REPORT

High level resistance to phytopathogenic fungi and oomycetes conferred by the use of a novel anti-microbial peptide

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ABSTRACT

Fungi and oomycete-borne plant disease constitute the largest limiting factor for crop productivity and yields both in Cuba and worldwide. Vast amounts of fungicidal chemicals have to be spent every year for their control, despite considerable environmental damage and the risk such chemicals pose for human health. Searching for biotechnological disease control alternatives is, therefore, a priority of current research on this subject. Plant defensins are small cysteine-rich peptides forming part of the defense mechanisms of plants that inhibit the growth of a wide range of phytopathogenic microorganisms. The present work describes a new cDNA coding for a defensin, isolated by looking at genes induced by the inoculation of *Nicotiana megalosiphon* with *Peronospora hyoscyami* f. sp. *tabacina*, the causative pathogen of tobacco blue mould. *NmDef02*, the isolated gene, was expressed in the yeast *Pichia pastoris*, and the purified recombinant protein exhibited a strong anti-microbial activity to major fungal and oomycetal plant pathogens. In addition, the constitutive expression of the *NmDef02* gene in transgenic tobacco and potato plants increased their resistance to economically important diseases.

*I*ntroduction

Plant defensins are a family of small, basic, highly stable cysteine-rich peptides typically 45 to 54 aminoacids long, distributed throughout the plant kingdom. These peptides are major players of the natural defense systems of vegetables, where they inhibit the growth of a disparate range of pathogenic microorganisms [1].

The present work describes the isolation of gene *NmDef02*, coding for a new defensin from the wild, non-cultured tobacco species *Nicotiana megalosiphon*. This gene was obtained following a strategy aimed at the obtention of an anti-microbial peptide able to confer high levels of protection against plant pathogens, comprised of two stages: 1) Expression in recombinant yeast, purification and *in vitro* evaluation of the anti-microbial activity of defensin NmDef02; and 2) Evaluation of the efficacy of the expression of this gene into transgenic tobacco and potato plants within the context of agricultural disease control [2].

*R*esults

Expression in recombinant yeast, purification and in vitro evaluation of anti-microbial activity of defensin *NmDef02*

A suppression subtractive hybridization cDNA library from leaves of *N. megalosiphon* inoculated with *Peronospora hyoscyami* f. sp. *tabacina* was used to isolate a clone bearing a 219 sequence denominated *NmDef02*, exhibiting sequence homology with a plant defensin. The sequence would code for a polypeptide with a length of 73 aminoacids, including a 27 aminoacid leader peptide. By means of alignments with plant defensin sequences from public databases and publications, it was possible to determine that the leader peptide cleavage site is conserved among all 74 studied polypeptides. There is strict conservation for eight cysteine residues thought to be involved in disulphide bonding, an aromatic residue at position 11 and glycines in positions 13 and 14 (ordinates are relative to those of defensin Rs-AFP2). A highly conserved serine at position 8 is not, however, present in *NmDef02*. Even though *NmDef02* clusters with this group of 74 plant defensins (62% bootstrap support), it is likely to belong to a new defensin subgroup because only a poor bootstrap support (19%) links this sequence to its closest homolog from *Triticum aestivum*.

The cDNA sequence coding for the mature portion of *NmDef02* was also fused in-frame with a modified α-factor leader peptide devoid of its last four aminoacids and transformed into the yeast *Pichia pastoris*. After inducing the expression of *NmDef02* through the addition of methanol for 72 hours into shake flask cultures, it was possible to detect, by SDS-PAGE of culture supernatants, a protein species with an approximate molecular weight of 5.1 kDa, matching that of processed *NmDef02* (Figure 1).

This recombinant species constitutes approximately 30% of all protein in culture supernatants, as estimated by densitometric analysis. It was not possible to detect this peptide in recombinant yeast transformed with the empty pPIC9 vector, used as negative control for the study. Recombinant *NmDef02* was purified from culture supernatants, and the purified fraction was shown to be constituted by a single protein species of the expected size by mass spectrometry (MALDI-TOF).

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1. Portieles R, Ayra C, Borrás O. Basic insight on plant defensins. Biotecnol Apl. 2006;23:75-8.

2. Portieles R, Ayra C, Gonzalez E, Gallo A, Rodríguez R, Chacon O, *et al*. *NmDef02*, a novel antimicrobial gene isolated from *Nicotiana megalosiphon* confers high-level pathogen resistance under greenhouse and field conditions. Plant Biotechnol J. 2010;8(6):678-90.

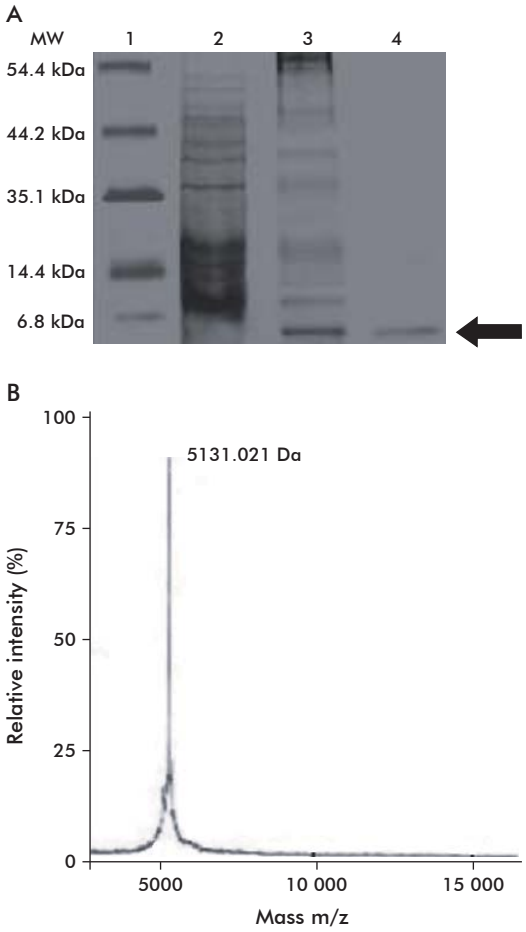

Figure 1. Expression and partial purification of recombinant defensin *NmDef02* from culture supernatants of *Pichia pastoris*. A) SDS-PAGE (18%) showing the recombinant protein and the purification profile of *NmDef02*. MW: Low molecular weight marker; 1: *Pichia pastoris* transformed with the pPIC9 empty vector (vector without insert); 3: *P. pastoris* transformed with pPIC9 containing *NmDef02*; 4. *NmDef02* (arrow) purified using Superdex 75. B) MALDI-TOFF mass spectrometry of purified recombinant *NmDef02*.

The anti-microbial activity of recombinant *NmDef02* was evaluated *in vitro* on different plant pathogens. As shown in table 1, the recombinant molecule exhibits varying median growth inhibitory concentrations (IC_{50}), ranging from 1 µM for *Fusarium oxysporum var. cubense* to 2.8 µM for *A. solani* after 48 hours of incubation.

In the particular case of *Phytophthora parasitica* var. *Nicotianae*, recombinant *NmDef02*, at a concentration of 4 µM, was sufficient to inhibit hyphal growth after 48 hours of incubation. These experiments confirmed the efficacy and width of the *in vitro* anti-microbial activity of recombinant *NmDef02*, as it is able to inhibit the growth of both fungi and oomycetes.

Table 1. *In vitro* anti-microbial activity of *NmDef02* against five plant pathogens

Plant pathogen	IC_{50}[a] (µM)
Phytophthora infestans	1.4
Phytophthora parasitica var. nicotianae	4
Alternaria solani	2.8
Fusarium oxysporum var. cubense	1
Verticillium dahliae	2

[a] Molar concentration of the protein (µM) required to inhibit growth by 50% (IC_{50}) after an incubation of 48 h, as determined from a dose-response curve (growth inhibition in % *vs.* protein concentration). Five replicates (n = 10) were used per experiment.

Efficacy of the introduction of the defensin gene in transgenic tobacco and potato plants for the control of agriculturally significant diseases

Given that recombinant *NmDef02* obtained from yeast exhibited anti-microbial activity *in vitro* against all five assayed plant pathogens, it was decided to generate transgenic tobacco and potato plants to determine whether expressing the 35S::*NmDef02* transgene confers resistance against plant pathogens *in vivo*. Relative expression of the *NmDef02* mRNA was measured before inoculation, by quantitative RT-PCR of 50 individual tobacco and potato lines homozygotic for the transgene. This followed the objective of identifying the best expressing clones for future research.

Although obtaining polyclonal antisera against *NmDef02* would have allowed a more direct estimation of the levels of this molecule than measuring its transcript by quantitative RT-PCR, all efforts in this direction were fruitless, and therefore the latter technique was selected for this purpose. Not all tobacco and potato homozygotic lines, grown under greenhouse conditions, expressed the 35S::*NmDef02* transgene at the same level, and transcript numbers were in general higher in tobacco than in potato plants. Twenty transgenic lines from each, having relatively high transcript levels of the *NmDef02* cDNA, were chosen for further experimentation on resistance to pathogens under greenhouse and field conditions.

Twenty transgenic tobacco lines expressing the 35S::*NmDef02* transgene, as well as control plants, were inoculated with the pathogenic oomycetes *P. parasitica var. nicotianae* or *P. hyoscyami f.sp. tabacina* (Figure 2). Five days after inoculation there were mild disease symptoms typical of *P. parasitica var. nicotianae* in the control plants, but the transgenic defensin-expressing plants remained healthy. Ten days after the inoculation there were severe disease symptoms in all control plants (stem rot and withering of the leaves), which died 5 days later. In the case of the transgenic plants expressing the 35S::*NmDef02* gene, however, only 6.7% of the plants developed mild disease symptoms, while the remainder stayed healthy.

A similar effect was observed in transgenic tobacco lines inoculated with *P. hyoscyami f.sp. tabacina*. Transgenic plants expressing the defensin remained healthy 10 days after inoculation, whereas control plants died after the same period. Interestingly, there was a high correlation between relative *NmDef02* transcription, as determined by quantitative RT-PCR, and resistance against *P. parasitica var. nicotianae* or *P. hyoscyami f.sp. tabacina* in several transgenic lines (data not shown). Lines 4, 5, 8, 28 y 49, expressing *NmDef02* at relatively low levels, developed symptomatic infections.

There is strong evidence suggesting *NmDef02* is involved in plant defense mechanisms, given the increased resistance of tobacco and potato transgenics expressing this defensin constitutively. The analysis

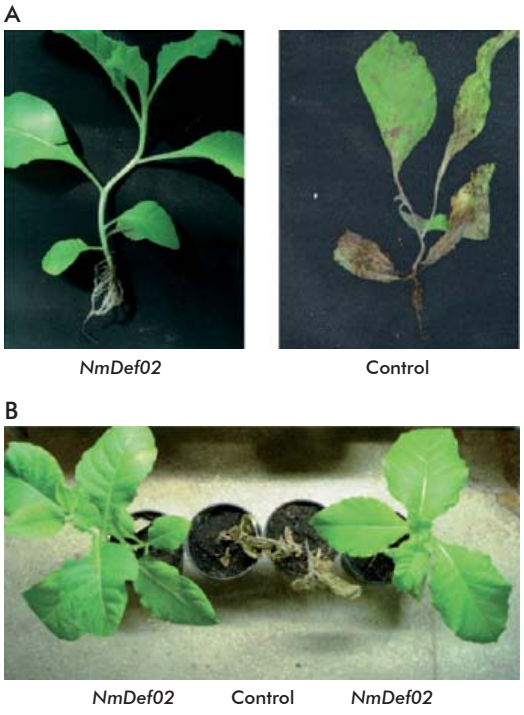

Figure 2. Greenhouse evaluation of transgenic *NmDef02* tobacco plants for resistance to *Phytophthora parasitica* var. *nicotianae* (A) and *Peronospora hyoscyami* f.sp. *tabacina* (B). Phenotypes of plants transgenic for control vector or the defensin, grown in greenhouses, 10 days after inoculation.

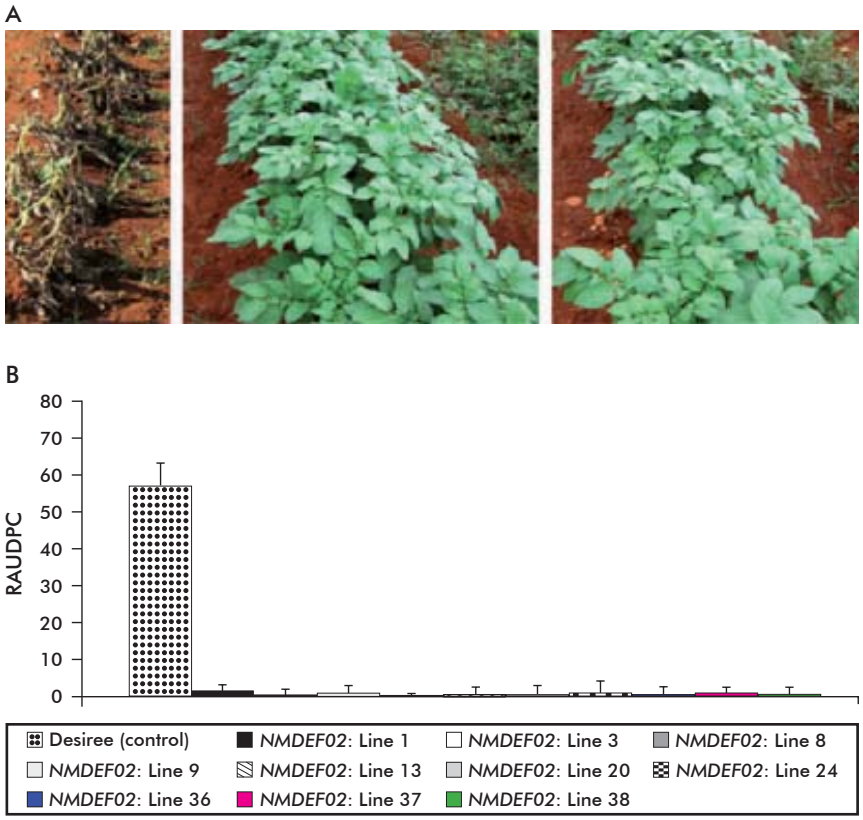

Figure 3. Evaluation under field conditions of transgenic *NmDef02* potato plants (cultivar 'Désirée') for resistance to *Phytophthora infestans*. A: phenotypes of plants transgenic for the control vector (left panel) or *NmDef02* (center and right) in a potato field with a high incidence of late blight, 35 days after planting. B) Relative area under the disease progression curve (RAUDPC). Each bar represents the mean with the corresponding standard error.

of disease resistance of the transgenic lines of tobacco and potato evidenced that they were more resistant to *P. parasitica var. nicotianae*, *P. hyoscyami f.sp. tabacina*, *A. solani* and *P. infestans*, respectively than their wild-type counterparts or transformants containing solely the empty vector. Ours is the first report of high-level resistance of tobacco plants expressing *NmDef02* to *P. infestans*, both under greenhouse and field conditions (Figure 3).

Both tobacco and potato transgenic plants remained healthy, without morphological changes or abnormalities even in cases where the accumulation of *NmDef02* was high. The anti-microbial activity provided by the expression of this defensin was reached, therefore, without toxic effects on its plant hosts. Finally, it should be stressed that the present investigation further corroborates the efficacy of defensins in mediating resistance to agricultural disease, and points to these molecules as strong candidates for further work in the area of crop improvement.