

Protective effect of an antimicrobial peptide from *Mytilus edulis chilensis* expressed in *Nicotiana tabacum* L.

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A "defensin-like" antibacterial peptide from *Mytilus edulis chilensis*, was sub-cloned into a binary vector for expression in plant tissues. The resulting new clone was electroporated into *A. tumefaciens* to transform tobacco plants. The presence of the construct in transgenic tobacco lines was demonstrated through RT-PCR, Northern and Western blots. Transformed positive

plants were selected and grown for challenging. Tobacco leaves were infiltrated with *Pseudomonassyringae* pv. *syringae* and visual lesions determined at different times post-exposure. Of seven plants exposed, four gave variable protection up to seven days post-infection while one of them appears to be fully protected. These results suggest that defensin-

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like antimicrobial peptides from molluscs are a good source to provide resistance of tobacco plants to *Pseudomonassyringae* pv. *syringae*.

Antibacterial peptides and proteins are part of the innate response, and as such constitute the first line of defence against microbial infections in most living organisms (Zasloff, 2002; Yeaman, 2003; Tossi, 2005). They have been reported at all levels, from mammals (Ackermann et al. 2004; Bardan et al. 2004) to insects (Otvos Jr., 2000), marine invertebrates (Haug et al. 2004; Tincu and Taylor, 2004) and plants (Castro and Fontes, 2005). Most of these peptides have been successfully cloned, expressed and tested both *in vitro* and *in vivo* (Wu et al. 2000; Metlitskaia et al. 2004; Ingham et al. 2005) in their ability to neutralize bacterial infections. At present, more than 500 different molecules have been reported (<http://aps.unmc.edu/AP/main.php>). In mussels, the defensins constitute the most important peptide family characterized considering their primary structure and their consensus cysteine array (Charlet et al. 1996; Hubert et al. 1996; Mitta et al. 2000; Marshall and Arenas 2003).

In plants, genes encoding defensins are developmentally regulated, with a predominant expression in outer cell layers (Moreno et al. 1994; Terras et al. 1995), and can be induced above basal levels in response to pathogen infection and other stresses (Chiang and Hadwiger, 1991; Moreno et al. 1994; Terras et al. 1995; Penninckx et al. 1996; Epple et al. 1997). Additionally, certain defensin genes are down-regulated by some pathogens (Moreno et al. 1994). Gene expression patterns of defensins (similar to

plant thionines) are thus consistent with a hypothetical role in plant defence (Broekaert et al. 1995; Broekaert et al. 1997).

There are a number of reports demonstrating the expression of heterologous antimicrobial peptides in plants with different degrees of success (Huang et al. 1997). The most abundant are those relating transferring these molecules from plant to plant (Sharma et al. 2000; Kanzaki et al. 2002). Notwithstanding, there are a few reporting transgenic expression of peptides derived from insect and amphibians (Sharma et al. 2000; Ponti et al. 2003). In this report, we demonstrate the protective expression of a defensin-like gene from *Mytilus edulis chilensis* as a stable transgene in *Nicotiana tabacum* plants when challenged with the phytopathogen *Pseudomonassyringae* pv. *syringae*.

MATERIALS AND METHODS

Clone description

The 148 bp fragment was obtained from genomic DNA from *Mytilus chilensis* using a set of degenerate primers constructed upon the DNA sequence from *Mytilus galloprovincialis* (Mitta et al. 1999). The pair of primers which efficiently amplified the putative gene was: SEN-A, 5'-CCGCATCKATACCANGTRCANC-3' and ANT-A2, 3'-CCGCATCKATARCANGTRCANCT-5'. The amplified sequence was cloned into pCX-TOPO, a maintenance vector described elsewhere (Topo TA Cloning, Instruction Manual, Version P 051302. 25-0184, Invitrogen, USA).

Construction of the expression vector

The pCX-TOPO-containing clone was digested with *Xho*I and *Bam*HI enzymes to release the defensin-like coding sequence. Prior to cloning the target sequence into the pBinAR plant tissue expression vector (Rocha-Sosa et al. 1989), the fragment was subcloned in the intermediate vector pBS in order to leave the target sequence in a "sense" orientation under the control of the 35 S CaMV promoter. pBS was linearized with *Xho*I and *Bam*HI, ligated with the defensin ORF, and positive clones detected through agarose gel electrophoresis. Restriction with *Kpn*I -*Xba*I enzymes released the coding sequence from the pBS vector which was exposed to the already *Kpn*I -*Xba*I linearized pBinAR vector. Ligation resulted in the recombinant expression vector. Positive clones (*MCh-Def*) were selected through standard bacterial transformation and restriction gel analysis as well as transformation and selection into *Agrobacterium tumefaciens*.

Agrobacterium tumefaciens-mediated plant transformation

The prepared construct MCh-Def was introduced in *A. tumefaciens* strain C58C1 containing plasmid pGV2260 (Deblaere et al. 1985) via electroporation. Transformation of tobacco (*Nicotiana tabacum* var. Xanhi NN) was

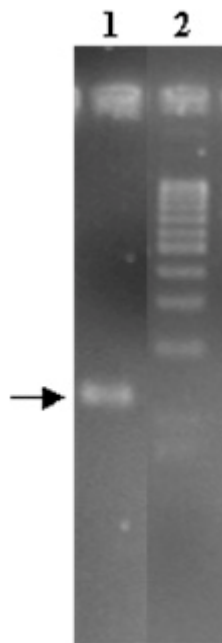


Figure 1. 2.5% Agarose gel electrophoresis demonstrating the expected size of the defensin-like clone. The 148 bp-specific insert (lane 1) was recovered from the amplified vector pCX-TOPO after digestion with *Xho*I and *Bam*HI enzymes. Lane 2, 100 bp ladder.

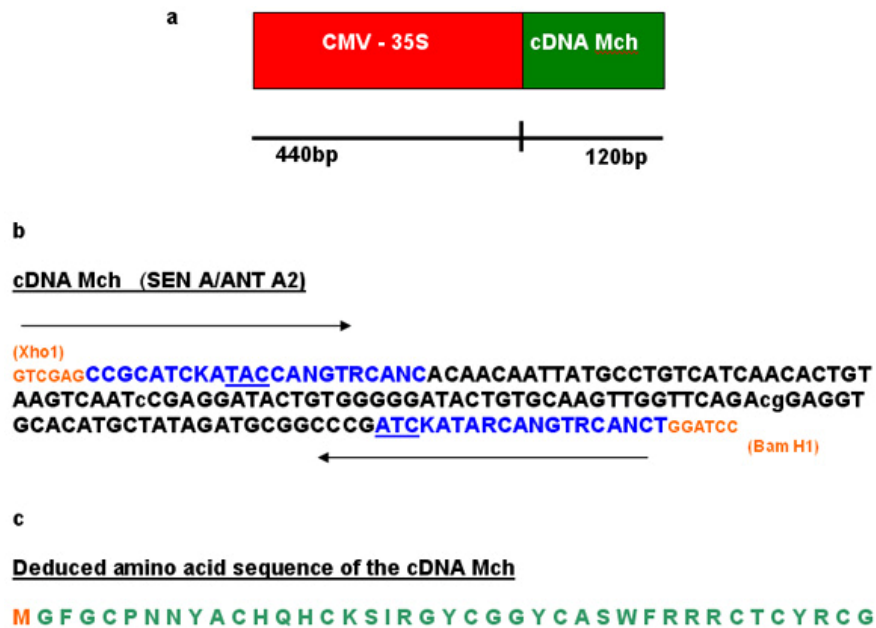


Figure 2.

(a) Diagram of the structure and the specificity of the defensin-like insert in the pBinAR vector.

(b) Nucleotide sequence of the defensin-like gene: underlined the beginning and end of the ORF (blue: primers; black: ORF).

(c) Amino acid sequence of the defensin-like peptide.

performed using *A. tumefaciens*-directed gene transfer essentially as described (Fillatti et al. 1987).

Presence of the defensin-like gene in transgenic plants

Plants in tissue culture were kept under a 16-h light/8-h dark period on Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 2% (w/v) sucrose at 22°C. In the greenhouse the plants were grown in potting compost soil at 22°C during the light period (16 hrs) and 15°C during the dark period (8 hrs). Leaves were used for genomic DNA extraction to confirm the presence of the defensin-like transgene using specific primers directed

either towards the cauliflower mosaic virus promoter 35S (5'-gCA CgA CAC gCT TgT CTA C-3') or towards the defensin-like gene sequence (Def1: 5'-CgC ATC gAT AgC Agg TgC AC-3'). The resulting amplicon had the expected bp size.

Expression of the defensin-like gene in transgenic plants

Northern blots. Total RNA from control and transgenic tobacco plants were resolved in formaldehyde agarose gels and transferred to a nylon membrane (Harms et al. 1998). The membrane was hybridized to a ³²P-labeled defensin-like probe.

RT PCR. The procedure described by Invitrogen SuperScript III First-Strand Synthesis System for RT-PCR (Cat. No: 18080-051) was used. The usual precautions for avoiding RNA degradation were taken until the reverse transcription reaction was complete. A solution of 10 µg total RNA in 9 µl of 1X annealing buffer was prepared and complemented with 1 µl of the oligodeoxynucleotide degenerate primer mix (100 pmol/µl). The mix was heated for 3 min at 65°C and allowed to cool to room temperature. To 10 µl of each annealed RNA sample, 15 µl of a cDNA synthesis mix was added, allowing the reverse transcriptase reaction to go for 1 hr at 37°C. The reaction was stopped by heat inactivation for 10 min at 75°C. Two µl of this cDNA mixture was used as a template for each 50 µl PCR amplification reaction, following standard procedures.

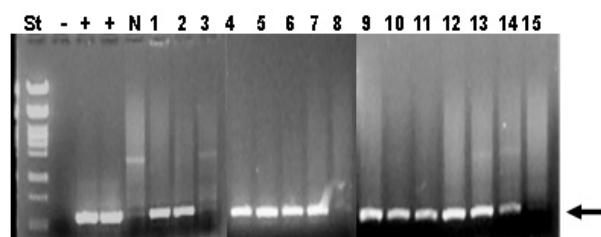


Figure 3. Presence of *Mytilus edulis chilensis* defensin-like gene in putative transgenic plants. Leaf genomic DNA was amplified by PCR using the primers described in Figure 1. The arrow indicates the expected size of the fragment (560 bp). Lanes: St, bp size standard; (-), no DNA in the amplification reaction; (+), PCR amplification of the chimeric gene-containing plasmid used to transform plant cells. N, non-transformed controls; 1-15, transgenic tobacco plant lines: lines 3, 8 and 15 were not transformed.

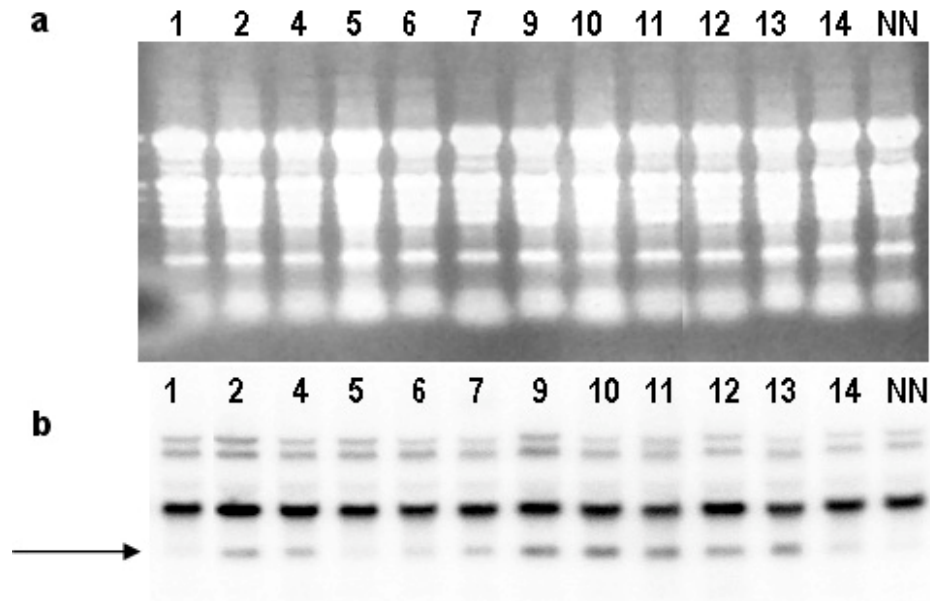


Figure 4. Expression of the defensin-like transgene in transformed whole tobacco plants.
(a) Agarose-formaldehyde gel stained with ethidium bromide.
(b) Northern blot analysis. Arrow indicates the expected position of the hybridization band specific for the defensin-like probe. Total RNA from 12 independent lines (lanes 1-14) were analysed. Lanes 2, 4, and 6 – 14 were considered positive lines. NN = Control, corresponding to non transformed plants.

Western blots. Total proteins from control and transgenic tobacco plants were determined by the bicinchoninic acid kit (Sigma Co. USA, B 9643), resolved in a 10% Laemmli PAGE-SDS gel, and transferred to a nitrocellulose membrane. The membrane was then exposed to a 1/5000 dilution of the polyclonal antibody MGD1, elicited in rabbits against a *Mytilus* defensin (Mitta et al. 1999) as the first antibody followed by an HRP-labelled anti rabbit IgG fraction and developed with DAB (Towbin et al. 1979).

Challenge with *Pseudomonas syringae* pv. *syringae*

Leaves from transgenic and control tobacco were infiltrated by mechanical injury with the phytopathogen *Pseudomonas syringae*pv. *syringae* at a concentration of approximately 10^5 - 10^6 CFU/ml in 10 mM MgCl₂ (Bi et al. 1995). Putative protective effect was measured by naked-eye observation after 2-7 days post-infection.

RESULTS

Generation of the *MCh-Def* clone

Figure 1 shows the DNA insert corresponding to the defensin-like ORF from *Mytilus edulis chilensis*. This was subcloned into the pBinAR plant tissue expression vector as shown in Figure 2.

Detection of the defensin-like transgene in transformation-positive tobacco plants

PCR. As shown in Figure 2, a set of primers specifically directed against the construct should resolve only transformation-positive clones which contain the whole

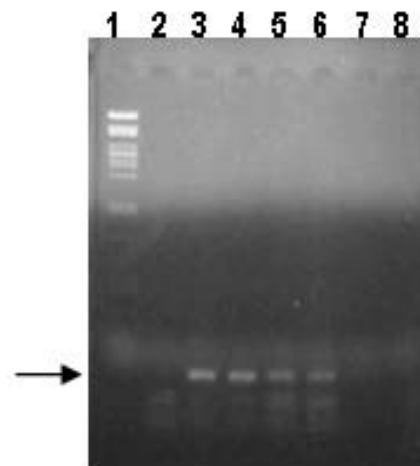


Figure 5. Expression of the defensin-like transgene in tobacco plants leaf tissue. Ethidium bromide stained agarose gel resolving the RT-PCR reactions of four of the selected positive lines shown in Figure 4 (lines 9 – 12). Lanes: 1, *Pst*1-digested lambda DNA; 2, no template control reaction; 3-6, positive lines 9-12; 7-8, two untransformed lines. Arrow indicates the expected amplicon size.

transgene when amplification occurs using primers against the flanking regions of the 35S-CaMV promoter from pBinAR (a) and from the defensin-like gene (b). Figure 3 shows that 12 out of 15 putative transformed lines amplify a 560 bp band corresponding to the whole construct when leaf genomic DNA from transformed and non transformed plants were amplified using the fore mentioned primers. Genomic DNA from tobacco leaves either from plants transformed with *MCh-Def* construct (lanes 1-15) as well as non transformed controls (N) was amplified using the standardized primers described in Figure 1.

Northern Blot. Selected lines, grown under axenic conditions, were the source for total RNA extraction in order to detect the putative transcript from the defensin-like transgene. Figure 4a shows the stained profile of the material resolved on the agarose gel, while Figure 3 shows the result of the transferred material hybridized to the defensin-like specific probe. Ten out of the 12 selected lines gave different degree of hybridization to the probe indicating differential transcription in the lines which correspond to the expression of *MCh-Def* gene (lanes 2, 4 and 6-14, respectively).

RT PCR. In order to reconfirm the results from Northern blot analysis, four out of the seven transcription positive lines were selected to evaluate specific transcription on leaf tissue which will be the target of challenging with pathogen bacteria. Figure 5 shows the RT-PCR results, confirming that lines 9-12 do express the defensin-like transgene.

Western Blot. Further, in order to detect recombinant peptide in the selected lines, crude protein extracts of leaves were resolved on denaturant gels, transferred to a nitrocellulose membrane and exposed to a defensin-like specific antibody (Figure 6).

Challenge with *Pseudomonas* pv. *syringae* and evaluation of resistance

Transgenic as well as control tobacco leaves were infected with *P. syringae* to evaluate the degree of resistance provided by the expressed defensin-like transgene. Figure 7 shows the degree of damage observed.

DISCUSSION

There have been a number of attempts to use both heterologous and even homologous peptides as tools to increase disease-resistance in transgenic plants (for a review, see Castro and Fontes, 2005). Although most of them have been unsuccessful, some of them have been inspiring in the search for new alternatives (Sharma et al. 2000; Kanzaki et al. 2002; Ponti et al. 2003). Some of the reasons for the lack of success in the past have been estimated to be due to either a low level of expression of the transgene (Halpin, 2005), a putative inactivation of the gene product by constitutive proteases in the new host environment, or a low half-life of the expressed peptide in the host plant (Owens and Heutte, 1997).

In this report, the use of a vector containing the defensin-like ORF from *Mytilus edulis chilensis* in frame with the 35S promoter from the cauliflower mosaic virus (Figure 2) allowed us to get an 80% efficiency of transformation and a reasonable good expression which offers a detectable, protection to tobacco leaves when challenged with *Pseudomonas syringae* pv. *syringae*. We selected 12 positive transgene lines containing the *M. edulis chilensis* defensin-like cDNA to demonstrate disease-resistance (Figure 3). Notwithstanding not all tissues of the positive lines either had the transgene and/or express it. Northern blot analysis indicated that only 7 out of 12 independent transgenic lines gave a significant expression and one of them, a faint one (Figure 4, lanes 2, 4, and 9-13). We selected lines 9-12 to further verify RNA expression in leaves of the transgenic plants, the target of our challenging test. Indeed all four positive lines gave significant transcription of the transgene (Figure 5). Additionally, the

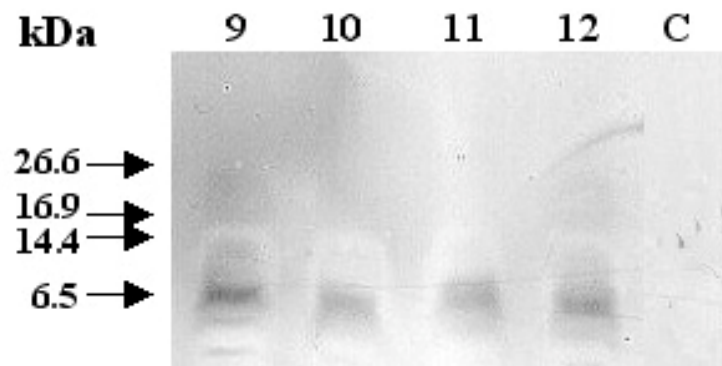


Figure 6. Western blot analysis of non-challenged crude extracts from leaves of the selected transgenic lines 9-12, shown in Figure 5, using polyclonal antibodies against *Mytilus* defensin. Protein load, 30 µg/lane. Lane C corresponds to an untransformed tobacco plant as control.

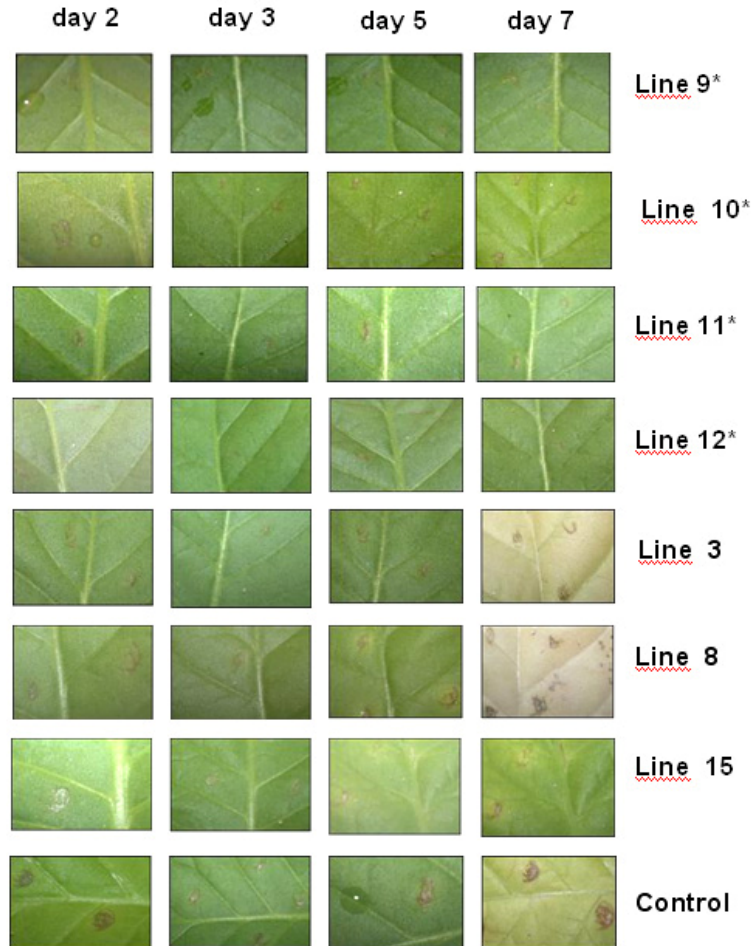


Figure 7. Tobacco leaves infiltrated by mechanical injury with the phytopathogen *Pseudomonas syringae* pv. *syringae* at a concentration of approximately 10^5 - 10^6 CFU/ml. Observation after 2-7 days post-infection indicates a putative protective effect for lines 9, 10, 11 and especially 12 (*), particularly evident at day 7 with respect to control leaves. Lines 3, 8, 15 are transformed non-expressing lines. Control leaves were derived from non-transformed plants. The figure is one of two identical duplicates.

protein was expressed in all four lines (Figure 6), providing a strong argument to support the resistance to infection shown by challenged leaves measured by two unequivocal parameters: number and/or size of the lesions observed (Figure 7). In this frame, protective responses were detected in the positive lines up to 7 days post-challenge, well above to those described for other peptides in previous works on tobacco plants (Okamoto et al. 1998; Koo et al. 2002; Li et al. 2001).

These positive preliminary results are encouraging. Considering that the transgene product is a defensin-like molecule expressed in a system which has natural counterparts (Castro and Fontes, 2005), endogenous proteases do not consider it a target. In addition, the transgene product could act synergistically with endogenous AMPs, natural defensins and/or alike, which might explain the observed protective effect over challenging in selected transgenic lines. Because endogenous defensins are expressed in minute amounts in tobacco leaves, over-expression of the transgene, even

under non-challenged conditions, transforms it in a helper-like tool to overcome putative encounters with aggressive pathogens.

In conclusion, this report is a contribution to further design strategies to evaluate the impact of family-related peptides in commercially relevant plant species which might be optimized through their enhanced disease-resistance potential.

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