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Expression of insect cystein-rich antifungal peptides in transgenic tobacco enhances resistance to a fungal disease

Nathalie Banzet [a], Marie-Pascale Latorse [b], Philippe Bulet [c], Evelyne François [a], Christine Derpierre [a], Manuel Dubald [a,*]

[a] *Aventis CropScience – Biotechnology Department, 14/20 rue Pierre Baizet, BP 9163, 69263 Lyon Cedex 09, France*
[b] *Aventis CropScience – Fungicide Biology Department, 14/20 rue Pierre Baizet, BP 9163, 69263 Lyon Cedex 09, France*
[c] *IBMC, UPR 9022 CNRS, 15 rue René Descartes, 67084 Strasbourg Cedex, France*

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Abstract

Heliomicin (from *Heliothis virescens*) and drosomycin (from *Drosophila melanogaster*), two cystein-rich antifungal peptides are produced by insects in response to septic injury. Both exhibit in vitro a rather similar broad-spectrum activity on phytopathogens, but microscopic observations indicate different modes of action. Heliomicin and drosomycin have been expressed in transgenic tobacco under the control of strong constitutive promoters, and targeted to the apoplast using signal peptides of plant origin. A significant enrichment of well-cleaved peptides with the expected molecular weight has been detected in the intercellular space of transgenic leaves. The recombinant peptides, partially purified from transgenic tobacco, exhibited in vitro the same antifungal properties on germination of spores of *Botrytis cinerea* and germ tube elongation as the recombinant peptides produced in *Saccharomyces cerevisiae*. Heliomicin and drosomycin expressed in transgenic tobacco confer a minor but statistically significant enhanced resistance to the fungal pathogen *Cercospora nicotianae*.

Keywords: Transgenic tobacco; Antifungal cystein-rich peptides; Disease resistance

1. Introduction

Fungal pathogens are responsible for considerable yield losses in agriculture, and there is a growing concern about food contamination with mycotoxins. Progress in understanding basic mechanisms of plant–pathogen interactions, coupled to the development of plant genetic engineering, are providing strategies for crop protection, as a complement to classical breeding [1,2]. Promising results have been obtained by the constitutive or inducible expression in transgenic plants of single or combined genes encoding antimicrobial proteins from plant origin, such as chitinases, glucanases, ribosome inactivating proteins [3–6], osmotin [7], oxalate decarboxylase [8], cystein-rich antifungal peptides [9–12], as well as antimicrobial proteins from fungi [13] or viruses [14].

Antimicrobial peptides (AMPs) constitute a heterogeneous class of low molecular weight proteins (<10 kDa), which are part of the immune system of both animals and plants [15,16]. A wide repertoire of such molecules is already described in the databases, and many new ones are being discovered each year. In plants, all the AMPs characterized so far are cyclic, stabilized by disulfide bridges [16]. Similar peptides have also been isolated from the hemolymph of insects in response to septic injury [17,18], some of them having antifungal activity. Transgenic approaches with AMPs are still a challenge, and results appear unpredictable since strong in vitro inhibitors of microbial growth can display a weak or no in planta efficacy. Increasing resistance may not be sufficient for field applications, as compared to the action of classical fungicides, and failures have often been correlated to peptide degradation by plant proteases [19,20] or inhibition of antimicrobial activity by divalent cations [21,22]. The challenge is now to improve the in planta efficacy of existing genes, to use or to discover molecules with

* Corresponding author. Tel.: +33-4-72-85-28-19; fax: +33-4-72-85-22-97.
E-mail address: manuel.dubald@aventis.com (M. Dubald).

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exceptional activity and stability towards plant cell composition. Recent efforts have focused on the use of mutated, hybrid or synthetic peptides with reduced sensitivity to plant proteases [20,23–26].

AFPs from animal origin may be particularly effective in crop protection since phytopathogens were never exposed to their action. In order to validate this concept, we have focused on two insect AFPs, which have been recently characterized. Drosomycin and heliomicin are 44 residue long cystein-rich AFPs, produced in the hemolymph of the diptera *Drosophila melanogaster* [27] and the lepidoptera tobacco budworm *Heliothis virescens* [28]. They share significant structure homology with plant defensins. In the present study, we report their in vitro activity on phytopathogens of agronomic interest, and analyze their expression in transgenic tobacco under the control of strong constitutive promoters. We also show that both insect peptides are expressed in planta in a biological active form and that their expression results in an increased resistance against a fungal pathogen.

2. Methods

2.1. Biological materials

Tobacco plants (*Nicotiana tabacum* cv Petit Havana) were grown in the greenhouse at 26 °C under natural and additional artificial light (15/9 h photoperiod, 200 μE/m^2/s). Transformation was performed by *Agrobacterium tumefaciens*-mediated leaf disk transformation system according to Horsch et al. [29], using strain EHA105 containing vectors pRPA-RD241 and pRPA-NP13. Resistance to kanamycin (100 mg/l) was used on T1 generation to identify transgenic monolocus lines, and on T2 generation to select monolocus homozygous material.

Economically important pathogens (Table 1) were chosen from Aventis internal biological collections for in vitro tests. *Botrytis cinerea* was chosen to study the morphogenic effects of heliomicin and drosomycin on spore germination and germ tube elongation. In vivo infections were performed with *Cercospora nicotianae.* Recombinant heliomicin and drosomycin, produced in the yeast *Saccharomyces cerevisiae*, and purified as previously described [28,30], were used for in vitro antifungal spectrum determination on plant–pathogens, as antigens for antibody production, and as positive controls in Western blot and ELISA experiments.

2.2. Construction of the plant expression vectors

The pRPA-RD241 vector encoding heliomicin (Fig. 2A) was constructed as described in patent application WO09953053 [31]. Expression is driven by the strong constitutive double 35S promoter followed by the TEV 5′-UTR [32], the open reading frame of the fusion signal peptide–AFP and the polyadenylation signal from CaMV-35S [33]. The synthetic sequence encoding heliomicin was obtained using an optimized codon usage for plant expression, and was fused to the sequence encoding the 30 amino acid long signal peptide of protein PR1a (Genbank 218303).

The sequence corresponding to the mature peptide drosomycin (Fig. 2B) was amplified from the cDNA (Genbank 7241182) with the proofreading *Pfu* polymerase (Stratagene) using primers 5′-gactgcctgtccggaagatacaagg and 5′-ggggtaccttagcatccttcgcaccag. The PCR product was digested with *KpnI* and cloned in pRPA-NP4 [31], cut with *SfoI* and *KpnI*, allowing the fusion between a synthetic sequence encoding the 22 amino acid long signal peptide of maize polygalacturonase 1 [34] and the sequence encoding drosomycin. This coding region was then finally subcloned using *XbaI* and *KpnI* in a binary vector derived from pBin19, between the cassava vein mosaic virus (CsVMV) promoter [35] and the polyadenylation signal from nopaline synthase (Genbank 1256363), resulting in plasmid pRPA-NP13.

2.3. Protein extracts

Total protein extracts were performed with TRIZOL (Life Technologies) according to the instructions of the manufacturer. Proteins were dissolved in 1% SDS.

Apoplastic proteins were extracted from leaves by infiltration under vacuum of 1 cm wide leaf strips with an ice cold extraction buffer (50 mM Tris, 250 mM NaCl, pH 6.8). The leaf material was then dried superficially with absorbent paper, loaded into empty PD10 columns and centrifuged 30 min at 7000 rpm and 4 °C in order to recover the apoplastic fluid. Protein concentrations were quantified with Coomassie Plus Protein assay (Pierce).

2.4. Analysis of peptide expression in transgenic plants

2.4.1. Antibodies

Guinea pig anti-heliomicin polyclonal antiserum was raised against denatured heliomicin and used for Western blot analyses. Rabbit anti-drosomycin and rabbit anti-heliomicin polysera were raised against recombinant refolded peptides and immunopurified by affinity chromatography on CNBr-activated Sepharose 4B linked to native antigens. These antibodies were used for Western blot analyses of drosomycin expression, and for ELISA on partially purified plant protein fractions. Alkaline phosphatase conjugated antibodies from goat were used as secondary antibodies (Sigma A-3687 and Sigma A-5062). All antibodies used in Western blot or ELISA experiments were previously diluted in 0.5%

Table 1
In vitro antifungal activity spectrum of recombinant heliomicin and drosomycin produced in *S. cerevisiae* on plant–pathogens

Fungal strain	% Efficacy					
	Heliomicin (μg/ml)			Drosomycin (μg/ml)		
	10	20	40	10	20	40
Botrytis cinerea (M)	0	0	30	0	0	0
Botrytis cinerea (S)	0	5	96	0	95	99
Cercospora nicotianae (M)	50	70	70	70	70	70
Fusarium culmorum (S)	95	95	95	95	96	96
Fusarium nivale (M)	75	75	90	30	50	55
Fusarium graminearum (M)	38	31	63	0	0	60
Fusarium monoliforme (M)	10	10	10	10	15	15
Fusarium monoliforme (S)	5	5	5	0	0	0
Fusarium oxysporum (S)	0	0	67	0	57	99
Fusarium oxysporum radici (S)	0	0	0	0	0	65
Pyricularia oryzae (S)	44	84	95	0	0	0
Phoma lingam (M)	0	0	0	0	0	0
Phoma lingam (S)	0	0	40	0	0	0
Phomopsis helianthi (M)	40	65	85	40	50	55
Phytophthora nicotianae (M)	15	15	20	15	15	15
Phytophthora cinnamomi (M)	0	0	0	0	0	0
Rhizoctonia solani (M)	60	70	90	30	50	90
Sclerotinia sclerotiorum (M)	90	99	98	81	97	94
Septoria nodorum (S)	40	40	65	20	45	65
Septoria tritici (S)	80	80	85	85	85	85

Effects on spores (S) or mycelium (M) are expressed as percentage of efficacy.

Western blocking reagent (Roche Molecular Biochemicals) in TBS (0.1 M Tris–HCl, 0.9% NaCl, pH 7.45).

2.4.2. Western blot analysis

Fifty microgram of total or apoplastic proteins were separated in 10–26% gradient acrylamide gel using a Tris–Tricine system [36]. One hundred nanogram of purified recombinant peptides were used as positive standards. Heliomicin samples were denatured by boiling in Laemmli buffer (0.1 M Tris–HCl, pH 6.8, 25% glycerol, 200 mM DTT, 8% SDS, 0.02% bromophenol blue) before loading. Drosomycin samples were analyzed under non-reducing conditions, i.e. diluted in the loading buffer without DTT, and loaded without previous boiling. Proteins were electroblotted on 0.2 μm nitrocellulose membrane. These membranes were incubated 1 h at room temperature with 1% Western blocking reagent in TBS, then overnight at 4 °C with primary antibody. They were washed twice with TBS-T (0.1% Tween 20 in TBS), twice with 0.5% blocking reagent in TBS, then incubated 1 h at room temperature with the secondary antibody. Finally, membranes were washed four times with TBS-T, and alkaline phosphatase activity revealed with the Chemiluminescent Immun Star Substrate (Biorad), according to the instructions of the manufacturer.

Fifty microliter of each protein fraction collected after gel filtration chromatography were diluted four times with TBS and incubated overnight at 4 °C in wells of an ELISA plate (Greiner). Wells were then washed three times with 0.05% Tween 20 in TBS, and unspecific sites were blocked for 1 h at room temperature with 0.5% Western blocking reagent in TBS. Rabbit immunopurified antibodies were added to each well and incubated 1 h at room temperature. Wells were washed three times with 0.05% Tween 20 in TBS, and the second antibody was added and incubated for 1 h at room temperature. Wells were washed again three times with 0.05% Tween 20 in TBS before adding the substrate (*p*-nitrophenyl phosphate, Sigma) for alkaline phosphatase, diluted in detection buffer (0.1 M glycine, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, pH 10.4) according to the instructions of the manufacturer. Activity was scored after 1 h at room temperature by measuring the optical density at 405 nm.

2.4.3. MALDI–TOF analysis

A sandwich method preparation was performed for MALDI–MS analysis. Briefly, 0.5 μl of a saturated solution of alpha-cyano-4-hydroxycinnamic acid (HCCA, Sigma) in acetone, were placed on the probe tip, and 0.5 μl of 5% TFA was deposited on matrix crystallized bed. Then 0.5 ml of the sample to analyze was loaded and finally 0.5 μl of a saturated solution of

alpha-cyano-4-hydroxycinnamic acid prepared in 50% acetonitrile in water was added. After drying, the target was washed with 2 μl of 0.1% TFA in H_2O and removed after a few seconds using forced air and dried again under vacuum.

This study was carried out on a Bruker (Bremen, Germany) BIFLEXIII™ Matrix-Assisted Laser Desorption/Ionization Time of Flight mass spectrometer, equipped with a SCOUT™ high-resolution optics and a gridless reflector. This instrument has a maximum acceleration potential at 20 kV and is operating in a linear mode. Ionization is accomplished with the 337 nm beam from a nitrogen laser with a repetition rate of 3 Hz. A camera mounted on a microscope allows visualization of the sample crystallization homogeneity before measurements. Spectra were obtained in the linear positive mode and externally calibrated with three standard peptides: non-glycosylated drosocin, metchnikowin and heliomicin with respective average molecular masses at m/z 2199.5, 3046.4, and 4784.3, respectively [37].

2.4.4. Partial purification of active drosomycin and heliomicin from transgenic tobacco

About 10 mg of apoplastic proteins isolated from lines 241F and NP13G were concentrated to 4 ml by partial freeze-drying, and fractionated by gel filtration on a 75 × 1.6 cm column with Superdex 30 prep grade matrix (Pharmacia). Elution was performed at 1 ml/min, with 50 mM Tris–HCl, pH 6.8; 250 mM NaCl. Fractions of 2.5 ml were collected, and submitted to an ELISA test for peptide detection. Selected fractions were acidified with 200 μl of acetic acid, and loaded on C_{18} Sep-Pak cartridges (Waters) equilibrated with 0.05% TFA. The cartridges were washed with 4 ml of 5% acidified acetonitrile (0.05% TFA) and eluted with 3 ml of 60% acidified acetonitrile. After freeze-drying, samples were diluted in 100 μl of sterile distilled water and tested for in vitro antifungal activity on *B. cinerea* spore germination.

2.4.5. In vitro tests

Microtiter biotests were performed in vitro to evaluate the antifungal spectrum and efficacy of the peptides studied. One microtiter plate per fungus was prepared: the peptide solution (10, 20 and 40 μg/ml as final concentrations, three replications per doses) was added to 150 μl of fungal spore suspension (10^5 spores/ml) or crushed mycelium (initial OD = 0.1 at 630 nm) in Potato Dextrose Broth (Difco). The microtiter plates were then incubated in the dark at room temperature (20–22 °C). The absorbance at 630 nm was measured 5 days after inoculation, with a spectrophotometer (DYNATECH Laboratories MRX). The fungal growth was expressed as the difference between the optical density at 630 nm at a given time ($OD_{(630)t}$) and at the beginning of the experiment ($OD_{(630)i}$). The inhibition efficacy of a product (peptide or fungicide) was calculated as follows:

$$\text{Efficacy}\% = 100(OD_{(630)t} - OD_{(630)i})_{(\text{UTC})} - (OD_{(630)t} - OD_{(630)i})_{(\text{product})}/(OD_{(630)t} - OD_{(630)i})_{(\text{UTC})}$$

where $(OD_{(630)t} - OD_{(630)i})_{(\text{product})}$ is the growth of the fungus in presence of peptide and $(OD_{(630)t} - OD_{(630)i})_{(\text{UTC})}$ is the growth of the same fungus without product (untreated control).

In vitro morphogenic effects on spore germination and germ tube elongation were studied in drop test. Twenty microliter drops, composed of spores of *B. cinerea* in PDB (50 000 spores/ml as final concentration) and peptide dilutions (final concentrations of 0, 10, 20, 40 and 100 μg/ml) or partially purified plant protein fractions from transgenic tobacco, were placed on hydrophobic slides (vinyl slides, 76 × 26 mm, RINZL, Polylabo). The fungicide Iprodione (2 μg/ml final concentration) was used as positive control product for antifungal activity. Slides were incubated overnight (16 h) at 20–22 °C, in the dark in a saturated humid atmosphere, into a 14 cm diameter sterile Petri dish, whose bottom was covered by sterile absorbing paper wetted with sterile distilled water. Drops were fixed with 2 μl of glutaraldehyde-Mac Ilvain (40% glutaraldehyde, 0.2 M Na_2HPO_4, 0.2 M citric acid 0.2 M, pH 6.8) and spores were observed (400-fold magnification) by reversed microscopy (Olympus CK 40), according to the morphological status and length of the germ tube.

2.4.6. Phytopathology tests

Tobacco plants were grown in a greenhouse at 26 °C under natural and artificial light (15/9 h photoperiod, 200 μE/m^2/s). Inoculation of each plant (16 plants/line) was done at the age of 2 months (6–8 leaves developed) by spraying an aqueous suspension of spores (100 000 spores/ml) of *C. nicotianae* obtained from artificial medium. After infection, the plants were incubated at 25 °C in a saturated humid atmosphere. Infected plants previously treated with a fungicide (Chlorothalonil) were used as reference. Disease symptoms were evaluated 14 days after infection, in terms of percentage of the necrotic area versus total leaf area, on six leaves infected for each plant. Mean and standard deviations were established, as well as the normal statistical distribution for each plant population. Statistical analysis of difference for each transgenic line, with respect to the wild type (WT) line, was established by the parametric Student's t-test, Kolmogorov Smirnov, and confirmed with the nonparametric K-Wallis test. Significant difference was admitted for $P < 0.01$.

3. Results

3.1. In vitro antifungal activity of heliomicin and drosomycin on major fungal phytopathogens

In vitro antifungal activity of recombinant heliomicin and drosomycin produced in *S. cerevisiae* was evaluated on seventeen fungal strains (spores and/or mycelium) responsible for major field damages (Table 1). Both peptides exhibited at least at the highest concentration of 40 ppm a maximal activity on mycelium growth of *Sclerotinia sclerotiorum, Rhizoctonia solani* and germination of spores of *Septoria tritici, Fusarium culmorum* and *B. cinerea*.

Peptide activity was weak or undetectable on spores of *Fusarium oxysporum radici, Fusarium monoliforme* and *Phoma lingam* or on mycelium of *B. cinerea* and *Phoma lingam*. In spite of an apparent similar spectrum of activity, heliomicin presented a strong activity on *Pyricularia oryzae* spores and *Fusarium nivale* mycelia on which drosomycin had no and only a moderate activity, respectively. On the contrary, drosomycin had a complementary effect on spores of *F. oxysporum.* Considering the whole antifungal spectra, heliomicin exhibited a higher activity on most of the pathogens listed than drosomycin. We decided to study heliomicin and drosomycin as two new candidates for enhanced plant resistance to fungal pathogens, through a transgenic approach.

3.2. Morphogenic alterations induced in vitro on Botrytis cinerea spore germination

Spores of *B. cinerea*, which are susceptible to heliomicin and drosomycin, are easy to collect from mycelium grown on PDB solid media, and germinate uniformly in 16 h in 20 μl droplets of liquid media. This simple test was first applied to observe morphological alterations in spore germination, and then used to quickly evaluate the potential antifungal activity of transgenic plant extracts. *B. cinerea* spores in PDB media (Fig. 1A) were incubated overnight at room temperature with 10, 20, 40 or 100 μg/ml of heliomicin or drosomycin (Fig. 1C–H). Distilled water or the fungicide Iprodione (2 μg/ml) were used, respectively, as negative and positive controls for fungicide activity. Germination of spores and germ tube elongation were examined by microscopy, and compared to control samples incubated with water (Fig. 1B). Two microgram per milliliter Iprodione totally inhibited spore germination without morphologic alterations or cell lysis (not shown). Ten microgram per milliliter of heliomicin (Fig. 1C) did not affect spore germination or germ tube development. Branching of the germ tubes associated with a strong disturbance of the elongation were observed from 20 μg/ml (Fig. 1D). At higher concentrations, 40 μg/ml (Fig. 1E) and 100 μg/ml (Fig. 1F), germinating spores appeared swollen, and release of cytoplasmic content occurred, shown by arrows. Ten microgram per milliliter of drosomycin did not affect spore germination (Fig. 1G), but inhibited the swelling of the hyphal extremity observed in control sample, resulting in a linear and continued elongation. At 20 μg/ml, intact spores appeared granular and swollen, germination was totally inhibited and disruption occurred (shown by arrows in Fig. 1H).

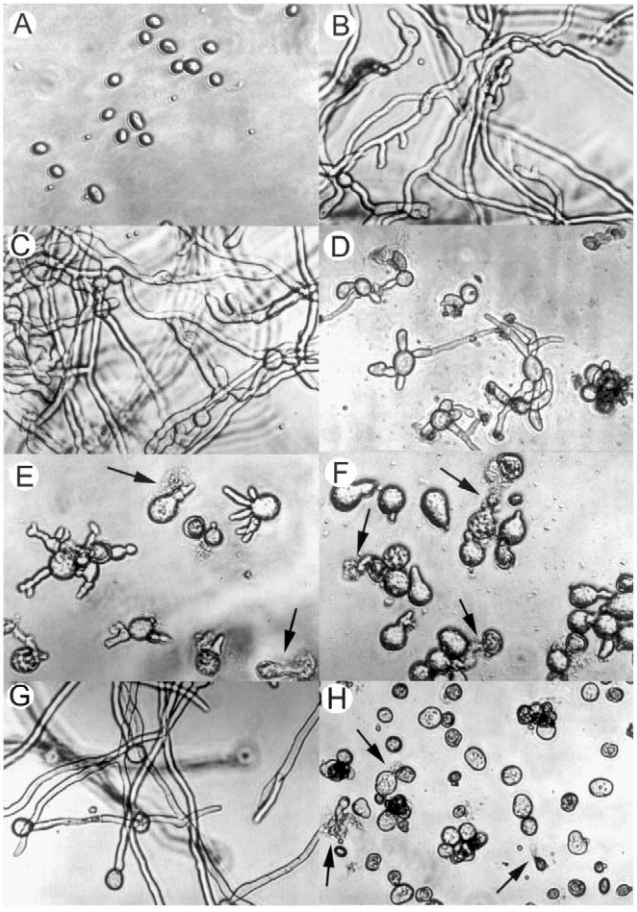


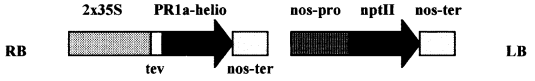
Fig. 1. Morphogenic alterations induced in vitro on *B. cinerea* spore germination. *B. cinerea* spores in Potato Dextrose Broth (PDB) media were observed before (A) and after overnight incubation (B–H) with heliomicin or drosomycin. (B) Spores incubated in PDB without peptide. (C–F) Spores incubated in PDB with heliomicin at a final concentration of 10, 20, 40 and 100 μg/ml, respectively. (G) and (H) spores incubated in PDB with drosomycin at a final concentration of 10 and 20 μg/ml, respectively. Arrows indicate lysis of spores.

3.3. Vectors for in planta constitutive expression

Insect AFPs were constitutively expressed in transgenic plants. Expression cassettes, nucleotide and amino acid sequences are presented in Fig. 2. Heliomicin and drosomycin were targeted to the apoplast, via the fusion

A

(1)



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tgcggttccttcgctaacgtgaactgctggtgcgagact

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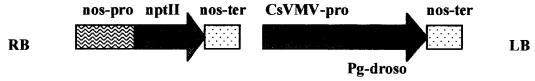
B

(1)



(2) atggcctgcaccaacaacgccatgagggccctcttcctcctcgtgctcttctgcatcgtgcacggcgactgcctgtccggaaga
tacaagggtccctgtgccgtctgggacaacgagacctgtcgtcgtgtgtgcaaggaggagggacgctccagtggccactgca
gccccagtctgaagtgctggtgcgaaggatgc

(3) MACTNNAMRALFLLVLFCIVHGDCLSGRYKGPCAVWDNETCRRVCKEEGRSSGHCSPSL
KCWCEGC

Fig. 2. Diagrams of the T-DNA regions and encoded antimicrobial sequences. (A) Cassette of the pRPA-RD241 vector for heliomicin expression. (B) Cassette of the pRPA-NP13 vector for drosomycin expression. (1) T-DNA regions, (2) Nucleotide sequences of the signal peptide-AFP open reading frame, with AFP sequences underlined, (3) Amino acid sequences of the signal peptide–AFP fusion protein, with AFP sequences underlined. RB, right border; LB, left border; nos-ter, nopaline synthase polyadenylation signal; tev, tobacco etch virus translation enhancer; 2 × 35S, double promoter from Cauliflower Mosaic Virus (CaMV); PR1a–helio, fusion between the signal peptide of tobacco PR1a protein and mature heliomicin; nos-pro, nopaline synthase promoter; nptII, neomycin phosphotransferase conferring kanamycin resistance; CsVMV-pro, promoter from CsVMV; PG–droso, fusion between the signal peptide of maize polygalacturonase 1 and mature drosomycin.

of the antifungal peptide sequence to the signal peptide sequences from tobacco protein PR1a and from maize polygalacturonase (PG), respectively. Their constitutive expression was regulated with strong promoters. The double CaMV 35S promoter associated with the translation enhancer from Tobacco Etch Virus [32] was used for heliomicin expression, and the CsVMV promoter [35] for drosomycin expression. Both expression cassettes were introduced into the T-DNA of plant transformation vectors, resulting in vector pRPA-RD241 and pRPA-NP13, respectively. Constructs were transferred to *Agrobacterium tumefaciens* and used for leaf disk transformation of tobacco with kanamycin as selection agent.

3.4. Analysis of heliomicin and drosomycin expression in transgenic tobacco

More than 40 different tobacco primary transformants were produced with the vector pRPA-RD241 for heliomicin expression (241 lines) and 8 lines with the vector pRPA-NP13 for drosomycin expression (NP13 lines).

Analyses of heliomicin and drosomycin expression were performed by Western blot on total and apoplastic protein extracts. MALDI–TOF experiments were carried out on the same apoplastic samples. Results from one representative line for each construct (241F and NP13G, respectively) are presented in Fig. 3.

Immunodetection was performed, on the transgenic line 241F with an antiserum raised against denatured heliomicin (Fig. 3A). No usable serum could be generated in rabbit or guinea pig after immunization with denatured drosomycin. For this reason, detection of drosomycin in the transgenic line NP13G (Fig. 3B) was performed with immunopurified antibodies raised against native drosomycin.

Recombinant peptides were used as positive standards (lanes 1). Absent from WT tobacco total (lanes 2) or apoplastic (lanes 4) protein extracts, 5 kDa peptides were detected in protein extracts from the transgenic lines (lanes 3 and 5), with an apparent higher molecular weight, attributed to the delay in peptide migration that occurs in highly loaded lanes (50 μg of plant proteins versus 100 ng of pure peptides). Apoplastic fractions (lanes 5) showed a significant enrichment in peptides compared to total extracts (lanes 3), suggesting an

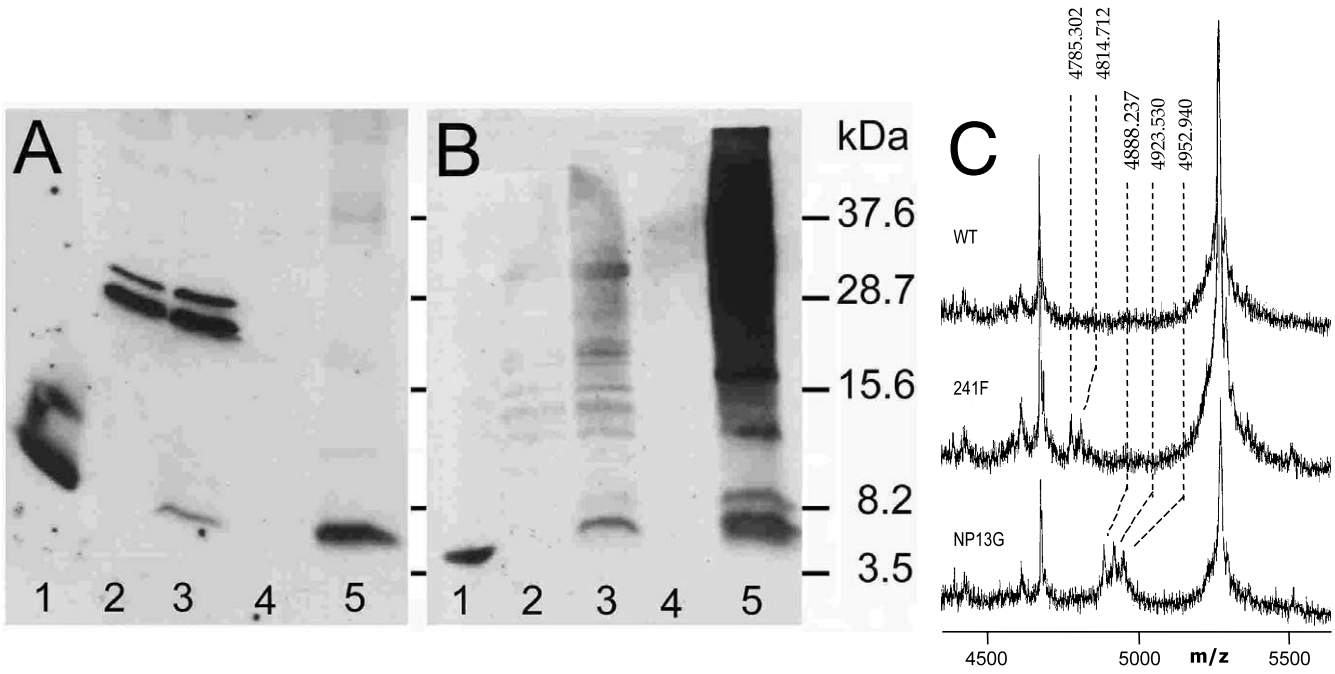


Fig. 3. Analysis of heliomicin and drosomycin expression in transgenic homozygous tobacco lines. Western blot analysis performed on 50 μg of total or apoplastic. (A) Detection of heliomicin under reducing conditions: (lane 1) 100 ng of heliomicin, (lane 2) total proteins from WT tobacco, (lane 3) total proteins from transgenic line 241F, (lane 4) apoplastic proteins from WT tobacco, (lane 5) apoplastic proteins from transgenic line 241F. (B) Detection of drosomycin under native conditions: (lane 1) 100 ng of drosomycin, (lane 2) total proteins from WT tobacco, (lane 3) total proteins from transgenic line NP13G, (lane 4) apoplastic proteins from WT tobacco, (lane 5) apoplastic proteins from transgenic line NP13G. (C) MALDI–TOF analysis on apoplastic fluids. Spectra from WT, transgenic lines 241F and NP13G expressing heliomicin and drosomycin, respectively, are focused on molecular masses (m/z) of both peptides (4500–5500 Da).

efficient targeting in the intercellular space. The intense double band at around 20–25 kDa (Fig. 3A, lanes 2 and 3) observed for total protein extracts from WT and tobacco transgenic line expressing heliomicin (Fig. 3A, lanes 2 and 3) is not present in apoplastic extracts (lanes 4 and 5), indicating aspecific interaction of the crude serum with inknown cytosolic proteins.

Interestingly, a smear was observed in the electrophoretic pattern of total and especially apoplastic fractions for the transgenic line NP13G analyzed under non-reducing conditions (Fig. 3B, lanes 3 and 5), with an immunopurified antibody. No such signal was detected with WT apoplastic extract (Fig. 3B, lane 4), indicating that no aspecific immunodetection occurs for apoplastic extracts. Similar results have been obtained for line 241F, using immunopurified antibodies raised against native heliomicin (not shown). These results suggest that AFPs expressed in plants establish before or after extraction strong interactions with other cell components, possibly other proteins via disulfide linkages.

The expression level of heliomicin was estimated in more than 40 lines transformed with pRPA-RD241. The highest level was approximately 0.1% of total proteins. The drosomycin level in transgenic NP13 lines was more difficult to estimate by Western blot under non-reducing conditions. However, while taking into account the high proportion of the peptide bound to other proteins, the expression level reached several percents of total proteins in our best line NP13G.

MALDI–TOF analyses were performed on the same apoplastic extracts. Spectra for WT, 241F and NP13G lines are overlapped in Fig. 3C, with scale focused between 4500 and 5500 Da. Specific profiles were obtained for the transgenic lines, with additional peaks as compared to WT control sample. A peak at m/z 4785.3, which corresponds to the exact molecular weight of heliomicin, was detected in the apoplast of line 241F. The peak at m/z 4814.7 might result from the oxidation of the two tryptophan residues. Drosomycin with the exact molecular mass (m/z 4888.3) was detected in the apoplast of the line NP13G, as well as two putative oxidized forms at m/z 4923.5 and 4952.9. Thus, MALDI–TOF analyses showed that the cleavage of the two distinct signal peptides PR1a and PG occurs efficiently at the junction with the AFP sequence, resulting in the release in the apoplast of peptides with the expected molecular mass and the cysteins engaged in the formation of the disulfide bridges.

3.5. In vitro antifungal activity on Botrytis cinerea of partially purified protein fractions from transgenic plants

In order to determine if the AFPs expressed in transgenic tobacco were folded in their antifungal active conformation, partially purified plant protein fractions

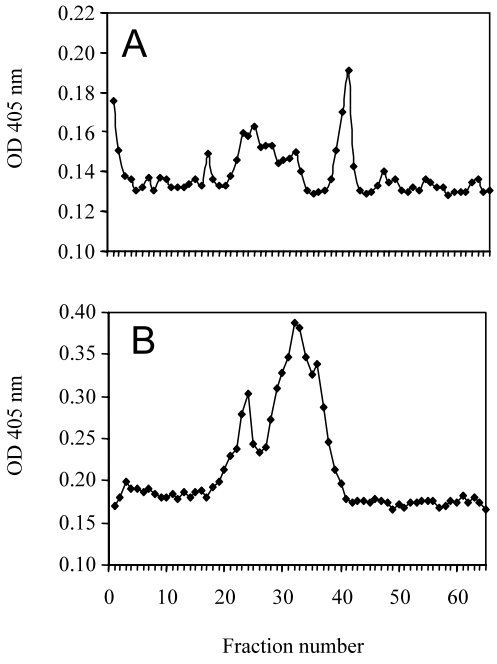


Fig. 4. ELISA on partially purified plant protein fractions. About 10 mg of apoplastic proteins were fractionated by gel filtration from line 241F expressing heliomicin (A) and from line NP13G expressing drosomycin (B). Immunoassay was performed under native conditions with immunopurified antibody.

were isolated from lines 241F and NP13G. Around 10 mg of apoplastic proteins were separated on a gel filtration column, and the eluted fractions submitted to an ELISA test (Fig. 4) with the corresponding immunopurified anti-heliomicin (Fig. 4A) or anti-drosomycin (Fig. 4B) antibodies raised against the native peptides. Since no aspecific immunodetection was previously observed for apoplastic extracts with immunopurified antibodies (Fig. 3B lane 4 for drosomycin—not shown for heliomicin), signals in ELISA tests were attributed to the specific recognition of the AFPs. In both experiments, under native conditions, antibodies reacted with various fractions (Fig. 4A and B). This complex profile reminds the smear observed under native conditions on our immunoblot (Fig. 3B, lane 5), suggesting again interactions with other components of tobacco extracts. For each separation, about 20 fractions were selected, desalted, concentrated, and tested in vitro in 20 μl droplets for their antifungal activity on spore germination of *B. cinerea* (Fig. 5). Concerning the heliomicin line 241F, antifungal activity was restricted to the thin isolated peak in the corresponding ELISA profile (Fig. 4A); i.e. fractions 39–43 (Fig. 5B–E), and displayed the characteristic features of genuine heliomicin, (hyphal branching and limited elongation). The other fractions were totally inactive on *B. cinerea* spores germination, as exemplified for fractions 24 (Fig. 5A) and 47 (Fig. 5F). Activity of fractions isolated from the NP13G line was restricted to fractions 32–34 (Fig. 5H),

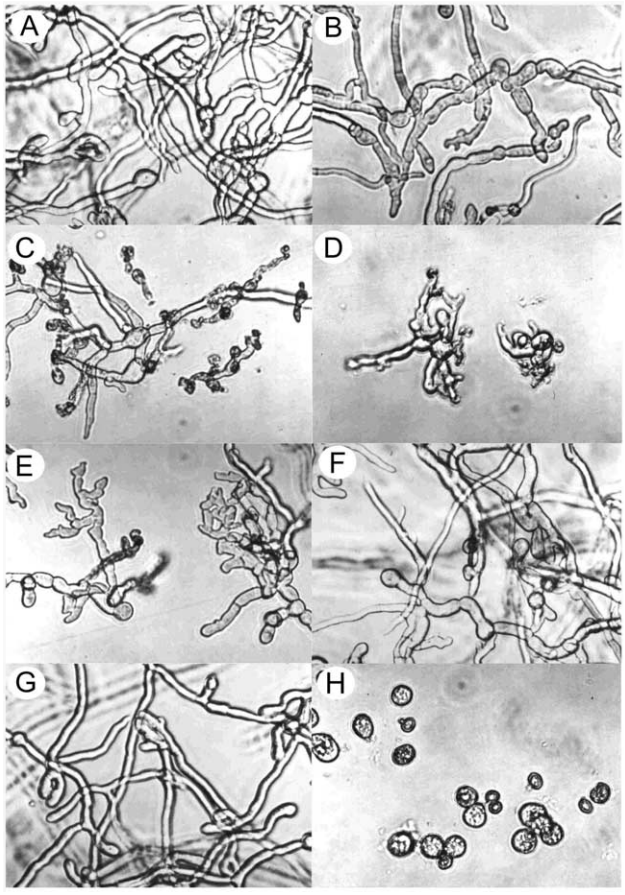


Fig. 5. In vitro antifungal activity of partially purified plant protein fractions. Fractions were desalted, concentrated, and evaluated for their antifungal activity by overnight incubation with spores of *B. cinerea*. Numbers refer to the fractions tested by ELISA (Fig. 4). (A–F): spores incubated with fractions 24, 39, 40, 41, 43 and 47, respectively, isolated from line 241F. (G) and (H): spores incubated with fraction 29 and 34 isolated from line NP13G.

that correspond to the top of the major peak in the ELISA profile (Fig. 4B), and resulted in a complete inhibition of spore germination as observed with genuine drosomycin. The other fractions were inactive, as exemplified for fraction 29 (Fig. 5G).

These results show that a fraction of the insect recombinant AFPs produced in transgenic plants is in an active, well-cleaved and efficiently targeted conformation, crucial for in planta efficacy.

3.6. Evaluation of disease resistance to *Cercospora nicotianae*

T2 homozygous monolocus lines expressing heliomicin and drosomycin were further evaluated for resistance to *C. nicotianae*. Foliar infection was measured 14 days after infection of 16 plants for each line. Results obtained from two independent experiments performed on four lines 241 (241C, 241D, 241F and 241G), and three lines NP13 (NP13B, NP13E, and NP13G) are

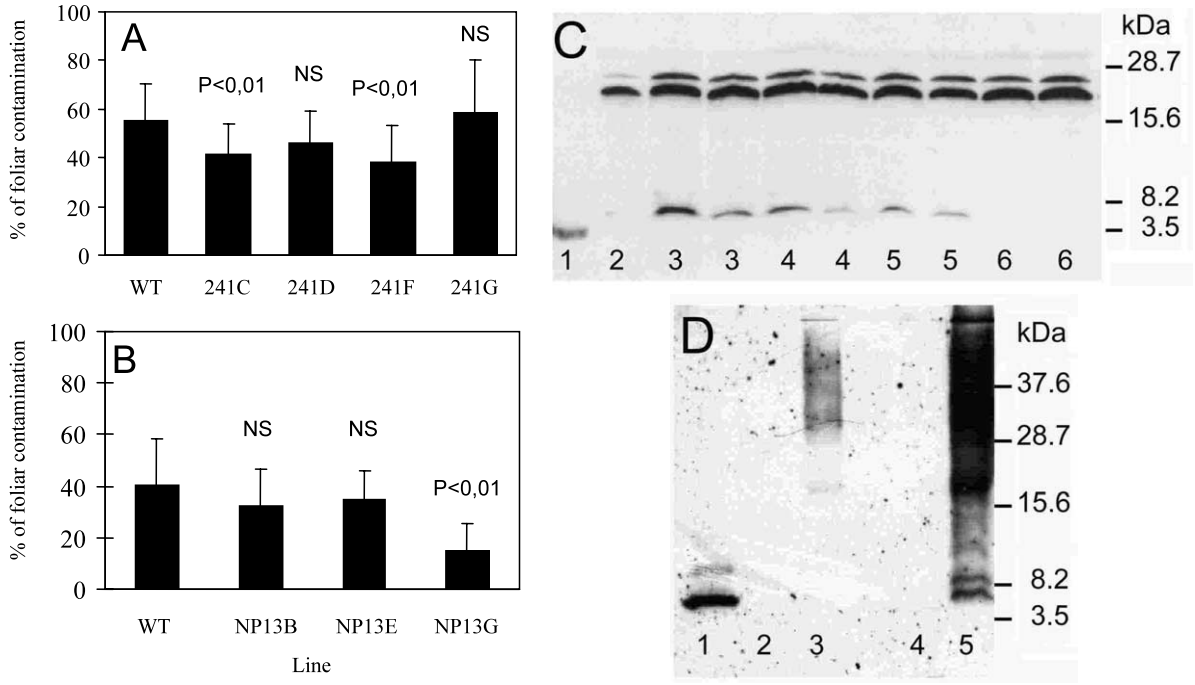


Fig. 6. Resistance against *C. nicotianae* and correlation with AFP accumulation (A) and (B) Plant disease symptoms evaluated 14 days after infection for WT or lines expressing heliomicin and drosomycin, respectively. Diagrams indicate mean values and standard deviation resulting from two independent experiments. Significance of difference (P) with respect to WT line (student's t-test) is indicated (NS = nonsignificant when $P > 0.01$). (C) Western blot analysis for heliomicin of reduced total protein extracts from transgenic lines 241C (2 plants-lanes 3), 241D (2 plants-lanes 4), 241F (2 plants-lanes 5), and 241G (2 plants-lanes 6). Lane 1 corresponds to 100 ng of recombinant heliomicin and lane 2 corresponds to WT tobacco. (D) Western blot analysis for drosomycin of unreduced apoplastic protein extracts from transgenic lines NP13B (lane 3), NP13E (lane 4) and NP13G (lane 5). Lane 1 corresponds to 100 ng of recombinant drosomycin and lane 2 corresponds to WT tobacco.

shown in Fig. 6A and B, respectively, and expressed as a mean of foliar infection for each transgenic or WT line, with the respective standard deviation. Plants treated with the fungicide chlorothalonil did not exhibit any disease symptom. For infected WT and transgenic lines, disease scores (number of plants versus level of infection) followed a normal distribution for each plant population (not shown), justifying the use of parametric Student's t-test to validate a significant difference between each transgenic line and the WT line in the corresponding experiment.

Among heliomicin transgenic plants (Fig. 6A), lines 241C and 241F exhibited a significant decrease of disease symptoms as compared to the WT line ($P = 0.009$ and $P = 0.004$, respectively). The apparent decrease in line 241D sensitivity was not significant ($P = 0.077$), and line 241G was clearly not resistant to *C. nicotianae*. Visual observations on line 241F revealed a pronounced decrease in leaf yellowing and in the size of the necrosis (not shown). For the NP13 lines (Fig. 6B), the mean infection was lower than for the 241 lines (40% of infection versus 55% for the WT line). The line NP13G exhibited a significant resistance to *C. nicotianae* as compared to WT ($P < 0.001$). Both NP13B and NP13E lines were not significantly different from WT.

For heliomicin, the resistance level was correlated to the presence of the antifungal peptide (Fig. 6C). Heliomicin was detected in the T2 lines 241C, 241D and 241F (Fig. 6C, lanes 3, 4, 5), whereas no expression was detected (Fig. 6C, lane 6) in the sensitive line 241G. Important variation in heliomicin expression levels were observed between the extracts from two plants of the same progeny, which have an identical genotype (Fig. 6C, lanes 3, 4, 5). This variation could be attributed to differences in the physiological state of the plants or introduced later during extraction and analysis. It may explain the lack of correlation between resistance (analyzed in 16 plants, Fig. 6A) and expression levels (analyzed in 2 plants, Fig. 6C) for lines 241C, 241D and 241F.

For drosomycin, lines NB13B and NP13E, which exhibited no resistance to *C. nicotianae*, showed in T0 generation a low level of the bound form of drosomycin for NP13B (Fig. 6D, lane 3), and no expression for NP13E (Fig. 6D, lane 4). However, the high level of disease resistance reached by the line NP13G was associated with a high level of peptide expression (Fig. 6D, lane 5), and a particularly high level of the expected 5 kDa form. Even though the level of resistance is only partial, it is clearly correlated to peptide accumulation in plant tissues. Such a level of resistance to *C. nicotianae* has not been observed in our conditions with other transgenes in tobacco (not shown). These results show that it is possible to enhance disease resistance to the

fungal pathogen *C. nicotianae* by expressing in transgenic plant antifungal peptides such as heliomicin and drosomycin.

4. Discussion

In this study, heliomicin and drosomycin, two 5 kDa cystein-rich antifungal peptides from insects, were analyzed as candidates to increase plant disease resistance to fungal pathogens, by the way of genetic engineering. We have evaluated their in vitro potential against plant–pathogens, as well as their in vivo expression and efficacy. Heliomicin and drosomycin exhibit in vitro a broad-spectrum of activity against phytopathogens of agronomic interest, in the micromolar range. Their efficacy, scored 5 days post-treatment, is favorably comparable to that of many other AMPs, although data are usually expressed as IC_{50} or MIC after various time periods [9,22,25,38,39]. The in vitro spectra of activity for both peptides are similar. However, heliomicin exhibits a higher activity on most of the strains tested.

Microscopic observations revealed differences in the dose-dependent morphogenic effects induced by heliomicin and drosomycin on germination and growth of *B. cinerea* spores. Spore lysis was observed for high peptide concentrations, a lytic effect frequently assumed for AMPs. Heliomicin did not totally block spore germination, but induced, at moderated concentrations, hyphal branching and swelling associated with limited elongation of the germ tube. These characteristic features of 'morphogenic' defensins, already observed for plant defensins on *F. culmorum* [38] and *A. longipes* [9], have also been described with thionins on *F. oxysporum* [10], but were not observed on *B. cinerea* spores submitted to drosomycin action (Fig. 1). Drosomycin at low concentration inhibited the swelling of the germ tube extremity, leading to 'unlimited' linear hyphal growth. Inhibition of appressoria formation but not mycelium elongation was also observed on *P. oryzae* (not shown). These results, as well as antagonist sensitivities to salts [28], suggest that the interactions of heliomicin and drosomycin with membranes of pathogens, and the resulting antifungal activity, involve two different modes of action as proposed for plant defensins [40].

Data on in vitro activity and in planta expression consolidate our choice to express cystein-rich antifungal peptides in plant. Heliomicin and drosomycin which accumulated in transgenic tobacco appear structurally stable in respect to proteolytic degradation. Both peptides purified from recombinant yeast keep their in vitro activity on *B. cinerea* when pre-incubated in crude plant extracts from tobacco, maize or rice (results not shown). Their 3-D structure presents a 'cystein-stabilized alpha-helix-beta sheet motif' [41] that may confer an extreme stability once expressed in planta. Moreover, their molecular mass and in vitro activity on *B. cinerea* were preserved in protein extracts from transgenic tobacco, and the enhanced resistance to *C. nicotianae* strongly suggest that the recombinant peptides are correctly folded. Apoplastic protein fractions, enriched in well-cleaved mature peptides, indicate the efficacy of the association of strong promoters with the plant signal peptides used in the two constructs to address the AFPs in the secretory pathway.

In vitro antifungal activity suggests that peptide expression in plant tissues must reach a threshold for in vivo activity. The highest level of the 5 kDa form of heliomicin expressed in our transgenic lines was estimated around 0.1% of total proteins. A similar moderate level has been measured for the defensin *Rs*AFP2 expressed in transgenic tobacco, providing a 7-fold enhanced resistance to *A. longipes* [9], and for the knottin-type *Mj*AMP2 and hevein-type *Ac*AMP2 antifungal peptides [21]. Expression of drosomycin reaches several percents of total proteins for our best transgenic line (NP13G), when also considering all the bound forms observed under non-reducing conditions. Thus, as described for thionins [42], drosomycin (and to a lesser extent heliomicin, not shown) seems to interact strongly with other cell components, possibly proteins through disulfide linkage, which might be a limiting factor for in vivo peptide expression and activity. Bound forms of peptide isolated from plant extracts have no in vitro activity on *B. cinerea*. Whereas no phenotypic alteration was observed in transgenic tobacco lines expressing heliomicin, the drosomycin line expressing the highest level of peptide (NP13G) exhibits a lower growth rate (reduction of about 10% of height at the flowering stage as compared to WT or heliomicin lines), more lateral ramifications, and morphologic alterations in leaf development (not shown). These phenotypic modifications were not observed in tobacco line NP13B expressing a low level of peptide, and therefore seem to be correlated with the level of peptide expression in tobacco.

Heliomicin appears to be the best candidate among the two peptides studied here, for transgenic applications in plants. Resistant to proteases (not shown) and salts [28], heliomicin exhibits a larger in vitro antifungal spectrum of activity than drosomycin, and a lower peptide expression level that confers enhanced disease resistance in tobacco without phenotypic alterations. Heliomicin presents the advantage of a better stability towards plant proteases, as compared to cecropin B and sarcotoxin IA, the first insect AMPs used in a transgenic approach [39,43].

The resistance conferred to transgenic tobacco against *C. nicotianae* by heliomicin or drosomycin is only partial and does clearly not match the efficacy of a chemical fungicide such as chlorothalonil. Similar ex-

periments in transgenic tobacco with AMPs such as *Rs*AFP2 [9], sarcotoxin IA [39] or a magainin analog [44] also conferred only a partial protection against pathogens. It seems therefore difficult to obtain a robust resistance through the overexpression in plant of a single gene coding for antimicrobial proteins or peptides, although this has been achieved with an alfalfa defensin in transgenic potato [12]. We believe that the best strategy to adopt in the future will be the co-expression of different molecules with complementary modes of action that act at different stages of fungal invasion or development. The choice must consider the in vitro activity of the molecule, but also its stability in plant cell environment, and the cell compartment where it should be addressed to fully exert its biological activity. With all existing genes already described, the best combinations have now to be found.

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