An amphibian antimicrobial peptide variant expressed in *Nicotiana tabacum* confers resistance to phytopathogens[1]

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Esculentin-1 is a 46-residue antimicrobial peptide present in skin secretions of *Rana esculenta*. It is effective against a wide variety of micro-organisms, including plant pathogens with negligible effects on eukaryotic cells. As a possible approach to enhance plant resistance, a DNA coding for esculentin-1, with the substitution Met-28Leu, was fused at the C-terminal end of the leader sequence of endopolygalacturonase-inhibiting protein, under the control of the cauliflower mosaic virus *35S* promoter region, and introduced into *Nicotiana tabacum*. The antimicrobial peptide was isolated from the intercellular fluids of healthy leaves of transgenic plants, suggesting that it was properly processed, secreted outside cells and accumulated in the intercellular spaces. The morphology of transgenic plants was unaffected. Challenging these plants with bacterial or fungal phytopathogens demonstrated enhanced resistance up to the second generation. Moreover, transgenic plants displayed insecticidal properties.

Key words: amphibian skin, disease resistance, esculentin-1, insecticidal, transgenic.

INTRODUCTION

Plant disease is one of the leading causes of crop loss which reduces the food production of the world. With the increased public concern about the use of chemicals on food crops, there is a clear need for alternatives, including biotechnological ones. Advances in genetic engineering have made possible the development of plants with new predictable phenotypes [1,2]. In particular, to increase resistance against diseases, different genetic strategies have been proposed, which include utilization of antibacterial proteins of both plant [3–5] and non-plant origin [6,7].

A large number of antimicrobial peptides from different organisms have been characterized [8–11]. Esculentins are a family of antimicrobial peptides from skin secretions of *Rana esculenta* displaying potent antimicrobial activities, with negligible effects on eukaryotic cell membranes [12]. In particular, esculentin-1 is a 46-amino-acid residue peptide, with a C-terminal disulphide bridge, effective against a wide variety of human pathogens (*Staphylococci*, *Yersinia*, *Pseudomonas* and *Candida* species) with lethal concentration values ranging from 0.1 to 1.5 μM. Preliminary experiments indicated esculentin-1 and its analogues as ideal candidates for genetic engineering of plants, as they display *in vitro* a potent activity against *Pseudomonas syringae* pv. *tabaci* [13] and *Phytophthora nicotianae* (M. L. Mangoni, D. Ponti, M. Simmaco and D. Barra, unpublished results). The finding that a defensin subfamily from spinach shares the N-terminal sequence Gly-Ile-Phe-Ser [14] with esculentin reinforced the possibility that esculentin can be properly processed in plants, thus conferring an enhancement of the physiological response to infections.

In the present study, we report the transgenic expression in *Nicotiana tabacum* of esculentin-1, carrying the substitution Met-28Leu (Figure 1A). The expression of this peptide in plants confers enhanced resistance to pathogens. These results further support the assignment of a defence role to esculentin-1 and highlight its biotechnological potential in plants.

EXPERIMENTAL

Expression vector construction

The leader sequence of the gene of *Phaseolus vulgaris* endopolygalacturonase-inhibiting protein (PGIP) *PGIP-1* was fused upstream of an esculentin-1-coding DNA to obtain an extracellular localization of the mature product. The construct was obtained by PCR as follows. The *PGIP-1* signal peptide (87 bp) was amplified by PCR from *pBI-PGP-1* DNA [15] as template using primers A (5′-CCGGATCCATGACTCAATTCAATATCCCA-3′) and B (5′-AGAGAGTGCAGTTCTCAA-3′). A *Bam*HI recognition site was incorporated into primer A. The coding region of esculentin-1 (138 bp), carrying the substitution Met28Leu (the sequence of the mature peptide is shown in Figure 1A), was amplified by PCR from *pET-Esc-GABA-T* DNA [13] using primers C (5′-GGAATCTTCTCCAAATTGGCC-3′) and D (5′-CGAGAGCTCTTAACATTCACCTTTAATTTT-3′). A *Sac*I recognition site was included in primer D. The two PCR products were ligated into the *Bam*HI and *Sac*I restriction sites of pBlueScript-SK (Stratagene, La Jolla, CA, U.S.A.). The generated *L-esc* fragment was further amplified using primers A and D. For *Agrobacterium* transformation, the PCR product was digested with *Bam*HI and *Sac*I restriction enzymes (Boehringer Mannheim Corporation, Indianapolis, IN, U.S.A.) and subcloned into pBI-121 binary vector, carrying the *NptII* (neomycin phoshotransferase) gene to confer kanamycin

Abbreviations used: CFU, colony-forming units; ICF, intercellular fluid; LB, Luria–Bertani; PDA, potato dextrose agar; PGIP, endopolygalacturonase-inhibiting protein; PVX, potato virus X; RH, relative humidity.

[1] Dedicated to the memory of Eraldo Antonini, eminent biochemist, prematurely deceased twenty years ago, on March 19th 1983.

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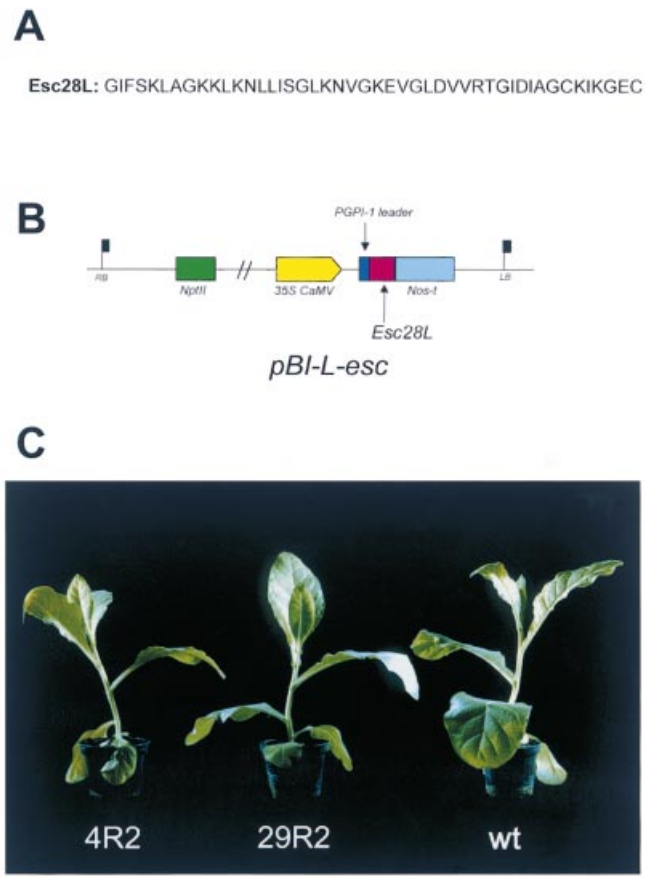

Figure 1 Expression of the antimicrobial peptide Esc28L in *N. tabacum*

(**A**) Sequence of the peptide; (**B**) schematic drawing of the expression construct *pBI-L-esc*; (**C**) morphological characteristics of *N. tabacum* wild-type (wt) and transgenic plants of lines 4 and 29 at the second generation.

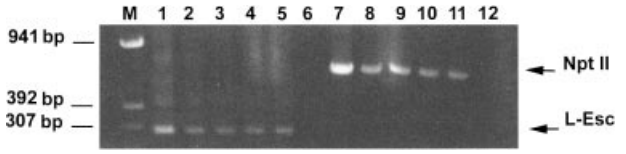

Figure 2 Integration of *L-esc* gene into the plant genome

Genomic DNA from transformed plants was amplified by PCR using suitable oligonucletotides and electrophoresed on agarose gel. Lane 1, pBI-L-esc amplified with oligonucleotides A and D from the *PIGP-1* leader and esculentin genes (see the Experimental section); lanes 2–6, genomic DNA from lines 4, 16, 28, 29 and wild-type, amplified as in lane 1; lane 7, pBI-L-esc, amplified with oligonucleotides from the 5′ and 3′ regions of the *NptII* gene; lanes 8–12, genomic DNA from lines 4, 16, 28, 29 and wild-type, amplified as in lane 7; M, markers of DNA molecular mass. The two products of PCR amplification are 225 and 792 bp, respectively.

resistance, and under the control of the cauliflower mosaic virus *35S* (*CaMV35S*) promoter region and the *Agrobacterium* nopaline synthase terminator (*NOS-t*; Figure 1B) [16]. The fidelity of the construct pBI-L-esc was confirmed by DNA sequencing (Sequenase version 2.0; USB Corporation, Cleveland, OH, U.S.A.).

Plant transformation and regeneration

Leaf disks of *N. tabacum* cv. petit havana SR1 were inoculated with *Agrobacterium tumefaciens* LBA4404 strain and selected on kanamycin (100 mg/l; Sigma) [17]. Regenerants (R0) were grown on Murashige-Skoog (Sigma) medium for 4 weeks in a chamber at 25 °C, 75 % relative humidity (RH), exposed to 16 h of light and 8 h of darkness. Integration of the transgene was confirmed by PCR using A and D primers and genomic DNA, isolated from 5 g of fresh leaves according to Clark [18], as template (Figure 2). Regenerants up to the second generation (R1 and R2) were grown on non-sterile soil under the same conditions (Figure 1C).

Tests with pathogens

P. syringae pv. *tabaci* (*P. tabaci*) and *Pseudomonas aeruginosa* A.T.C.C. 15692 were grown in Luria–Bertani (LB) medium up to absorbance $A_{600} = 1$ and then diluted to 10^5 colony-forming units (CFU)/ml. Spores of *Phytophthora nicotianae* were collected by water washes of 1-week-old sporulating culture grown on potato dextrose agar (PDA) plates in reduced light at 25 °C.

The *in vitro* antibacterial activity of leaf extracts was evaluated using an inhibition zone assay on LB medium–agarose plates [19]. For transgenic plants, 9-mm diameter leaf discs of *N. tabacum* regenerants grown on sterile soil were weighed and put on LB medium/1 % agarose (Sigma-6013) in 3 cm Petri dishes. Each of them was covered with 1.5 ml LB medium/agarose containing 20 μl of 10^5 *P. tabaci* cells/ml. After overnight incubation at 30 °C, the inhibition zones were measured. Non-transformed controls were also grown in sterile soil.

For the *in vivo* tests, the sixth leaf from the bottom of each plant was inoculated with 15 μl of the bacterial culture (10^5 and 10^4 CFU/ml LB medium for *P. tabaci* and *P. aeruginosa* respectively) by infiltrating bacteria into the lamina with a syringe without needle at 5 (*P. tabaci*) and 6 (*P. aeruginosa*) spots/leaf [20]. Plants were grown at 25 °C, 80 % RH and 16 h light. After 10 days, the necrotic zone around each site of inoculation was measured with a calliper and the area of lesion was calculated. Then, leaf squares were cut from a single inoculated area (1 cm²) of each plant and quickly ground in 1 ml of LB medium. The homogenates and two dilutions (10^{-1} and 10^{-2}) were diluted with 3 ml of LB medium–1 % agarose (Sigma-6013) and plated on LB medium. The colonies were counted after incubation at 27 °C for 48 h.

Antifungal assays with *P. nicotianae* were performed on plant leaves by placing 10 μl of an aqueous suspension containing 10^6 spores/ml (two spots/leaf). Plants were maintained in highly humidified conditions (100 % RH) at 25 °C, 16 h light in a growth chamber. After 10 days, 1 cm² of the infected area of each leaf was homogenized in 1 ml of 10 mM PBS, pH 7.4, containing 20 mM glucose, to which 3 ml of PDA/1 % agarose was added and plated on PDA. Plates were incubated at 25 °C and fungal cells were counted after 3 days.

Alternatively, 2 week-old rooted plants, grown in Magenta jars in Murashige-Skoog medium, were incubated for 4 weeks at room temperature (22 °C) with two slices (1 cm² each) of PDA in which *P. nicotianae* was allowed to grow for 10 days.

All experiments were repeated at least three times. Statistical analysis was performed with GraphPad Prism software and the standard errors of the means are indicated on the bar graphs.

Immunological detection

Intercellular fluids (ICFs) were collected from leaves subjected to vacuum three times for 20 s in 0.5 M NaCl, 20 mM Tris/HCl,

1 mM PMSF and 1 mM dithiothreitol. After centrifugation at 800 ***g*** for 5 min, the extracted fluids were concentrated by a Microcon-3 (Amicon, Beverly, MA, U.S.A.) at 7000 ***g*** for 40 min. ICF aliquots containing 20 μg of proteins were electrophoresed on an acid/urea gel [21] and then electroblotted on to PVDF membranes (BioRad, Hercules, CA, U.S.A.) using a semidry transfer apparatus (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). Membranes were incubated for 1 h with a 1:500 dilution of mouse antiserum raised against recombinant esculentin-1 [13]. Goat anti-mouse IgG (1:12500 dilution) was used as secondary antibody. Detection was performed using a commercial BM chemiluminescence Western-blotting Kit (Boehringer Mannheim).

Esculentin detection

ICFs were subjected to reverse-phase HPLC under the conditions reported [12]. The antimicrobial activity of collected fractions was evaluated using an inhibition zone assay against *Bacillus megaterium* BM11 and *P. syringae* pv. *tabaci*. The active fractions were analysed by a matrix-assisted laser-desorption ionization–time-of-flight MS (PerkinElmer Voyager-DE, Wellesley, MA, U.S.A.). Similar experiments were performed on supernatants obtained after boiling of leaves for 10–15 min in 0.01 % acetic acid.

Insecticidal assay

Both ICF or boiled extract of control and transgenic plants (lines 4R1 and 4R2), at the same protein quantity (340 μg), were tested for antibacterial activity against *B. megaterium* BM11 with the inhibition zone assay [19] and then incorporated into an artificial diet (Standard corn meal medium; Sigma) of *Drosophila melanogaster* Oregon R. Fifty insects were placed in ventilated and humidified plastic boxes containing 0.5–1 g of diet. Mortality was recorded after 2–3 days [22].

RESULTS

Previous experiments on the recombinant expression of esculentin-1 cyclic and linear analogues in *Escherichia coli* showed that both peptides retain the biological activity of the natural one [13]. Furthermore, it was demonstrated that the infiltration of the cyclic peptide in *N. tabacum* leaves does not produce toxic effects. Therefore, for these studies, the cDNA coding for the cyclic analogue with the Met-28Leu substitution was used [13]. After *Agrobacterium* transformation, 32 independent transgenic lines resistant to kanamycin were selected, of which four lines contained the appropriate DNA fragment (Figure 2). Transgenic plants were self-pollinated to obtain the second generation (R2). Their morphological characteristics were comparable with those of non-transformed plants (Figure 1C).

Phytopathogen resistance of Esc28L-expressing plants

Two bacterial strains were used, *P. syringae* pv. *tabaci* and *P. aeruginosa* A.T.C.C. 15692. To obtain a rapid *in vitro* estimation of the production of the peptide in transgenic plants, the inhibition zone assay [19] against *P. tabaci* was performed directly on leaves from transformants (R0, R1 and R2) and non-transformed controls. The highest growth inhibition was displayed by leaf discs of lines 4 and 29. Surviving bacteria were observed on leaves of non-transformed plants only (in Figure 3, the inhibition zone assays on line 4R2 and the control are reported as an example).

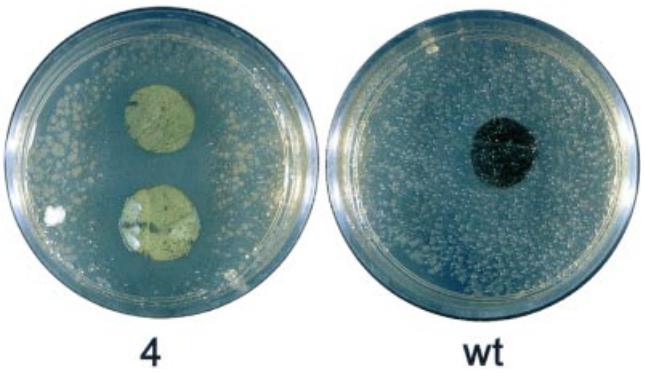

Figure 3 ***In vitro*** **antimicrobial activity of** ***N. tabacum*** **leaf discs**

Leaf discs (9 mm diameter) were put in LB medium/1 % agarose seeded with *P. syringae* pv. *tabaci* and incubated overnight at 30 °C. Left, transgenic line 4 at the second generation; right, wild-type (wt) plant.

Transgenic plants from the progenies of transformants 4, 16, 28 and 29 were used for *in vivo* experiments with *P. tabaci*. Ten days after bacterial infiltration on leaves, the control showed major necrotic lesions, whereas bacterial growth was completely inhibited in lines 4 and 29 (Figure 4A). For lines 16 and 28, no significant difference from the control was noticed. A quantitative estimate of the lesions caused by the pathogen are presented in Figure 5(A). It is worth noting that the syringe infiltration by itself can cause a small necrotic area.

Reports in the literature indicate that specific strains of *P. aeruginosa* might function as plant and animal pathogens [23,24]. Leaf infiltration with *P. aeruginosa* A.T.C.C. 15692 was performed in transgenic lines 4 and 29 (R2). No disease symptoms were observed, whereas tissue maceration was evident in the

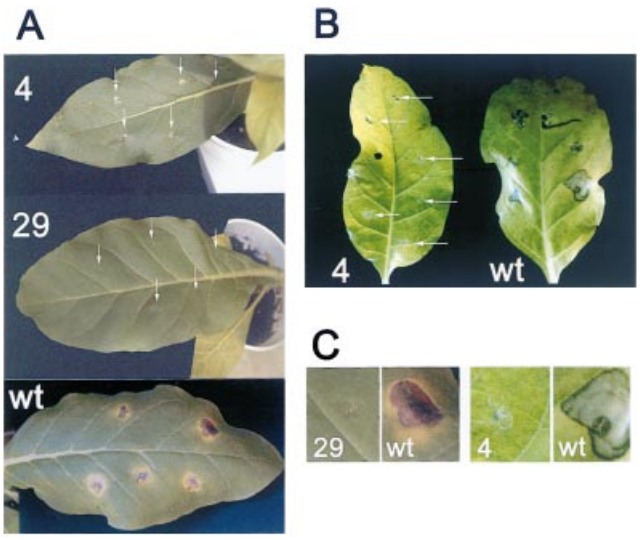

Figure 4 ***In vivo*** **antimicrobial activity of** ***N. tabacum*** **leaves**

Leaves from wild-type (wt) and transgenic plants were infiltrated with *P. syringae* pv. *tabaci* (**A**) and *P. aeruginosa* A.T.C.C. 15692 (**B**) (15 μl of LB medium containing 10^5 or 10^4 CFU/ml respectively). Arrows identify the sites of infiltration. The black hole in (**B**), line 4, indicates the syringe diameter. Enlargements are shown in (**C**): left, *P. tabaci*; right, *P. aeruginosa*.

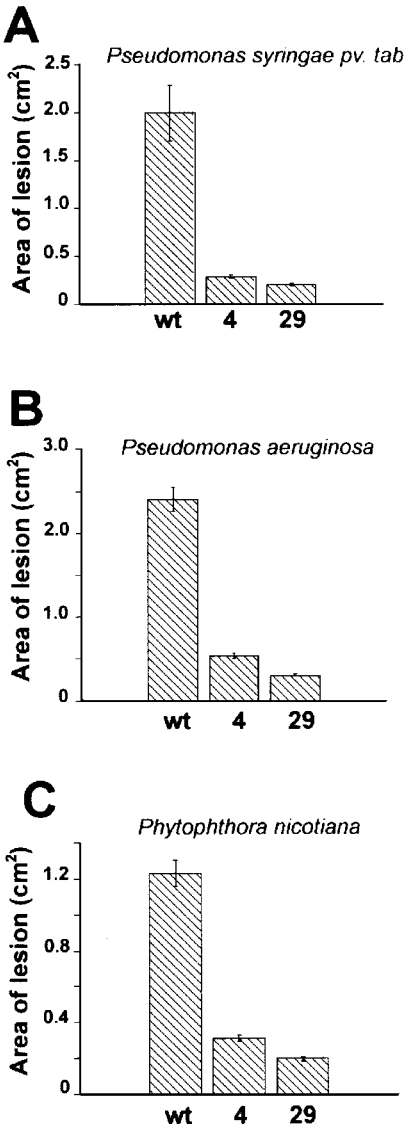

Figure 5 Lesions produced on leaves by pathogen infiltration

Area of lesions of plant leaves infiltrated with *P. syringae* pv.*tabaci* (**A**), *P. aeruginosa* A.T.C.C.15692 (**B**) and *P. nicotianae* (**C**). Values are means ± S.E.M. for three independent measurements.

Figure 6 Effect of *P. nicotianae* on 2-week-old plants

Two slices (1 cm^2 each) of PDA/1% agarose containing *P. nicotianae* were applied to both sides of wild-type (wt) and transgenic (4R2) *N. tabacum* plants incubated at room temperature (22 °C) for 4 weeks.

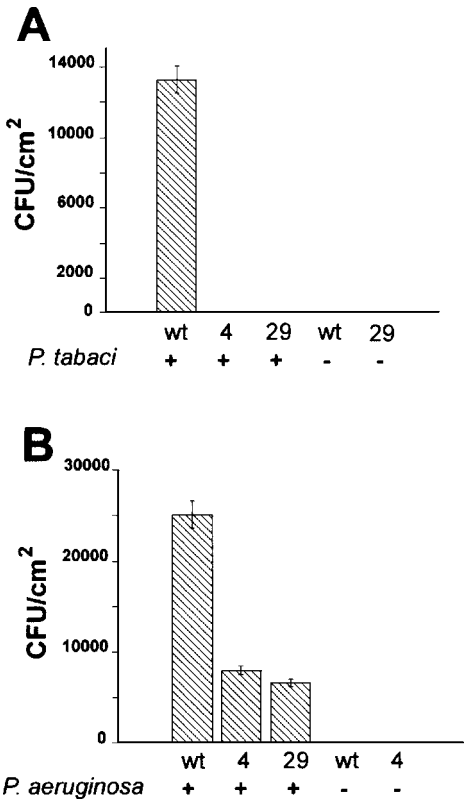

Figure 7 Survival of bacteria infiltrated in *N. tabacum* plants

The number of surviving bacteria after leaf infiltration with *P. tabaci* (**A**) and *P. aeruginosa* (**B**) was determined on 1 cm^2 leaf squares from a single inoculated area (see Figure 4), ground in LB medium and plated. Colonies were counted after 48 h incubation at 27 °C. Controls are leaf squares of non-infiltrated leaves from the same plant. Values are means ± S.E.M. for three independent measurements.

control (Figure 4B). Quantitative estimates of *P. aeruginosa* lesions are reported in Figure 5(B).

To evaluate the resistance potential to fungi, a similar experiment was performed by infecting leaves with a suspension of *P. nicotianae* spores. As shown in Figure 5(C), a significant reduction of the lesion area is displayed by both transgenic lines.

Challenging the transgenic plant 4R2 with *P. nicotianae* showed that, after 30 days, the fungus had grown extensively over the surface of the Murashige-Skoog medium, only in controls that were infected from the roots to the tips, resulting in yellowing of leaves and consequent death. In contrast, transgenic plants were still green and growing (Figure 6).

Bacterial growth on leaves infiltrated with *P. tabaci* and *P. aeruginosa* was measured after 10 days by counting colonies of 1 cm^2 leaf square ground in LB medium. The results are reported in Figure 7. Total absence of bacteria was evidenced following *P. tabaci* infiltration, whereas with *P. aeruginosa* a 70% reduction was observed. With *P. nicotianae*, the CFU reduction was approx. 50% (results not shown).

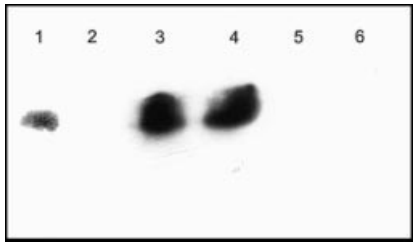

Figure 8 Immunological detection of Esc28L

Immunoblot analysis of the ICFs of transgenic plants electrophoresed on a acid/urea gel stained with anti-esculentin antibodies. ICFs were extracted from leaves by vacuum treatment (see the Experimental section). Lane 1, Esc28L; lane 2, wild-type; lanes 3–6, transgenic lines 4, 29, 16 and 28 respectively.

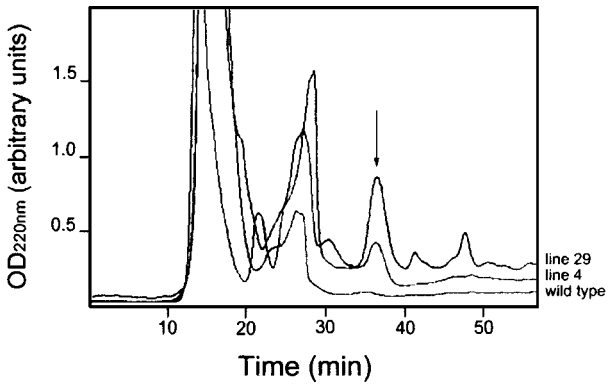

Figure 9 HPLC separation of ICFs of *N. tabacum* leaves

The fractionation was performed on an Aquapore RP-300 column (2.1 × 150 mm; Applied Biosystems, Foster City, CA, U.S.A.) eluted with a linear gradient of acetonitrile in 0.2% trifluoroacetic acid. ICFs of wild-type and lines 4 and 29 were analysed. The arrow indicates the elution position of the active fraction.

Esculentin purification

Esc28L was extracted from leaves by vacuum treatment. The expression levels of the peptide in transgenic plants were examined by immunoblotting using a specific anti-esculentin antibody. A positive band was clearly detectable in the ICF from lines 4 and 29, whereas no signal was detected in ICF from controls as well as from lines 16 and 28 (Figure 8).

ICFs were fractionated by reverse-phase HPLC (Figure 9). A similar HPLC profile was obtained from boiled leaf extracts. Antibacterial activity, associated with the peak indicated by the arrow in the figure, was only found in ICFs from lines 4 and 29. Active fractions were pooled and subjected to mass spectral analysis. A molecular mass of 5.51279 kDa, corresponding to that of Esc28L including the stretch SLRRRTALS of the PGIP-1 leader, was found. Other observed signals (m/z 3667.71, 3553.45, 2399.03 and 2338.88) corresponded to shorter, still active, forms of the peptide (1–35, 1–34, 1–23, 1–22 respectively), differently truncated at the C-terminus. This multiplicity of molecular forms can justify the broad bands observed in the immunoblotting (see Figure 8).

Insecticidal properties

Transgenic plants, left on the bench of the laboratory, presented progressively increasing numbers of dead insects on leaves. These were identified to be mainly hymenoptera but also diptera. Some of these environmental insects were captured, allowed to multiply under controlled conditions and then delivered into the plant chamber. Subsequently, dead insects were found on both sides of leaves, stem and flowers of transgenic plants more abundantly than on wild-type ones (Figure 10A). Therefore the effect on insect survival was assayed on ICFs or boiled leaf extracts of *N. tabacum* plants, incorporated into an artificial diet for *D. melanogaster*. Transgenic samples were more effective than controls in terms of insect mortality (see Table 1).

Figure 10 Insecticidal property of a transgenic plant and sequence comparison with an insecticidal peptide

(**A**) Front, a leaf of the transgenic line 4R2 covered with dead insects; back, wild-type leaf. The number of dead insects (± S.E.M.) was counted on different portions (1 cm^2 each) of the fourth leaf from the bottom of two sets of plants. (**B**) Comparison of the sequences of esculentin and ponericin G1 [29]. Identical residues (*) as well as conservative (:) and semiconservative (.) replacements are indicated.

Table 1 Antibacterial and insecticidal activity of transgenic plant extracts

The extracts (approx. 340 μg of total proteins obtained by boiling the leaf) were tested for antibacterial activity against *B. megaterium* BM11 with the inhibition zone assay [19], and then incorporated into an artificial diet of *D. melanogaster* oregon R. Fifty insects were fed with 0.5–1 g of diet. Mortality was recorded after 2–3 days. Antibacterial activity is given as cecropin units/g of leaf. One cecropin unit corresponds to the activity of 1 ng of cecropin A on *B. megaterium* [30]. Insecticidal activity is given as the number of dead insects and the ratio between the percentage of dead insects fed with leaf extracts of transgenic lines and that of the insects fed with wild-type leaf extracts. Results are means ± S.E.M. ($n = 3$); $P \leqslant 0.01$.

Transgenic line	Antibacterial activity	Insecticidal activity	
		Dead insects/50	Ratio
4R1	7700	31 ± 5.2	1.69 ± 0.6
4R2	5856	16 ± 1.8	1.70 ± 0.5

DISCUSSION

Attempts have been made to bolster plant defences against bacteria and fungi by genetically engineering plants to express insect peptides [25]. This approach failed because these peptides proved to be toxic. Hybrid peptides retaining the ability to kill bacteria without harming plant cells were also investigated. Plants expressing a cecropin–melittin cationic chimaera showed resistance to bacteria as well as fungi [7], although the efficacy of the chimaera was cultivar-specific.

In the present study, we have chosen a variant of the antimicrobial peptide esculentin-1 [12] as an interesting candidate for transgenic plant expression. The peptide-coding gene inserted into plant DNA carried the substitution Met-28Leu that does not alter the activity of the mature product [13]. This peptide was compatible with plant systems without producing the lesion-mimic effect [26,27]. Tests of infiltration on leaves of *N. tabacum* showed that Esc28L was well tolerated by plant cells at antimicrobial concentrations. In preliminary experiments, the coding sequence of Esc28L, with and without the PGIP-1 leader, was introduced into the expression cassette of a vector based on potato virus X (PVX) for transient expression [28]. Only plants transfected with PVX/leader-Esc28L produced the antimicrobial peptide. Therefore a suitably engineered fragment of DNA, carrying the leader-Esc28L coding region under *CaMV35S* constitutive promoter, was introduced into *N. tabacum* cv. petit havana SR1 via *Agrobacterium* transformation for stable expression. The peptide production was proved by immunoblot and MS analyses of the ICFs, suggesting a correct functioning of the construct. It is conceivable that the N-terminal sequence of esculentin, Gly-Ile-Phe-Ser, identical to that of a subfamily of plant defensins [14], protects the amphibian peptide from plant peptidases. However, degradation of the C-terminal region was found to occur, but the shorter peptides, purified by HPLC, retain antimicrobial activity. The amount of expressed peptide, estimated on the basis of its antimicrobial activity, is 1–2 μg/g of fresh leaf. This level of expression does not alter the normal phenotype of the plant up to the second generation.

Both transgenic plants and leaf extracts showed insecticidal activity. We suppose that this property is conferred to the plant by Esc28L expression but so far we have no indication on the mechanism of action. Recently, from the ant *Pachycondyla goeldii* a peptide family, the ponericins, was isolated [29], displaying antimicrobial, insecticidal or haemolytic properties. A 26 % sequence identity and 70 % sequence similarity were found between Esc28L and ponericin G1 in the region 18–46 of the former molecule (Figure 10B). The fragment 19–46 of esculentin-1, devoid of antibacterial activity, was actually found in *R. esculenta* skin secretion [12]. We have produced the 1–18 fragment of this peptide by chemical synthesis and found it to possess antimicrobial activity (M. L. Mangoni, D. Barra, M. E. Schinina and M. Simmaco, unpublished work). This confirms that the observed formation of shorter molecular forms is not detrimental for the biological activity of transgenic plants, and that their insecticidal property may be due to the C-terminal region of the molecule. This fact highlights another aspect of the defensive function of esculentin in frog skin secretion, being the antibacterial, antifungal and insecticidal activities exerted by the same class of molecules, possibly by the same molecule. The finding that Esc28L expression in plants strongly increases the resistance to micro-organisms makes this peptide a promising alternative defence tool with application in several fields, from floriculture to food production and storage.

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