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Inhibitory effect of a defensin gene from the Andean crop maca (*Lepidium meyenii*) against *Phytophthora infestans*

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Plant defensin

Summary

In this study, we report the isolation of a defensin gene, *lm-def*, isolated from the Andean crop 'maca' (*Lepidium meyenii*) with activity against the pathogen *Phytophthora infestans* responsible of late blight disease of the potato and tomato crops. The *lm-def* gene has been isolated by polymerase chain reaction (PCR) using degenerate primers corresponding to conserved regions of 13 plant defensin genes of the Brassicaceae family assuming that defensin genes are highly conserved among cruciferous species. The *lm-def* gene belongs to a small multigene family of at least 10 members possibly including pseudogenes as assessed by genomic hybridization and nucleotide sequence analyses. The deduced mature Lm-Def peptide is 51 amino acids in length and has 74–94% sequence identity with other plant defensins of the Brassicaceae family. The Lm-Def peptide was produced as a fusion protein using the pET-44a expression vector and purified using an immobilized metal ion affinity chromatography. The recombinant protein (NusA:Lm-Def) exhibited in vitro activity against *P. infestans*. The NusA:Lm-Def protein caused growth inhibition and hyphal damage at concentration not greater than 0.4 μM. In contrast, the NusA protein alone expressed and purified similarly did not show any activity against *P. infestans*. Therefore, these results indicate that the *lm-def* gene isolated from maca belong to the plant defensin family with activity against *P. infestans*. Its expression in potato, as a transgene, might help to control the late blight disease caused by *P. infestans* with the advantage of being of plant origin.

Abbreviations: Bp, base pair; CTAB, cetyl trimethyl ammonium bromide; EDTA, ethylene diamine tetra-acetic acid; *lm-def*, *Lepidium meyenii* defensin gene; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR; TBE, tris borate EDTA buffer

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Introduction

Phytophthora infestans, the causal agent of late blight disease, causes serious losses to potato and tomato crops worldwide and is probably the most important pathogen of both crops (William and Stephen, 1997). This disease was the cause of the Irish potato famine in the 1840s which resulted in about one million deaths and the emigration of about 1.5 million people to other parts of the world, particularly to the US. Even now, annual crop losses and fungicide costs amount to about US$4 billion throughout the world. While considerable efforts have been invested in plant breeding and genetic engineering, sources of resistance to late blight are still in great needs. Although recent successes in expressing an *R* gene from a Mexican wild potato species which confers partial and broad-spectrum resistance to late blight (Song et al., 2003; van der Vossen et al., 2003), most of genetic engineering strategies have used peptides and proteins from diverse origin. For instance, pathogenesis-related (PR) proteins such as chitinases (Nishizawa et al., 1999; Terakawa et al., 1997), 1,3-β-glucanase (Lusso and Kuc, 1996; Masoud et al., 1996), and osmotin-like proteins (Liu et al., 1994) have been used to engineer resistance to pathogens because of their capacity to degrade at least partially fungal and bacterial cell walls. Other plant proteins were shown to bear antifungal activities such as the case of the storage protein, ocatin, found in the Andean tuber crop oca *Oxalis tuberosa* (Flores et al., 2002), an isoform of patatin from potato (Sharma et al., 2004) and the sporamin storage protein from sweet potato (Yeh et al., 1997). In some cases, peptides from potato were constitutively expressed in potato and displayed antimicrobial activity such as the pseudothionin-*St*1 (Moreno et al., 1994) and snakin-1 (Segura et al., 1999).

Plant defensins comprise a family of small cationic, cysteine-rich peptides (45–54 amino acid) that are mostly found to contribute to broad-spectrum host defense against pathogens and are widely distributed among plants, including wheat, barley, spinach, pea, and several members of the Brassicaceae family (Broekaert et al., 1995, 1997; Lay and Anderson, 2005; Thomma et al., 2002). Many plant defensins can inhibit the growth of a broad range of fungi at micromolar concentrations but are non-toxic to both mammalian and plant cells (Broekaert et al., 1995; Moreno et al., 1994; Osborn et al., 1995; Terras et al., 1995). The first plant defensins that were demonstrated to possess antimicrobial activity were the two plant defensins isoforms Rs-AFP1 and Rs-AFP2 isolated from the radish seed from Brassicaceae family (Terras et al., 1992). Because at least some of the plant defensins were shown to be induced upon pathogen invasion, these are referred also as members of the family PR-12 of pathogenesis-related proteins (Van Loon and Van Strien, 1999). The use of defensin genes in genetic engineering has resulted in broad spectrum resistance against fungal pathogens (Gao et al., 2000; Kanazaki et al., 2002). Genome organization was in general reported as a small multigene family with, for example, up to 15 members in *Arabidopsis thaliana* (Thomma et al., 2002). However, a recent reassessment of defensin-like sequence in the near-complete genome sequence of *A. thaliana* revealed that 317 homologous sequences could be identified (Silverstein et al., 2005). Defensins may have evolved into such a large multigene family in order to provide non-host resistance to numerous pathogens in many different tissues and in addition seem also to be involved in non-pathogen resistance mechanisms (Silverstein et al., 2005). The precise mode of action of defensin is still under investigation but modification of plasma membrane has been reported as the most likely primary interaction, secondary activities in the cell may involve enzyme inhibition, ion channel inhibitors, and others (Lay and Anderson, 2005; Thevissen et al., 1999).

One member of the Brassicaceae family is the Andean crop maca (*Lepidium meyenii*) which is known to have good antimicrobial defense. Its cultivation is restricted today to the Departments of Junín and Cerro de Pasco of Peru at elevations above 3500 m and often reaching 4450 m in the central Andes of Peru (León, 1964; Tello et al., 1992). In this study, we report the isolation, cloning, characterization, expression and purification of a defensin gene from maca and the evaluation of its activity against *P. infestans*. Our results indicate that this defensin gene of maca is of potential use in the development of transgenic potato plants resistant to late blight disease.

Materials and methods

Plant material

Leaves of maca plants (*L. meyenii* Walp.) were obtained from greenhouse grown plants derived from seeds collected directly in the field where the crop is locally grown and consumed (central Andes of Peru, 3500 m altitude).

Primers design for defensin gene isolation from maca

Sequences of plant defensins from GenBank (http://www.ncbi.nlm.nih.gov) and EMLB (http://www.ebi.ac.uk) were used to design degenerated primers. The alignment of the sequences was performed using the algorithm ClustalW of the program Bioedit vs 7.0.1 (Hall, 1999). Primers were designed using the most conserved part of the nucleotide and protein sequence. Forward primers were as follows: N1 at the position of the initiation codon 5′ ATGGCTAAGTYTGCTTCYATC 3′ (Y = C or T), N2 at a position just upstream of the intron 5′ GCTCTTGTTCTYTTTGCTGCTTT 3′ (Y = C or T) which is present in 13 defensin genes of four species of the Brassicaceae family (*Raphanus sativus* Rs_AFP1, Rs_AFP2, Rs_AFP3, Rs_AFP4; *A. thaliana* pdf1.1, pdf1.2, AFP1_ARATH, T19L18.17, T19L18.18, Mfc16.8-At, Mfc16.9-At; *Wasabia japonica* gamma-thionin1; *Brassica napus* AFP3_Brana), and N3 and N4 at a position downstream of the intron and upstream (four amino acids) of the first amino acid of the mature protein 5′ TGGTGGAAGCNAAGTTGTG 3′ and 5′ TGGTGGAAGCNCAGAAG TTGTG 3′ (N = A, C, G, or T) respectively. The unique reverse primer C1 was designed at the position of the stop codon 5′ TTAACATGGGAMGTARCAGATGCA 3′ (M = A or C; R = A or G).

Polymerase chain reaction (PCR) amplification and cloning amplicons

Total DNA was extracted from leaves by the cetyl trimethyl ammonium bromide (CTAB) method adapted from Murray and Thompson (1980). PCR reactions were carried out following standard PCR protocol using an annealing temperature of 52 °C (Williams et al., 1993). PCR products were resolved by agarose gel electrophoresis in a tris borate EDTA (TBE) 1 × buffer (10 × TBE 0.89 M Tris-base, 0.89 M Boric Acid, 20 mM ethylene diamine tetra-acetic acid (EDTA)) and visualized by staining with 0.5 μg/mL ethidium bromide. PCR amplicons were purified from 0.8% agarose gel slices using the MinElute Gel Extraction kit (Qiagen, Germany) following manufacture's protocol. Purified DNA was cloned into pCR 2.1 vector (TA Cloning Kit, Invitrogen Life Technologies, USA) following the manufacture's instructions.

Reverse transcriptase PCR (RT-PCR) analysis

RNA was isolated from hypocotyls of maca plants by Trizol method according the manufacturer instructions (Invitrogen, USA). RT-PCR was carried out using the Acces RT-PCR System kit (Promega, USA). The RT-PCR conditions were carried out according to the manufacturer protocol using an annealing temperature of 55 °C.

DNA sequencing and analysis

DNA sequencing was done with the fmol DNA Cycle Sequencing System kit (Promega, USA) using [γ-32P] ATP (Amersham Biosciences, USA) to label the M13 universal primers. Sequencing reactions were then loaded on 6% denaturing polyacrylamide gels with 8 M urea at 1200 V for 4 h. DNA fragments were fixed in 10% acetic acid for 30 min and gels were subsequently air dried. Autoradiography was obtained using Kodak film BioMax. Sequences were analyzed using Bioedit and pDRAW software.

DNA gel blot hybridization

Genomic DNA (15 μg) was digested with the restriction endonucleases *Cla*I and *Eco*RV, separated on a 0.8% agarose gel and transferred to Hybond-N^+ nylon membranes (Amersham Pharmacia Biotech, USA) following standard procedures (Sambrook et al., 2001). DNA probe was separated by electrophoresis in agarose and purified using the MinElute Gel Extraction kit (Qiagen, Germany). Fifty nanograms of the probe was labeled with fluorescein-11-UTP using the Random Prime Labeling kit (Amersham Pharmacia Biotech, USA). The nylon membrane was then hybridized at 60 °C for 18 h and then washed with 1 × SSC, 0.1% SDS (w/v) and 0.5 × SSC, 0.1% SDS (w/v) at 60 °C for 15 min each with gentle agitation. Band detection was done with the Gene Images CDP-Star kit (Amersham Pharmacia Biotech, USA). The nylon membrane was exposed for 1 h using Kodak film and GBX reagents (Sigma-Aldrich, USA).

Construction, expression and purification of recombinant NusA-Lm-Def

The coding sequence of the mature peptide of the *L. meyenii* defensin (*lm-def*) gene was obtained by PCR amplification of a 153 bp fragment with a forward 5′ phosphorylated primer Np-44 F (5′ ACAAAAGCTGTGCGAGCGATCAAG 3′) and a reverse primer Cp-44 R (5′ CGGAATTCTATTAACATGGGACGTAGCAGATGC 3′). The product was digested with *Eco*RI and ligated into *Psh*AI and *Eco*RI sites of the vector pET44a (Novagen, USA) containing the NusTag sequence (NusA and 6XHis-Tag) under the control of the T7 promoter (Fig. 5a). A 200-mL

culture of transformed *Escherichia coli* BL21 (strain DE3) was grown to an optical density of 0.6 at 600 nm after which expression of the recombinant protein was induced by adding 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). After 4 h of induction, bacteria were harvested by centrifugation at 6000*g* for 5 min, lysed, and the protein fraction purified using Talon Purification kit (Clontech, USA). The purification of NusA:Lm-Def was performed using affinity chromatography with the 6XHis-Tag NusA-Def using an immobilized metal ion affinity chromatography resin provided with the kit. The recombinant protein NusA:Lm-Def of 609 amino acids long with a calculated molecular weight of 66 kDa was purified by gel electrophoresis. The protein was isolated from a 10% SDS-polyacrylamide gel stained with Coomassie Brilliant blue R-250 by dialysis in a Slide-A-Lyzer 3.5K Dialysis Cassette (Pierce, USA) in Tris 50 mM (pH 8.0) solution at 4 °C, with three changes of buffer every 6 h and then finally concentrated with polyethylene glycol. The protein concentration was measure by the Bradford method (1976) using bovine serum albumin fraction V as standard (Sigma-Aldrich, USA).

Antimicrobial activity assay

A spectrophotometric method was developed to quantify the activity of Lm-Def against *P. infestans* based on Broekaert et al. (1990). Sporangia or hyphal pieces were isolated from an aggressive strain of *P. infestans* (strain PHU82) grown on potato dextrose agar incubated at 18 °C for 3 days. After the incubation, the pathogen was harvested in sterile water and diluted to a concentration 250 sporangia in 100 μL of a dilution broth solution (2500 sporangia mL^{-1} by hemocytometry). Dilution broth solution was obtained by boiling fresh pea seeds for 20 min in distilled water which, then, was clarified by filtration and autoclaved for 15 min at 121 °C at 15 Psi. One hundred microliters (250 sporangia) of the spore suspension was mixed with 50 μL of the NusA-Lm-Def protein (0.2 and 0.4 μM) and completed to 200 μL with 50 mM Tris–HCl buffer (pH 8.0) into a sterile 96-well flat-bottom microtiter plate. As controls, both the NusA protein (0.4 μM) (protein control) and 50 μL of 50 mM Tris HCl buffer (pH 8.0) (blank control) were used. The plates were covered and homogenized by gentle shaking. Plates were incubated at 18 °C in darkness for 24 h. Each assay was performed in triplicate. Spore germination and growth were measured with a microtiter plate reader at 595 nm 24 h after inoculation. Hyphae were stained with lactophenol cotton blue. Microscopic observations were made at several time points during the incubation period using a light microscope.

Results

Isolation of a defensin gene from *L. meyenii*

Nucleotide sequences of different defensin genes of four species of the Brassicaceae family were aligned to design five degenerated primers to amplify homologous defensin sequences of *L. meyenii* (Fig. 1). The four forward primers (N1, N2, N3, and N4) and one reverse primer (C1) were designed to amplify different regions of the defensin gene corresponding to the full coding sequence (N1-C1), the sequence starting at the intron (N2-C1), and the sequence corresponding to the mature protein (N3-C1; N4-C1) (Fig. 1).

The first three primer pairs produced fragments of the expected size based on their position on the consensus sequence. The N4-C1 pair did not produce any amplicons. The product N1-C1 had an approximate molecular weight of 330 bp, N2-C1 of 290 bp, and N3-C1 of 160 bp estimated by gel electrophoresis. These PCR amplicons were purified and cloned for nucleotide sequence determination. The sequences of 6 clones from amplicon N1-C1 were all identical and exactly 330 bp long (Fig. 2). This sequence represents a full coding sequence of a defensin gene from *L. meyenii* and is referred to as *lm-def* gene (GenBank accession number AY829229). The transcriptional activity of this sequence was confirmed by RT-PCR using primers N1-C1 which produced a single band of 243 bp using total RNA from leaves (Fig. 4B). Two different sequences were obtained for the amplicon produced using the primers N2-C1, one corresponded exactly to the *lm-def* gene while the other, referred to as N2-C1, of 291 bp, displayed sequence variation from the *lm-def* gene (two frame shifts; one nucleotide addition in the first exon; and one deletion in the second exon in addition to single nucleotide substitution and an additional triplet). These changes leading to loss of half of the structurally conserved cysteine residues and the lack of a stop codon suggest that N2-C1 might not be functional. The nucleotide sequences obtained from sequencing the amplicon N3-C1 is also homologous to the *lm-def* gene but presents one additional codon and several single nucleotide changes which result in a premature termination of the putative peptide with a stop codon at position 247 (the numbering is respect to the

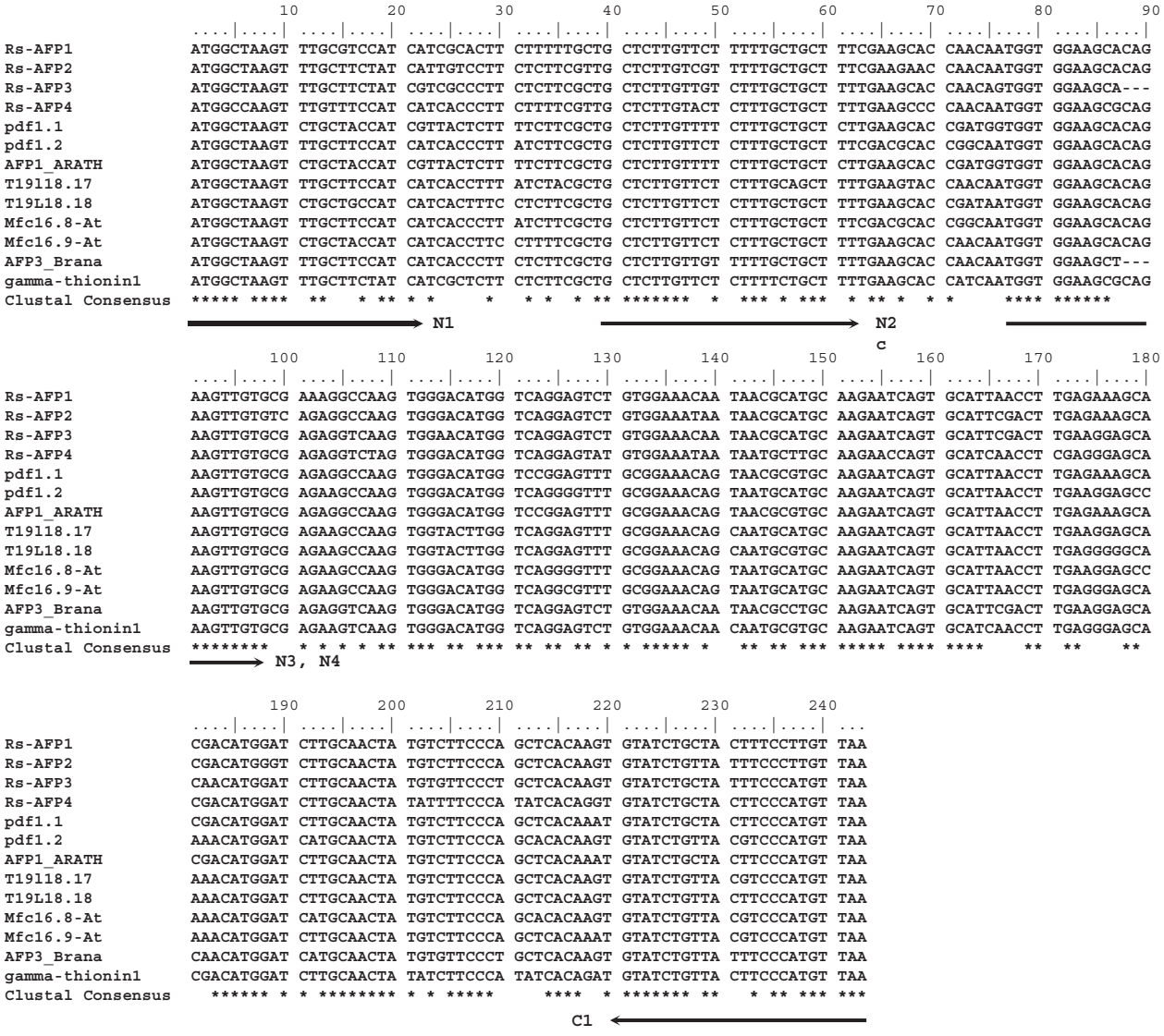

Figure 1. Design of degenerated primers from defensin cDNAs from four species of the Brassicaceae family: *Raphanus sativus*: Rs_AFP1 (U18557), Rs_AFP2 (genbank:U18556U18556), Rs_AFP3 (X97319), Rs_AFP4 (X97318); *Arabidopsis thaliana*: pdf1.1 (AF049870), pdf1.2 (A68647), AFP1_ARATH (AC007396), T19L18.17 (AC004747), T19L18.18 (AAC31234), Mfc16.8-At (NM_123809), Mfc16.9-At (NM_123810, AB01765); *Wasabia japonica*: gamma-thionin1 (AB012871); *Brassica napus*: AFP3_Brana (BNU59459). The arrows indicate the primers used in this study (N1, N2, N3, N4 (forward), and C1 (reverse).

lm-def sequence) (Fig. 2). Therefore, N3-C1 is also likely not to be functional. However, definitive proof of this sequence and the N2-C1 being part of pseudogenes will require additional transcriptional and genomic sequence analyses.

The comparison of the deduced amino acid sequence encoded by the *lm-def* gene with other defensins from the Brassicaceae family indicates that it is closely related to Rs-AFP4 and gamma-thionin1 with sequence similarity ranging between 74% and 94% (Fig. 3). These results indicate that the *lm-def* gene encodes a protein of the morphogenic defensin type I (De Samblanx et al., 1997) of 51 amino acids.

Genomic characterization of *lm-def* sequences

Genomic DNAs from three different maca accessions, chosen randomly, were analyzed for *lm-def* sequence by genomic DNA hybridization. Hybridization with the *lm-def* 330 bp sequence as a probe revealed high levels of polymorphism using

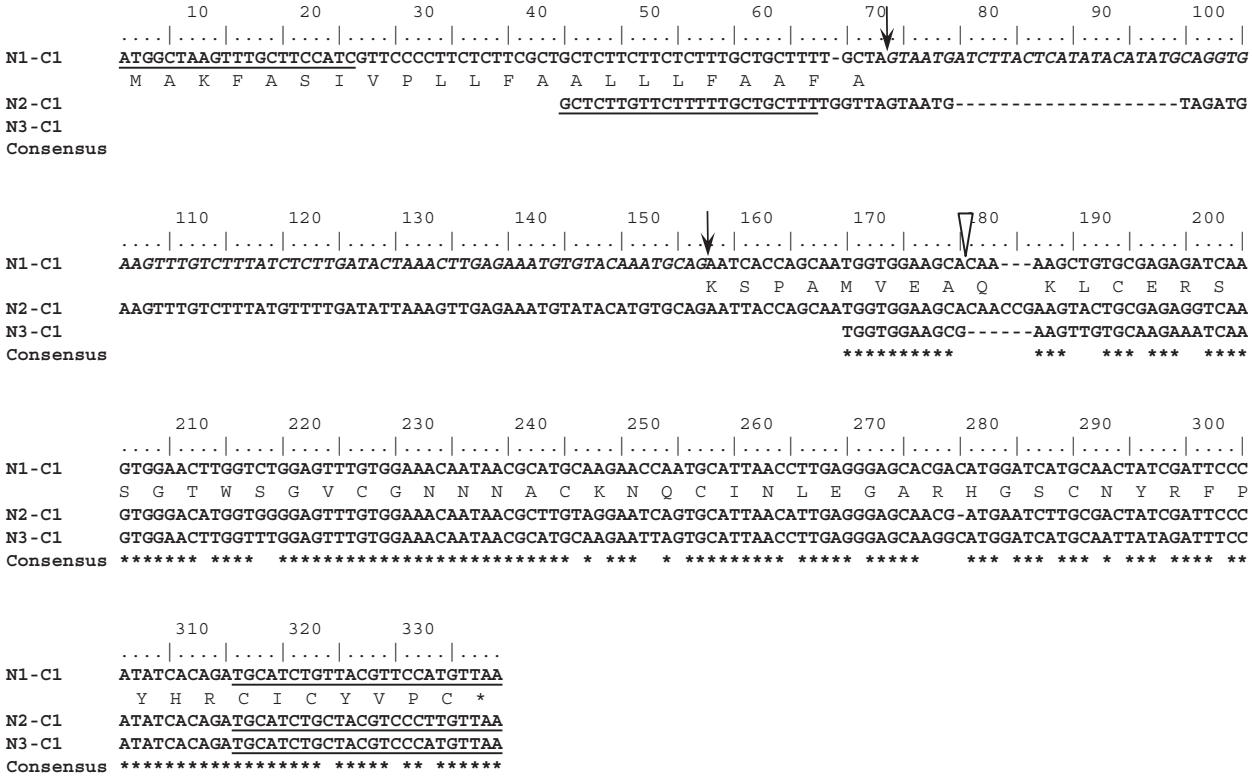

Figure 2. Defensin gene sequences isolated from maca (*Lepidium meyenii*) obtained with three primer combinations: N1-C1 (referred to as the *lm-def* gene), N2-C1, and N3-C1. Primer sequences are underlined. The *lm-def* gene starts with its first initiation codon, two arrows indicate the beginning (position 67) and end (position 152) of an intron, and ends with a stop codon. The putative initiation of the mature peptide is indicated by an inverted triangle. The deduced amino acid sequence is presented below the N1-C1 sequence (# Acc. AY829229).

restriction enzymes *Cla*I which digests one site within the *lm-def* gene and *Eco*RV which does not digest within this sequence (Fig. 4). DNA probe was obtained by PCR of the plasmid with N1-C1 fragment of 330 bp (complete coding sequence of *lm-def* gene). The multiple banding pattern as well as differences in band intensities reveals the existence of multiple copies of *lm-def* sequences. The estimation of number of copies is in range of 10 copies which coincides with the results observed from PCR sequencing of the amplicons.

Expression and purification of recombinant NusA:Lm-Def protein

Because purification of defensins from bacterial expression systems was reported to be problematic (Park et al., 2002), we pursued a fusion protein approach in order to test its activity against *P. infestans*. The coding sequence of the mature *lm-def* gene (153 bp) was cloned in frame with the NusA gene to produce a fusion protein referred to as NusA:Lm-Def (Fig. 5A). The fusion protein as well as the NusA protein alone were purified and eluted from polyacrylamide gels (Fig. 5B). The eluted fractions were pooled and dialyzed until approximately 1 mg of recombinant and non-recombinant proteins were obtained.

Antimicrobial properties of the recombinant NusA:Lm-Def protein

The purified recombinant NusA:Lm-Def protein was used to test activity against *P. infestans*. Inhibitory activity of *P. infestans* growth was observed in liquid medium and measured by absorbance at 600 nm. Around 93% growth inhibition was observed at approximately 0.4 μM NusA:Lm-Def protein concentration whereas the non-recombinant protein did not inhibit *P. infestans* growth at this concentration. The assay was repeated three times successfully (Table 1). Under microscopic examination, hyphae in presence of NusA:Lm-Def exhibited an unhealthy state at concentration of 0.4 μM where extensive septum formation and thinning of the unhealthy hyphae

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                    10         20         30         40         50         60         70         80
                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Lm-Def              MAKFASIVPL LFAALLLFAA FESPAMVEAQ KLCERSSGTW SGVCGNNNTC KNQCINLEGA RHGSCNYRFP YHRCICYVPC
Rs-AFP1             .......IA. .....V.... ..A.T..... .....P.... ........A. ........K. .......V.. A.K....F..
Rs-AFP2             .......IV. ..V..VV... ..E.T..... ...Q.P.... ........A. .....R..K. .......V.. A.K....F..
Rs-AFP3             ........A. .....VV... ..A.TV...- .......... ........A. .....R.... Q......V.. A.K....F..
Rs-AFP4             ....V..IT. ..V..V.... ..A.T..... .......... ........A. .......... .......I.. .......F..
pdf1.1              ...S.T..T. F....VF... L.A.MV.... .....P.... ......S.A. ........K. .......V.. A.K....F..
pdf1.2              ...S.T..T. F....VF... L.A.MV.... .....P.... ......S.A. ........K. .......V.. A.K....F..
AFP1_ARATH          ...S.T..T. F....VF... L.A.MV.... .....P.... ......S.A. ........K. .......V.. A.K....F..
T19L18.17           .......ITF IY...V.... ..V.T..... ....KP.... ......S.A. .......... K......V.. A.K.......
T19L18.18           ...S.A.ITF .....V.... ..A.I..... ....KP.... ......S.A. .......... K......V.. A.K....F..
Mfc16.9-At          ...S.T.ITF .....V.... ..A.T..... ....KP.... ......S.A. .......... K......V.. A.K.......
Mfc16.8-At          .......IT. I....V.... .DA..... ....KP.... ......S.A. .......... K......V.. A.K.......
AFP3_Brana          .......IT. .....VV... ..A.T....- .......... ........A. .....R.... Q......V.. A.K....F..
gamma-thionin1      .......IA. .....V..S. ..A.S..... ....K..... ........A. .......... .......I.. .......F..
Clustal Consensus   *** .:*: : ::.**:.*:* :: * :***  ***::.**** ******.*:* *****.** * :****** **  *:****.**
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Figure 3. Similarity analyses of the deduced amino acid sequence of the *lm-def* gene with 13 plant defensins from the Brassicaceae family. Dots indicate conserved residues. The sequences analyzed are the deduced Lm-Def (# Acc AY829229), Rs-AFP1 (# Acc. AAB22709), Rs-AFP2 (# Acc. AAA69540), Rs-AFP3 (# Acc. O24332), and Rs-AFP4 (# Acc. O24331) from *Raphanus sativus*; pdf1.1, pdf1.2 (# Acc. AAM91721/AAL36086), AFP1_ARATH (# Acc. P30224), T19L18.17 (# Acc. T02621), T19L18.18 (# Acc. T02622), Mfc16.9-At (# Acc. NP_199256), and Mfc16.8-At (# Acc. NP_199255) from *Arabidopsis thaliana*; AFP3-Brana (# Acc. Q39313) from *Brassica napus*, and gamma-thionin1 (# Acc. BAB19054) from *Wasabia japonic*a.

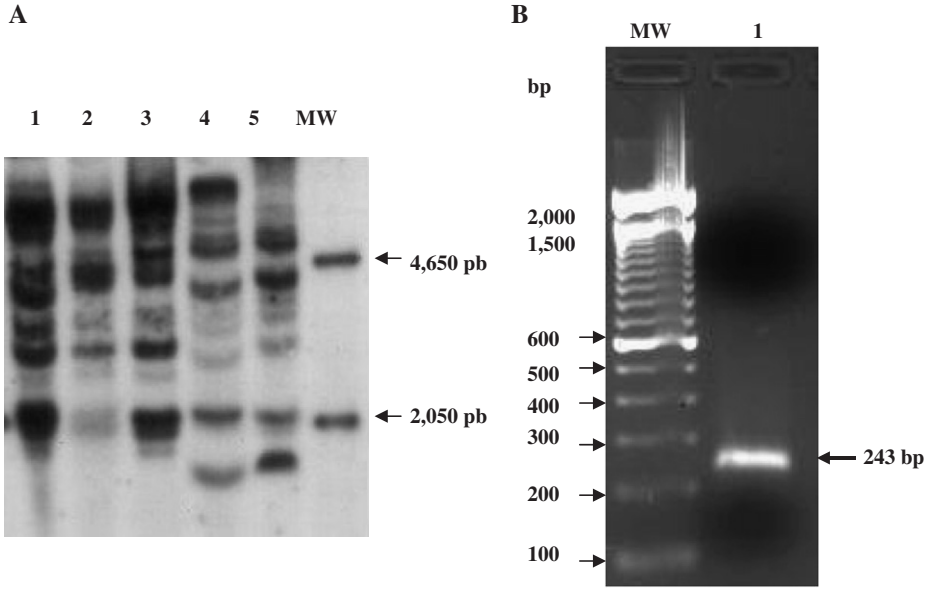

Figure 4. Molecular analyses of *lm-def* gene in *Lepidium meyenii*. (A) Genomic hybridization using the full *lm-def* gene of 330 bp as probe: Lanes 1, 2, and 3 are three different maca accessions digested with *Cla*I. Lanes 4 and 5 represent two of them digested with *Eco*RV. Lane MW represents the pET44a-Lm-Def digested by *Sph*I (one internal site in *lm-def* gene) to provide a size marker. (B) Reverse transcriptase PCR of *lm-def* gene using primers N1-C1 displays a band of 243 bp corresponding to the sequence amplified from RNA genome of maca.

could be observed (Fig. 6). The protein concentration required for 50% growth inhibition (IC_{50}) was estimated to be of 96.9 μg/mL.

Discussion

Defensins are a class of antimicrobial peptides found in several plant species including several members of the Brassicaceae family. Because the maca (*L. meyenii*) crop belongs to this family, we investigated whereas it possesses homologs of known defensin genes. Using a degenerated primer cloning approach, we were able to isolate an *lm-def* gene from maca. We observed by RT-PCR assay that this gene or very closely related copies are expressed in leaves. Hybridization analysis of genomic DNA from maca using the *lm-def* gene as

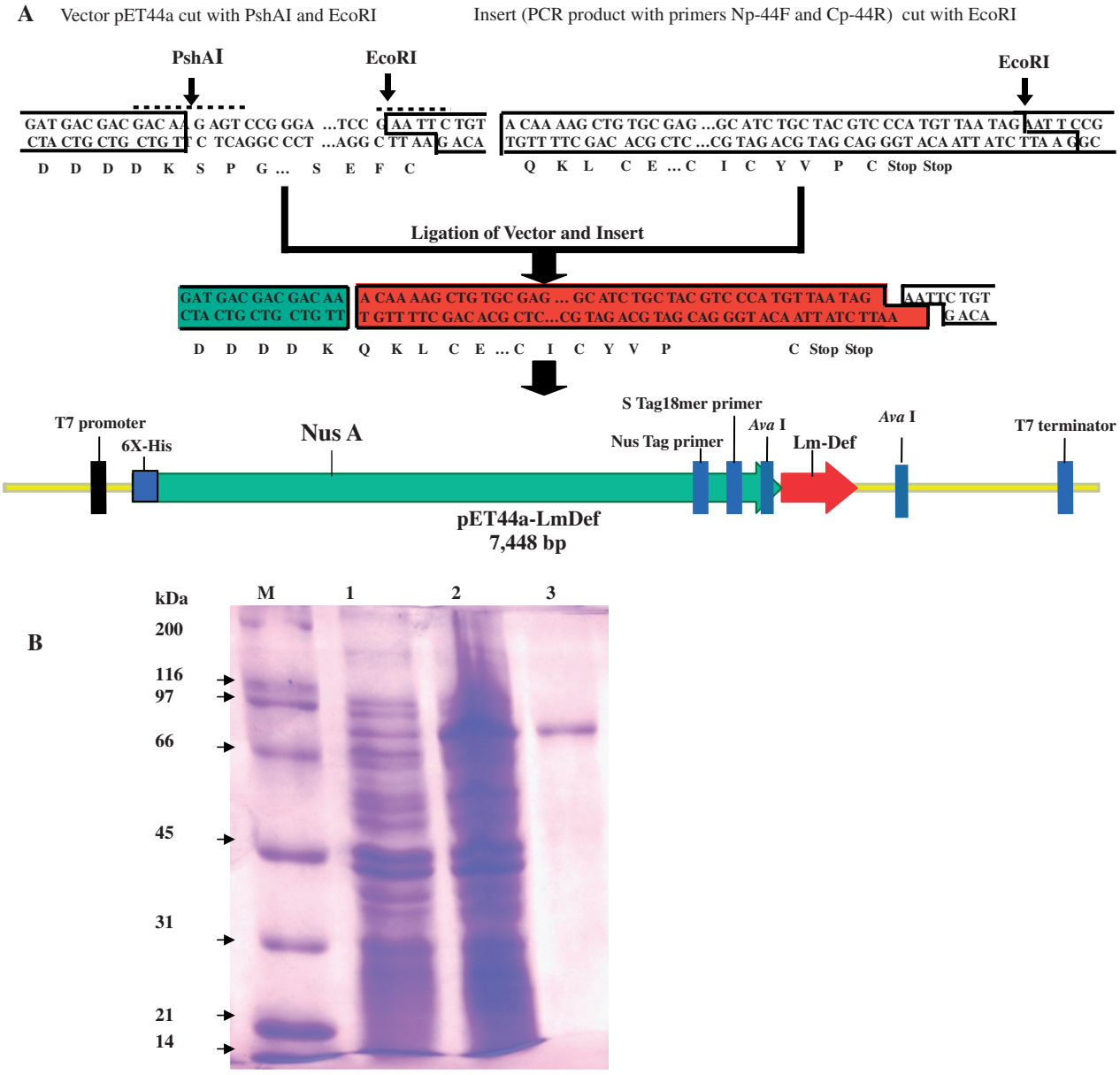

Figure 5. Production of the NusA:Lm-Def protein fusion. (A) Construction of the NusA:Lm-Def expression vector. (B) Purification of the fusion protein NusA:Lm-Def expressed in BLB21(DE3) *E. coli* cells and observed on a 10% SDS polyacrylamide gel stained with Coomassie blue. Lane M represent molecular weight, lane 1 the non-induced protein extract (10 μg), lane 2 the induced protein extract (10 μg), and lane 3 the affinity-purified NusA:Lm-Def protein (10 μg).

probe resulted in the identification of approximately 10 copies of *lm-def* sequences present in the maca genome with high level of polymorphism. Because the maca is an octoploid crop ($2n = 8X = 64$), with a haploid basic chromosome number of 8, several of these *lm-def* sequences could be allelic. The genetic mapping of these sequences would be needed to determine how many loci exist. Multiple genes were found in other plants as well. For instance, in *R. sativus*, four genes were isolated, Rs-AFP1 and Rs-AFP2 expressed in leaves and Rs-AFP3 and Rs-AFP4 expressed in seeds (Terras et al., 1995). Similarly in *A. thaliana*, the number of defensin genes was first reported to be 5 and designated as PDF1.1, 1.2, 2.1, 2.2, and 2.3 (Epple et al., 1997; Penninckx et al., 1996; Thomma and Broekaert, 1998) and increased to 15 by analyzing published genome sequence (Thomma et al., 2002). In the species *Brassica campestri* L. spp. *pekinensis*, a defensin BSD1 peptide was identified and DNA gel blot hybridization determined that this gene is likely to be present in multiple copies in the *B. campestris* genome (Park et al., 2002). However,

Table 1. Growth inhibition of *P. infestans* after 24 h incubation with NusA:Lm-Def fusion protein

	NusA alone 250 μg/mL (0.4 μM)	NusA:Lm-Def 150 μg/mL (0.2 μM)	NusA:Lm-Def 250 μg/mL (0.4 μM)
Absorbance mean (SD)	0.790 (0.05795)	0.386 (0.12578)	0.038 (0.00153)

Absorbance at 595 nm of *P. infestans* media with two concentrations of fusion protein NusA-Lm-Def and NusA protein alone (SD stands for standard deviation). Values represent means of three repetitions.

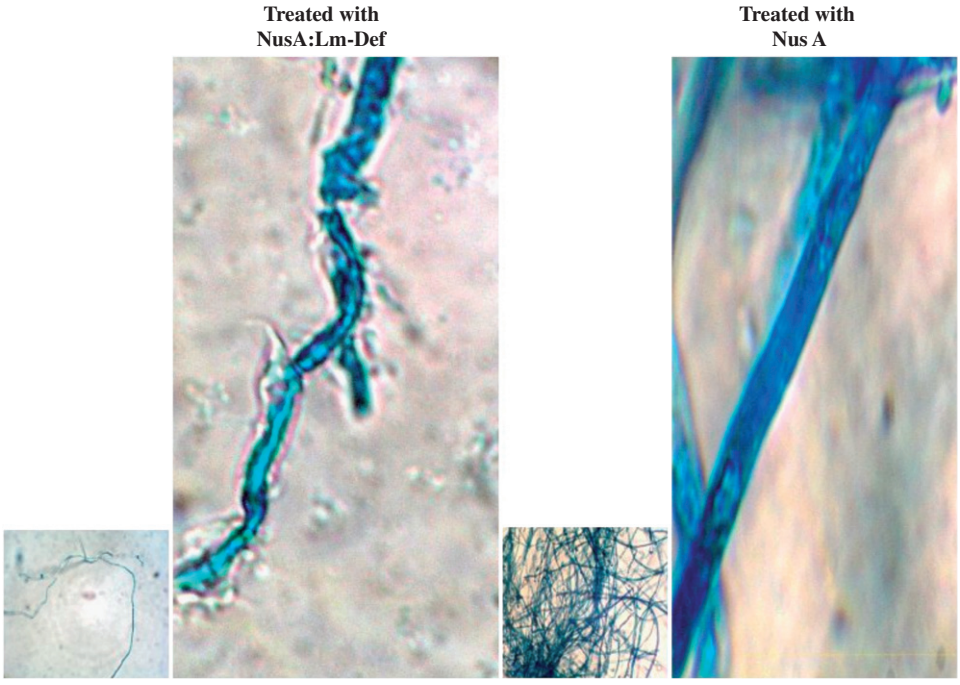

Figure 6. Damages of Lm-Def on *Phytophthora infestans*. Microscopic images of hyphae grown 24 h with 250 μg/mL of NusA-Lm-Def protein (left) and NusA alone (right).

recent search for defensin-like sequences in the near-complete genome sequence of *A. thaliana* resulted in 317 defensin-like sequences with 10% of them being pseudogenes (Silverstein et al., 2005). Therefore, it is possible that the *lm-def* defensin gene is a member of a large multigene family in maca as it is the case in *A. thaliana*.

Gene structure appears also to be remarkably conserved with an identical intron position and exon length as reported in the extensive gene structure analysis of Arabidopsis defensin-like sequences by Silverstein et al. (2005). However, a small number of floral defensins display an additional C terminal domain of 27–33 amino acids (Lay et al., 2003). Our cloning strategy does not allow ruling out the presence of such C terminal extension of the maca *lm-def* gene as well as minor sequence variation at the primer binding sites.

Amino acid sequences of plant defensins from 13 different plant species were found to bear only relatively few common residues apart from the cysteine conserved structure (Broekaert et al., 1995; Lay and Anderson, 2005). The Lm-Def amino acid sequence shares 74–94% sequence identity with group I defensins of the Brassicaceae family and between 30% and 70% with other defensins. The full peptide Lm-Def has 29 residue changes out of its 80 amino acids, two being unique, while the putative mature peptide sequence is more conserved. The conserved residues are restricted to the cysteine conserved residues [KRG]-x-C-x(3)-[SV]-x(2)-[FYWH]-x-[GF]-x-C-x(5)-C-x(3)-C typical of basic cysteine-rich defensin, and two glycines at position 13 and 34, a glutamate at position 29, and an aromatic residue at position 11 similar to most of the plant defensins (number relative to Rs-AFP1). Hence, as in the case of thionins, residues not related to peptide structure are variable and likely reflect distinct biological activities (Broekaert et al., 1997). The predicted molecular weight of Lm-Def is 5.7 kDa with a predicted isoelectric point of 8.73. The similarities of the *lm-def* gene

with known antimicrobial defensins lead us to test its activity against *P. infestans* which is one of the most important pathogen of the potato crop.

The mature peptide sequence was deduced from the *lm-def* gene sequence as of 51 amino acids assuming a conserved position of the cleavage of the signal peptide. Purification difficulties of defensin peptides from bacterial expression systems were reported previously (Park et al., 2002). Hence, to circumvent these potential limitations, we adopted a protein fusion strategy. The carrier protein, NusA, was chosen to fuse with Lm-Def because of its good solubilizing characteristics and high expression level (Harrison, 1999).

A growth inhibition assay using liquid medium was used to test Lm-Def activity against *P. infestans*. At the two concentrations tested, an inhibition of *P. infestans* growth was observed with the strongest being at 0.4 μM (only 3% growth compared with NusA alone after 24 h incubation). The observation of hyphae morphology in the presence of NusA-Lm-Def at 0.4 μM revealed clear damages compared to treatment with NusA alone. The IC_{50} was calculated using the fusion protein at two dose-response points and after only 24 h. The result, 96.9 μg/mL, is difficult to compare with other values reported using the purified defensins from radish seeds using different incubation medium and time (Terras et al., 1992). However, a fusion protein with BSD1 from *B. campestris* L. ssp. *pekinensis* was reported to have an IC_{50} of around 100 μg/mL against the oomycete *Phytophthora parasitica* (Park et al., 2002). Hence, it seems that the Lm-Def peptide is in the same range of growth inhibition of *P. infestans*.

Although conventional breeding and genetic engineering have recently developed resistance against late blight, the durability of such resistance is still under question. The use of antimicrobial peptides could be complementary to these other sources of resistance to late blight by increasing its stability. Very few antimicrobial compounds have been shown to inhibit growth of *P. infestans* despite numerous experiments and the importance of the pathogen. The expression of the cecropin B gene isolate from the insect *Hyalophora cecropia* was reported to confer resistance to *P. infestans* by in vitro assays (Owens and Heutte, 1997). More recently, the use of a synthetic peptide (msrA3) derived from the temporin A gene of the batra chian *Rana temporaria* in transgenic potato conferred resistance to *P. infestans* and *Phytophthora erythroseptica* (Misra et al., 2004). Peptides of plant origin that affect oomycetes are scarce. The constitutive expression of an hevein-like peptide (Pn-AMP2) from the seed of *Pharbitis nil* in transgenic tobacco under the control of the cauliflower mosaic virus 35S promoter conferred enhanced resistance against the oomycete *P. parasitica*, the causal agent of black shank disease (Koo et al., 2002).

Defensins have attracted a lot of interest recently because they are widely distributed in organisms from invertebrates, insects, mammalians, and plants, with an evident protective role against pathogens. In plants, their main action is towards fungal pathogens and less frequently bacterial pathogens. The precise mode of action of plant defensins is still unclear but may be primarily a specific interaction with membranes that alter its permeabilization (Thevissen et al., 1999). Transgenic approaches have shown that defensin genes expressed in plants can confer protection against pathogens. Indeed, transgenic plants carrying the Rs-AFP2 gene from *R. sativus* displayed resistance against the fungus *Alternaria longipes* (Terras et al., 1995). Another example is the expression of a defensin gene isolate from alfalfa (*alfAFP* gene) in transgenic potato which conferred resistance to the fungus *Verticilium dahliae* although this protein was shown to lack antimicrobial activity against *P. infestans* (Gao et al., 2000). More recently, activity against the oomycete *P. parasitica* was reported when the defensin BSD1 from *B. campestris* L. ssp. *pekinensis* was expressed in transgenic tobacco plants (Park et al., 2002).

Hence, our results showing a severe growth inhibition of the potato oomycete, *P. infestans*, suggest a transgenic approach might be successful using the *lm-def* gene. The effect observed at a concentration of 0.4 μM is quite encouraging when compared with other defensins with activities against oomycetes (Park et al., 2002; Thevissen et al., 1999). The use of native genes of plant origin is certainly advisable to reduce the difficulties and costs of risk assessment of food derived from transgenic potatoes. In addition, this research demonstrates, if it was still needed, the vast potential of the unexploited genetic resources of plant biodiversity. The discovery of valuable genes and alleles in tropical biodiversity may also be an alternative for developing country biotechnology research to the difficult access to existing transgenic solutions encumbered with complex patent restrictions.

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