized. Result revealed that insecticidal protein is glycosylated with either α-D-mannose or α-D-glucosyl residue in sugar moiety at the terminal position.

Example 3

Peptide mass finger printing and de novo sequencing of the insecticidal protein

[0085] Peptide mass finger printing: For the identification of purified insecticidal protein, peptide mass fingerprinting (PMF) was performed using Matrix-assisted laser/desorption ionization mass spectrometry (MALDI-MS) technique. Protein was resolved on the SDS-PAGE, band of interest excised and digested by trypsin (sequencing grade, Promega, USA). Digested peptides were extracted from the gel pieces, mixed with matrix (α-cyano-4-hydroxycinnamic acid), spotted on MALDI

plate and used for peptide mass fingerprinting. The data was analyzed by MASCOT search (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF) with reported protein in databases. PMF data generated from insecticidal protein did not show significant similarity with known proteins in protein database.

[0086] ***de novo*** **sequencing**: *de novo* sequencing of trypsin digested peptide was performed to find out possible amino acid sequence in purified protein. This was done to (a) identify the insecticidal protein, if possible and (b) design degenerate primers for cloning the gene. A total of 7 peptide sequences were obtained, none showed any significant similarity with any protein in data base. This conformed that the purified insecticidal protein is new entry in protein database.

[0087] Table 1 Amino acid sequences obtained from *de novo* sequencing

S.N	m/z of precursor	Amino acid sequence
1.	954.4897	WNTFSSGR
2.	1131.6035	LSLWGNGAGTR
3.	1314.7640	TGRWAGEWQKLR
4.	1366.6715	MYTTTGGTMMPK
5.	1510.7944	TKDVDSLGFLFLR
6.	1783.76	ASGSYSEYTFADGER
7.	2663.3374	(1562.83)PSTFYDSKR

Example 4

Cloning of the insecticidal protein encoding gene

[0088] Degenerate primers were designed and synthesized. Total RNA was isolated from the rhizome. The cDNA synthesis was performed for 5' and 3' rapid amplification of cDNA ends. For 5' RACE, RNA was reversely transcribed with the 5' RACE CDS Primer and SMART II A Oligonucleotide. The PCR was performed with gene specific primer (dGSP1: TAYTCNSWRTANSWNCCNSWNGC) that was

degenerate and Universal Primer A Mix (UMP, provided in the kit). The amplified product was visualized on agarose gel, cloned subsequently and several clones were sequenced. All the cloned were translated in 6 frames and matched with MS/MS data and *de novo* sequencing data. Desired clone was selected and used in making primers for 3'RACE. For 3' RACE, RNA was reversely transcribed with the 3' RACE CDS Primer A. The first round of PCR was performed with the primer designed on the basis of 5' RACE result (dGSP2:GAAAGATGGCGCTGTACCAGACACC) and Universal primer A mix. The PCR product was diluted 50-folds for second round of amplification with nested gene specific primers (dGSP3:ATCGGAGGGCAAGGTGGCAACTCG) and Nested Universal Primer A. The 3' RACE product was examined on agarose gel, cloned in TA cloning vector and several clones were sequenced. The desired clone was selected as discussed above.

[0089] End sequences of the clones of 5' and 3' RACE were used in designing primers and full-length cDNA was amplified. It was cloned in a TA vector and sequenced. The sequence was subjected to ORF finder and 1062 bp long ORF was identified (SEQ ID NO: 1). The protein sequence of ORF matched with MS, MS/MS data of the purified insecticidal protein (SEQ ID NO: 2). As set of primers were designed for the cloning of the insecticidal proteins in *E. coli* and plant expression vector (SEQ ID NO: 3 and SEQ ID NO: 4). After the cloning, the gene was sequenced.

Example 5

[0090] Against whiteflies (*Bemisia tabaci*): Bioassay was carried out with >1 day old whiteflies (*Bemisia tabaci*). Whiteflies were reared on potted cotton plants in the laboratory at 26 ± 2°C and 80% relative humidity. Plants having large number of nymphs and pupae were selected, adult whiteflies removed and plants kept in isolation for the emergence of fresh adults. Bioassays were carried out as per Upadhyay et al., 2011 (J. Biosciences. 36: 153-161). Whiteflies were collected into specimen tubes, directly. The leaves containing freshly emerged adults were kept close to the open end of the tube. Insects were forced gently to move inside the tube by tapping. After the

collection of insects, tubes were capped and kept in inverted position. Artificial diet (with/without insecticidal protein) was filter sterilized through syringe filter (0.22 µm) and sandwiched (100 µl) between the two layers of sterilized stretched parafilm onto the inner surface of the cap of tube, aseptically. The caps of insect containing tubes were replaced with the diet containing caps. The tubes were kept in upright position so that the caps faced light. The old caps were replaced with caps containing fresh diet of respective test sample on alternate days to minimize the possibility of degradation of test sample and contamination in diet. Perforations were made on the bioassay vial for air exchange.

[0091] Table 2 Insect bioassay with whitefly (*B. tabaci*).

Concentration of insecticidal protein (µg/ml)	Mortality After				
	1 day	2 days	3 days	4 days	5 days
15.00	*85*	*95*	*100*	*100*	*100*
12.00	*64*	*86*	*100*	*100*	*100*
9.00	*50*	*79*	*93*	*93*	*100*
6.00	*47*	*73*	*80*	*88*	*92*
3.00	*26*	*39*	*54*	*65*	*80*
0.75	*23*	*26*	*50*	*76*	*76*
0.37	*26*	*29*	*44*	*55*	*55*
0.18	*15*	*21*	*31*	*34*	*40*
Control	*10*	*15*	*15*	*15*	*15*

[0092] Table 3 LC_{50} of insecticidal protein against whitefly (*B. tabaci*).

	After one day	After two days	After three days	After four days	After five days
LC_{50}	7.750 µg/ml	4.120 µg/ml	1.044 µg/ml	0.602 µg/ml	0.438 µg/ml

[0093] Insect bioassay against *H. armigera*: Insect bioassay was carried out with the neonatal larvae by feeding them on semi-synthetic diet. The test proteins were uniformly spread on diet surface of each well in the 24 well insect-rearing trays and

air-dried. Neonate larvae of *Helicoverpa armigera* were release individually in each cell and a total of 25 individual larvae were tested for each concentration. Mortality and average larval weight was measured after 3 and 5 days of treatment and percent growth retardation calculated in comparison to the controls.

[0094] Table 4 Insect bioassay with *H.armigera*

Concentration of protein (µg/cm^2)	After five days	
	% mortality	% Growth reduction ± Std. Error
1.850	83.33	91.30 (0.12 ± 0.02)
0.920	44.4	76.81 (0.32 ± 0.05)
0.600	11.11	62.31 (0.52 ± 0.73)
0.230	0	42.02 (0.80 ± 0.10)
Control	0	00.00 (1.38 ± 0.11)

Means compared using DMRT at α=0.05; Means in the column carrying same letter are not significantly different.

Example 6

Expression of protein in *E. coli*

[0095] The insecticidal protein was expressed in *E. coli.* The gene was amplified from the cDNA of fern rhizome and cloned at the *NdeI* and *EcoRI* site (downstream of T7 lac promoter) in the expression vector. The vector was designed to express insecticidal protein having hexa- histidine tag at the N-terminus. The recombinant vector was transformed in BL21(DE3)pLysS strain of *E. coli.* Clones were screened for the expression of fusion protein, after induction with IPTG. Optimal growth and expression conditions were optimized at small scale culture. For the purification of recombinant fusion protein, 500 µl overnight grown culture was inoculated in 500 ml medium (in 2 liter flask, 4 flasks) and grown (200 rpm, 37°C) till OD_{600} reached 0.6-0.8. The expression of the insecticidal protein was induced by addition of IPTG (200

rpm, 20°C, 4h). The cells were harvested, lysed with lysozyme and sonicated. Total soluble proteins were collected after centrifugation (40,000xg, 20 min, 4°C) and loaded on Ni-NTA-affinity column. Column was washed and bound protein was eluted with 250 mM imidazole. Undesired proteins in affinity purified protein were removed by ion exchange chromatography. Purity and molecular size of the recombinant protein was analyzed on analytical size-exclusion column (Superdex 200). Purified protein was nearly 95% pure, and retained its desired biological property (toxic to whiteflies). The protein was confirmed with Peptide Mass Fingerprinting and western blot analysis.

[0096] Purified insecticidal protein from rhizome, leaf and *E. coli* were analyzed together by Immuno Blot Analysis. Primary antibody was developed in rabbit against insecticidal protein purified from rhizome and used in dilution 1:5000. HRP labeled antibody was used as secondary antibody at dilution 1:20000.

ADVANTAGES OF THE PRESENT INVENTION

[0097] Since none of the reported insecticidal proteins are toxic against whitefly at sub ppm level, we identified a protein toxic to whitefly at ng/ml concentration. The identified protein is novel in its sequence and function and has huge potential in pest management.

[0098] The identified protein also causes mortality and significant growth retardation in *H.armigera*. It can be used in combination with other known toxic proteins to delay the resistance development in target pests. Hence, it has potential use in resistance management in target insect pests.

[0099] The present invention is related to plant derived molecules (insecticidal protein) so its durability and acceptance is more compared to other molecules like chemical pesticides.

[00100] Bio-molecules are known for their selectiveness and have less adverse effect on beneficial and non-target pests as compared to chemical pesticides. These properties also enhance its applicability in pest management.

[00101] Since the same protein is toxic to both whitefly and *H.armigera*, which are important pests of several crops, its candidature for insect pest management is strong compared to other proteins toxic to single insect pest only.

I/We claim:

1. A cDNA fragment having nucleotide sequence as set forth in SEQ ID NO: 1, said cDNA fragment encoding a polypeptide with insecticidal properties having amino acid sequence as set forth in SEQ ID NO: 2.

2. A polypeptide having insecticidal properties having amino acid sequence as set forth in SEQ ID NO: 2.

3. A set of primers having sequence as set forth in SEQ ID NO: 3 and SEQ ID NO: 4.

4. A nucleotide fragment encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 2.

5. The nucleotide fragment as claimed in claim 4, wherein said nucleotide fragment sequence is as set forth in SEQ ID NO: 1.

6. A DNA construct comprising a cDNA fragment as claimed in claim 1; or a nucleotide fragment as claimed in any of the claims 4-5, operably linked to a promoter.

7. A DNA vector comprising a DNA construct as claimed in claim 6.

8. A recombinant host cell comprising a DNA construct as claimed in claim 6.

9. A recombinant host cell comprising a DNA vector as claimed in claim 7.

10. The recombinant host cell as claimed in any of the claims 8-9, wherein said recombinant host cell is of bacterial, fungal, or plant origin.

11. A method of production of a polypeptide as claimed in claim 2 said method comprising:

- obtaining a recombinant host cell comprising a DNA construct as claimed in claim 6 or a DNA vector as claimed in claim 7;
- subjecting said recombinant host cell to conditions which result in expression of said polypeptide in said recombinant host cell.

12. The method as claimed in claim 11, further comprising the step of isolation or purification of said polypeptide.

13. A cDNA fragment as claimed in claim 1; or a polypeptide as claimed claim 2; or a nucleotide fragment as claimed in any of the claims 4-5 for use in inhibiting whitefly, or *H.armigera*.

Sheet no 1

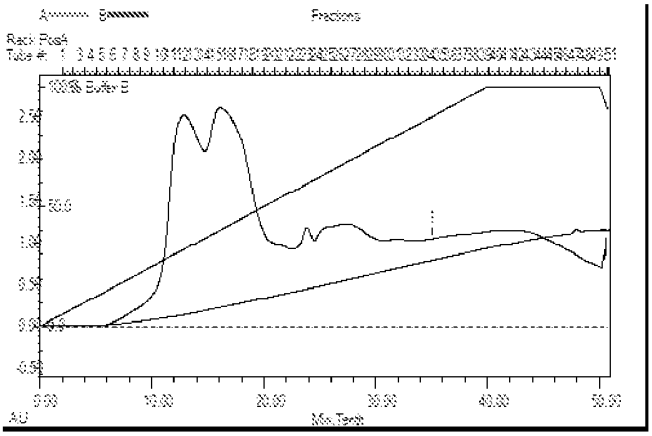

FIGURE 1

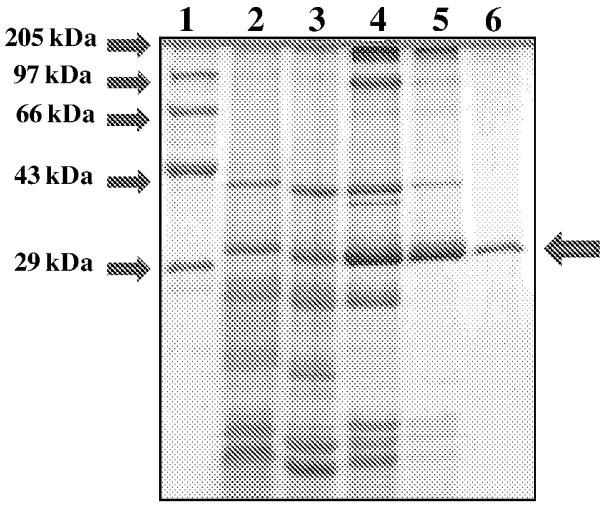

FIGURE 2

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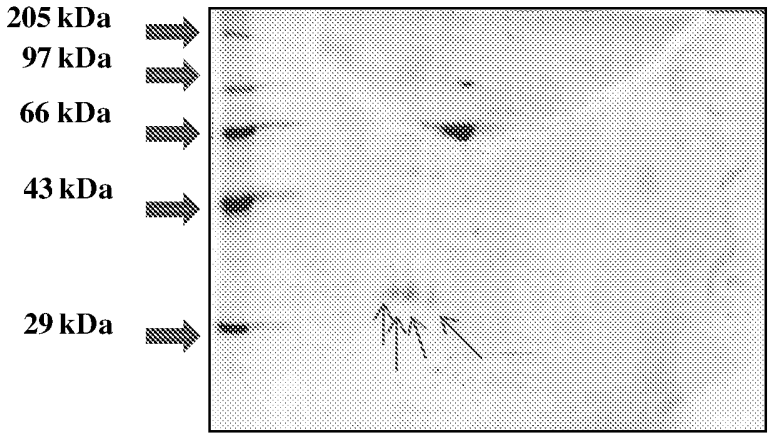

FIGURE 3

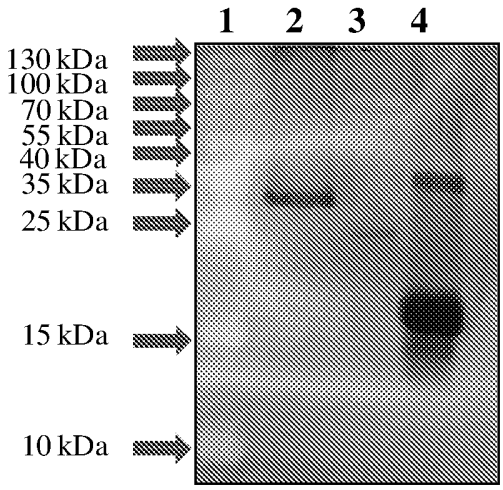

FIGURE 4

Sheet no 3

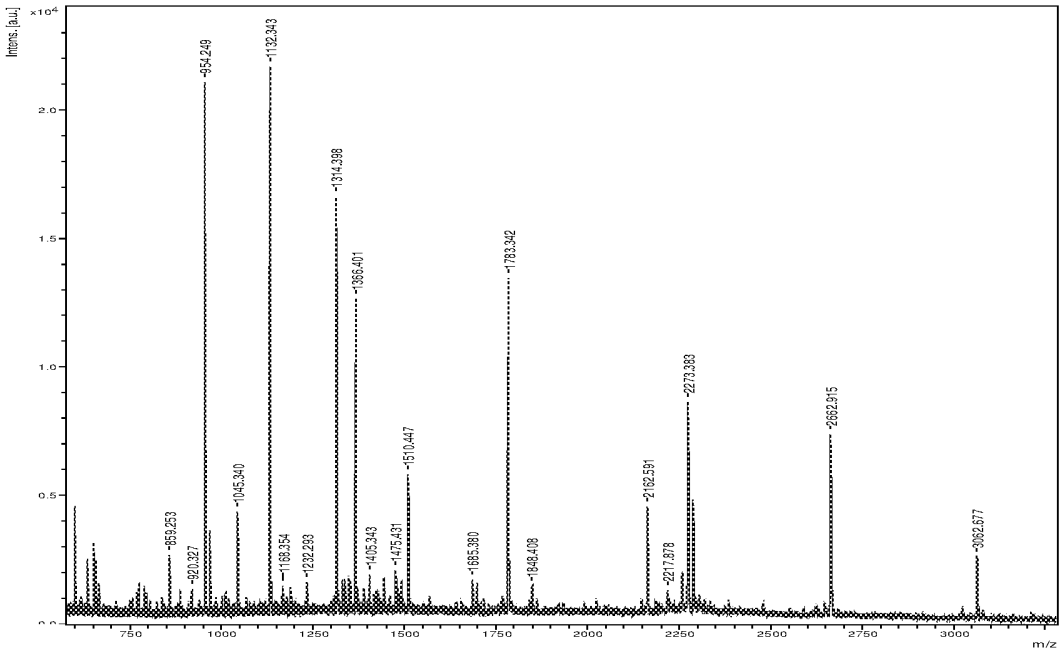

FIGURE 5

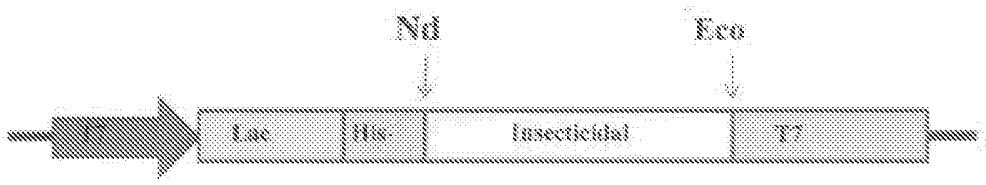

FIGURE 6

Sheet No 4

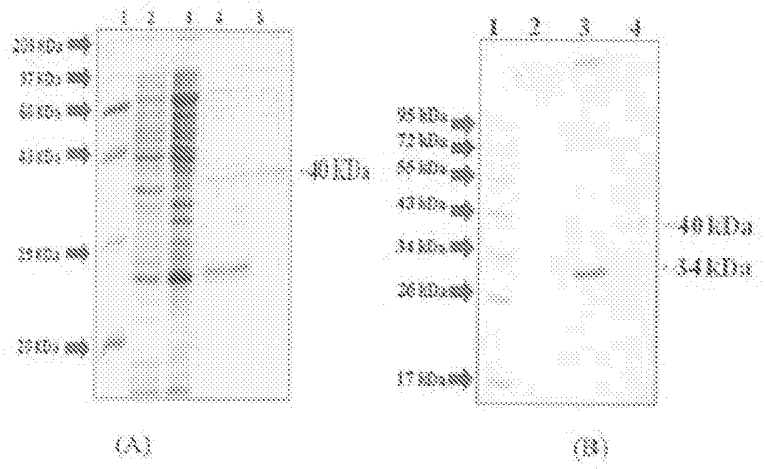

FIGURE 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/IN2015/050165

A. CLASSIFICATION OF SUBJECT MATTER
INV. A01N63/02 C07K14/415 C12N15/82
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A01N C07K C12N

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

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

EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2013/098858 A2 (COUNCIL SCIENT IND RES [IN]) 4 July 2013 (2013-07-04)	1-13
X,P	WO 2015/120270 A1 (PIONEER HI BRED INT [US]; DU PONT [US]) 13 August 2015 (2015-08-13) page 39, line 9 - line 14	1-13

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 March 2016	05/04/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Smalt, Rolf

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2015/050165

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

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

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

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

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

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

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

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

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IN2015/050165

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2013098858 A2	04-07-2013	AU 2012360018 A1	17-07-2014
		CA 2861194 A1	04-07-2013
		EP 2798061 A2	05-11-2014
		US 2015139976 A1	21-05-2015
		WO 2013098858 A2	04-07-2013
WO 2015120270 A1	13-08-2015	NONE	