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Expression of a Cecropin B lytic peptide analog in transgenic tobacco confers enhanced resistance to bacterial wilt caused by *Pseudomonas solanacearum*

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Cecropin B is a naturally-occurring lytic peptide found in *Hyalophora cecropia*, the Giant Silk Moth. It is thought to comprise part of an inducible humoral defense system that combats infection in the insect. Two 38 amino acid peptides, SB-37 and Shiva-1, were produced as substitution analogs of Cecropin B. SB-37 is 95% homologous to Cecropin B while Shiva-1 retains only 46% homology to the natural molecule. However, hydrophobic properties and charge density of the native structure were conserved at 100% in the synthetic peptides. The genes for both peptides were chemically synthesized and cloned into the binary vector pBI121 under the control of a constitutive or wound-inducible plant promoter. Transgenic tobacco plants (RO) were subsequently obtained via *Agrobacterium* transformation. Bioassays to test disease resistance of R1 progeny indicate that, compared to transgenic control and SB-37 plants, Shiva-1 seedlings exhibited delayed wilt symptoms and reduced disease severity and mortality after infection with a highly virulent strain of *Pseudomonas solanacearum*.

Key words: peptide design; genetic engineering; disease resistant plants

Introduction

It has been said that plant disease is the exception rather than the rule [1]. In effect, only the interplay between a virulent pathogen and a susceptible cultivar leads to disease. Despite this delicate interaction between host and pathogen, plant disease is one of the leading causes of crop loss in the world. Its economic import can be especially significant in the developing world where up to forty percent of crop destruction can be directly attributed to plant disease [2]. Also, their cultivation of just a few plant species within a localized area accelerates the spread of disease, exacerbating the problem.

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Plants under microbial attack elaborate several inducible defensive responses of a structural and biochemical nature. Among them are the synthesis of hydrolytic enzymes, such as chitinase and β-glucanase, and proteinase inhibitors; the modification of the plant cell wall by lignification and accumulation of callose, a β-1,3-glucan, and hydroxyproline-rich glycoproteins (HRGPs); and the synthesis of phytoalexins, low molecular weight compounds with antimicrobial activity [3].

Recent advances in genetic engineering have made it possible to develop plants with new predictable phenotypes. The expression of the TMV coat protein in transgenic plants was shown to cause a delay in the development of symptoms upon infection by the virus in concordance with the idea of cross-protection [4]. Also, expression of an insecticidal protein, the BT protein from *Bacillus thur-*

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ingiensis, has rendered plants resistant to insect attack [5]. Therefore, appropriate small modifications in the biochemistry of a plant may significantly augment the resistance of this plant to the action of a pest or pathogen. Jaynes et al. [6,7] and more recently, Casteels et al. [8] have proposed the idea of using the genes for proteins with antimicrobial activity, found in insects, to enhance bacterial disease resistance in plants. The results presented in this paper indicate that this notion can have practical application and suggest a different approach to development of new plant cultivars with increased microbial disease resistance.

Lytic peptides are small proteins that appear to be major components of the antimicrobial defense systems of a number of animal species including insects, molluscs, amphibians, and at least one mammal [9]. They consist of 23–39 amino acid sequences, which have potential for forming amphipathic α-helices. An amphipathic α-helix may be depicted as a cylinder with one curved face composed primarily of nonpolar amino acid side chains and the other face composed of amino acids containing polar side chains (the property of amphipathy can best be visualized by casting the amino acid sequence on a helical wheel projection [10]). The presence of lytic peptides in other higher organisms is under intensive investigation and it has been proposed that they will be found in all mammals, including humans [9]. Defensins, a family of small molecular weight antimicrobial peptides found in mammals and more recently in insects, are a separate kind of lytic peptide, because their action is intracellularly localized following phagocytosis and their in vivo conformation seems to be a β-pleated sheet [11].

The lytic peptides that have been described in the literature seem to fall into one of three different classes based on the arrangement of amphipathy and high positive charge density within the molecule: cecropins (35 amino acids in length and derived from the Giant Silk Moth), N-terminal half amphipathic while the C-terminal half mostly hydrophobic [12]; magainins (23 amino acids in length and derived from the African Clawed Frog), amphipathic the full-length of the molecule [13]; and melittin (26 amino acids in length and derived from the Honeybee), C-terminal half amphipathic with the N-terminal half primarily hydrophobic [14]. The conservation of these physical properties is requisite for activity, but the requirements seem to be somewhat nonspecific in terms of amino acid sequence (see Fig. 1A). For example, we have synthesized highly sequence divergent analogs for each of the peptide classes and have found some of them to be more active than their natural counterparts [15,16].

Materials and Methods

Peptide synthesis

Lytic peptides were synthesized using a Milligen/Biosearch Model 9050 automated peptide synthesizer which employs the Fmoc chemistry [17]. All reagents were purchased from Milligen/Biosearch. Upon completion of the synthesis, the peptide was cleaved from the resin and exhaustively extracted with ethyl ether, dissolved, frozen in a solid CO_2/acetone bath, and lyophilized. Sephadex column chromatography was then used to remove all remaining small organic molecules and to fractionate the peptide mixture according to size. The fractions were shell-frozen, lyophilized, and subjected to HPLC for initial analysis and preparative work. The samples were prepared in aqueous buffer containing 50% methanol and analyzed in a Varian 5000 HPLC System and a Waters μBondapak C18 column, 8 mm × 10 cm radial pak cartridge which employed the radial compression module. The appropriate fractions were then subjected to mass spectroscopy analysis to confirm the identity of the full-length peptides. The samples were analyzed using a 252Cf Plasma Desorption Mass Spectrometer (PDMS) BIO ION 20 (Bio Ion Nordic AB, Uppsala, Sweden). The operating conditions for analyses were as follows: the acceleration voltage was 15 kV with 8 K channels being monitored for a duration of 1 million counts (about 10 min). The samples were applied to a nitrocellulose-coated target in a 50:50 water/ethanol solution, allowed to absorb for 10 min and then loaded into the instrument (Note: only data for Shiva-1 are shown in Figs. 1B and 1C).

were kept at room temperature with constant agitation at 150 rev./min. The cell suspensions were maintained by subculturing 10 ml of filtered and washed culture in 50 ml of fresh medium fortnightly.

The expression of the Shiva-1 gene was tested at the RNA level by northern analysis [19]. Expression of these peptides in tobacco plants was analyzed using a modified version of the method described by Sanchez-Serrano [20]. Tobacco plants, about 60–80 cm tall, were wounded on one leaf by two consecutively applied dialysis clamps. RNA was isolated from the leaves of the wounded plants 24 h after the clamps were attached. Poly(A)-RNA was isolated using a FastTrack™ oligo-dT cellulose kit (from Invitrogen) and ~10 μg per sample loaded onto the gel. A 125-bp Shiva-1 DNA insert was used as probe. Total protein was extracted from tobacco leaf tissue with 80 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol and separated on 13% denaturing polyacrylamide gels, ~20 μg of total protein per sample [21]. The proteins were subsequently transferred to an Immobilon-P membrane (from Millipore). The membrane was incubated with antiserum raised against Shiva-1 peptide according to Blake et al. [22]. Detection of immunoreactive protein was carried out using alkaline phosphatase coupled to antirabbit IgG with 5-bromo-4-chloro-3-indolyl-phosphate as a substrate [22].

Single blind bacterial challenge

Pseudomonas solanacearum strain UW77, which is highly virulent on tobacco, was obtained from L. Sequeira (University of Wisconsin) and used in all inoculation experiments. After overnight growth in a rich broth medium, bacteria were harvested by centrifugation (5500 × *g*, 20 min, 4°C), washed once with water and suspended in water at various cell densities for use as inoculum. Kanamycin resistant tobacco seedlings were grown from R1 generation seeds of the control and selected transgenic plant lines and transplanted directly into 8-oz. styrofoam cups or 4-inch plastic pots containing an artificial potting soil (Pro-Mix), and grown in a greenhouse (control plants were transgenic for the vector without the Shiva-1 insert). Plants for the wounded root bacterial challenge assay were grown in cups until reaching a height of ~10 cm and then the margin of 4–5 leaves was marked by cutting a small notch. All roots on one side of the plant (half way between the stem and the wall of the cup) were cut, the cups were placed in saucers, and 5 ml of bacteria suspended in water at 10^7 cells per ml were poured slowly onto the soil. Controls received water alone. The plants were then randomly arranged and coded to create a single-blind experiment. The plants were incubated in a 30°C growth chamber and the soil kept constantly moist by watering into the saucers. The percentage of notched leaves that were completely wilted was recorded for each plant on a daily basis, and the mean percentage of leaves wilted for each treatment calculated. When all of the notched leaves were wilted the plant was considered completely wilted; new leaves that appeared during the assay were not counted even though they too were usually severely wilted. The results were analyzed using a one-tailed Wilcoxon two-sample test by comparing the set of mean values that spanned a range of days for the control to analogous sets for each transgenic line. Plants for the stem inoculation assay were grown in the pots until they were about 15 cm in height, and the margins of the 8–10 leaves to be monitored were notched as above. Each of the plants received 20 μl of inoculum containing 10^6cells/ml or 20 μl of water applied into a stab wound in the stem. The notched leaves on each plant were rated daily as being either 1/4, 1/2, 3/4, or completely wilted; the percentage of leaves and plants wilted was calculated as for the root inoculation method.

Results and Discussion

Our original intent was to utilize the gene encoding a close homolog of Cecropin B (SB-37) to augment bacterial disease resistance in plants. However, during our studies, a new highly sequence divergent peptide was synthesized (Shiva-1) in our laboratory (see Fig. 1) and was shown to possess a more potent lytic activity than SB-37 [23–27]. The enhanced bioactivity of Shiva-1 was the first indication that modifications made in the primary sequence of lytic peptides would not

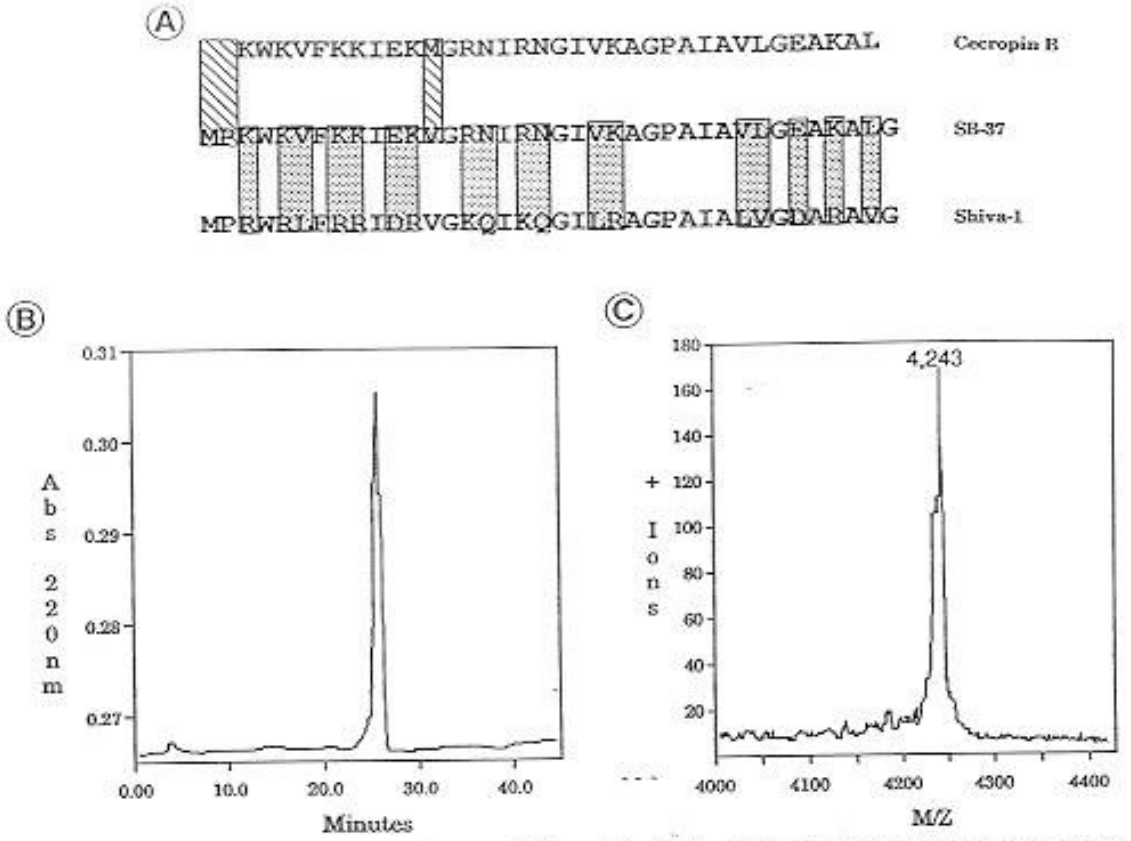

Fig. 1. Sequence comparison of Cecropin B with the novel lytic peptides SB-37 and Shiva-1, and physical characterization of synthesized Shiva-1. (A) The Cecropin B analog, SB-37, has minor changes from the parent molecule by substitution of Met11 with Val and addition of an NH_2-terminal, MetPro. The hatched rectangles denote the differences between cecropin B and SB-37 while the dotted rectangles show the differences between Shiva-1 and SB-37. (B) The peptides were subjected to HPLC for initial analysis and for preparative work. (C) The appropriate fractions from (B) were subjected to mass spectroscopy and full-length identity confirmed by this technique.

destroy the peptide's activity provided certain physical characteristics of the peptide were conserved.

We chose initially to examine the activity of the two peptides against a series of phytopathogenic bacteria. Significant cytotoxic activity in the micromolar range was observed for both peptides with Shiva-1 usually showing the lowest LC_{50} values (LC_{50} is the concentration necessary to kill 50% of the test organisms) on most test strains (Table I). Additionally, another research group testing the effectiveness of the peptides in killing bacteria, although assaying in a different way, have recently reported similar results to our own [28]. These findings prompted us to clone the genes for several of the peptides into transgenic tobacco plants in an attempt to increase disease resistance.

It was found that a significant decrease in LC_{50} was observed when the peptides were incubated in the presence of lysozyme. Loss of integrity of the peptidoglycan bacterial cell wall, combined

Table I. Sensitivity of plant pathogenic bacteria to lytic peptides. In vitro bactericidal activity of peptides against well known plant pathogenic bacteria are shown. Numbers shown are concentrations (μM) of peptide necessary to kill 50% of the cells. Thus, in each assay a 1-μM solution of SB-37 and Shiva-1 would contain 4.08 μg and 4.24 μg, respectively. The asterisk denotes the addition of lysozyme at concentrations which were not lethal to the bacteria (100 μg/ml). At least three separate assays were conducted for each treatment and variation was no greater than 15%.

Microorganisms	SB-37	SB-37*	Shiva-1	Shiva-1*
C. michiganense spp. *michiganense*	3	0.2	1.0	0.05
E. carotovora spp. *carotovora*	2	0.4	0.5	0.2
P. solanacearum	64	16	40	7
P. syringae pv. *tabaci*	5	0.2	2	0.1
X. campestris pv. *campestris*	0.6	0.03	0.4	0.02

with the lytic activity of the peptides, creates a synergistic interaction similar to the concerted action which has been reported for the humoral immune system of the cecropia moth [29]. It should be noted that in addition to the induction of lysozyme, the cecropia moth also synthesizes a class of bactericidal proteins called the attacins, although less active than the cecropins, it is thought that they also work in synergy with the other humoral immune response components and thus, enhance lytic peptide potency [29]. These data indicate that a synergistic effect may be seen in a plant transgenic for both lytic peptide and lysozyme and warrants further investigation.

We used a constitutive promoter, 35S cauliflower mosaic virus 5′ region-nopaline synthetase-3-polyadenylation cassette [30], for the less active peptide, SB-37, and an inducible promoter, proteinase inhibitor II (PiII) [20], for the more active Shiva-1. In non-wounded potato plants, PiII accumulates in the tubers with non-detectable levels of protein in leaves, stem or roots. When the leaves are wounded, expression of the gene is induced not only in the wounded leaves, but also in non-wounded upper and lower leaves and in the upper part of the stem [31]. Detailed histological analysis, using the β-glucuronidase gene under the control of the PiII promoter, has revealed that induced expression is strongest in cells closest to the vascular tissue, suggesting that the signal mediating the wounding response is transported via this tissue [32]. When the PiII gene is transferred to tobacco, it is regulated in the same way as it is in potato, indicating that tobacco plants contain similar trans-activating factors that recognize the cis-elements present in the promoter [32].

Fertile transgenic tobacco plants were obtained and tested for the presence of the intact 35S-SB37 and PiII-Shiva-1 genes by standard Southern analysis (data not shown). Those transgenic plants exhibiting a non-rearranged single-copy gene pattern were selected for further study. Preliminary bacterial challenge results, utilizing a highly pathogenic strain of *Pseudomonas solanacearum* (this organism is a vascular pathogen that causes severe wilting), indicated that Shiva-1 progeny exhibited a delayed appearance of symptoms which were less severe than those shown by the control plants. Furthermore, there was a dramatic difference in the mortality of Shiva-1 plants when compared to control plants three weeks after infection (see Fig. 2). No enhanced resistance was observed between the controls and plants producing SB-37, presumably because of its low bioactivity against this pathogen [18]. Therefore, we chose to focus on the Shiva-1 transgenic lines. However, it should be noted that other workers, utilizing these constructs, have obtained transgenic plants, including some transgenic lines expressing SB-37, resistant to several different bacterial diseases [33; W. Belknap, pers. commun.].

Northern analysis demonstrated expression of mRNAs of the expected size (Fig. 3A). Plants containing the Shiva-1 gene showed mRNA expression regulated at the transcriptional level as expected in wounded vs. non-wounded plants. Different levels of expression were found and the two best producers were selected for further study. The

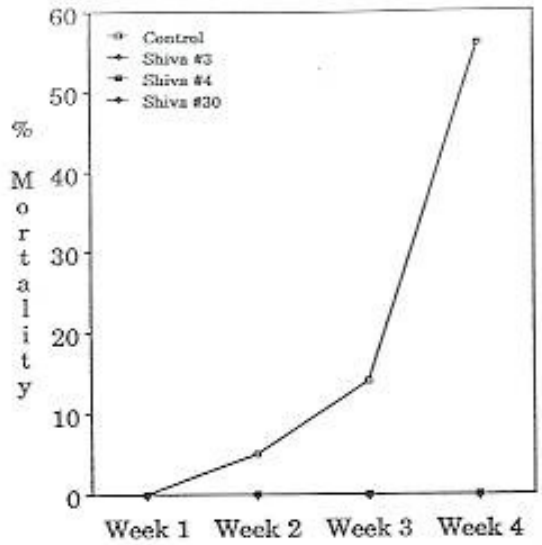

Fig. 2. Approximately fifty each of transgenic control and Shiva-1 peptide producing tobacco seedlings were infected with about 10^7 *P. solanacearum* by wounding of the stem. No Shiva-1 transgenic plants died.

expected mRNA length of about 340 bases, including the poly(A) stretch, was observed. Lanes 1 and 2 contain RNA isolated from Shiva-1 plants no. 3 and no. 4 while Lane 3 contains RNA prepared from the transgenic control.

Western analysis of total protein extracts (Fig. 3B) isolated from wounded plants revealed the presence of a peptide with a molecular weight similar to that of Shiva-1 when the blots were probed with antibody raised against chemically synthesized Shiva-1 peptide. The expression (level is no more than 0.1% of total protein) of this peptide did not have an obvious detrimental effect on the plants, since they proved to have an indistinguishable phenotype from non-transformed controls. No Shiva-1 band was detected in the transgenic control (Lane 4) while Shiva-1 plants no. 3 and 4 (Lanes 1 and 2, respectively) showed an immunoreactive protein in the 4000 molecular weight size which co-migrated with ~100 ng purified synthetic Shiva-1 peptide (Lane 3). The band that appears below Shiva-1 in all lanes, including the control, is an artifact resulting from the marker dye used in electrophoresis [21]. It should be noted that PCR-DNA sequence analysis was conducted on DNAs isolated from some of the no. 3 and 4 seedlings (data not shown). We found that the R1 plants were identical to their R0 counterparts in all aspects except that the Shiva-1 gene, in the R1 plants, was truncated. The sequences were all missing the codons for amino acids no. 37 and 38. However, the termination codon and poly-adenylation signal were intact. We know that from our design work that this modification would not affect lytic activity. At this time, we have no explanation for the apparent instability in the C-terminal sequence encoding Shiva-1 gene.

In a more rigorous set of single-blind bacterial challenge experiments, two inoculation methods were used to evaluate our best transformed tobacco plants, Shiva-1 lines nos. 3 and 4, for their susceptibility to *P. solanacearum*. In a wounded root assay, assessment of the percentage of leaves wilted revealed that both lines showed symptoms before the control (Fig. 4A). However, when examined over time, line no. 3 was significantly slower to wilt than was the control, whereas line no. 4 was only marginally different. A similar trend was observed in the percentage of plants wilted after inoculation (Fig. 4B). The same experiments performed with 100 times more inoculum gave similar results; line no. 3 wilted more slowly while line no. 4 wilted and died almost as fast as the control (data not shown).

When tested with a stem-inoculation assay, lines no. 3 and 4 were markedly less susceptible to the bacterial pathogen. Both lines showed significantly fewer wilted leaves in individual plants (Fig. 4C), and overall fewer plants wilted by the end of the experiment (Fig. 4D). Furthermore, line no. 4 plants showed no completely wilted plants compared to almost 85% wilted plants in the control. However, in contrast to the root assay, line no. 4 was far less susceptible than line no. 3 when stem inoculated. Here, too, experiments with 100 times more inoculum gave very similar results (data not shown). Prior wounding of the plants had no effect on the overall outcome of these experiments (unpublished observations).

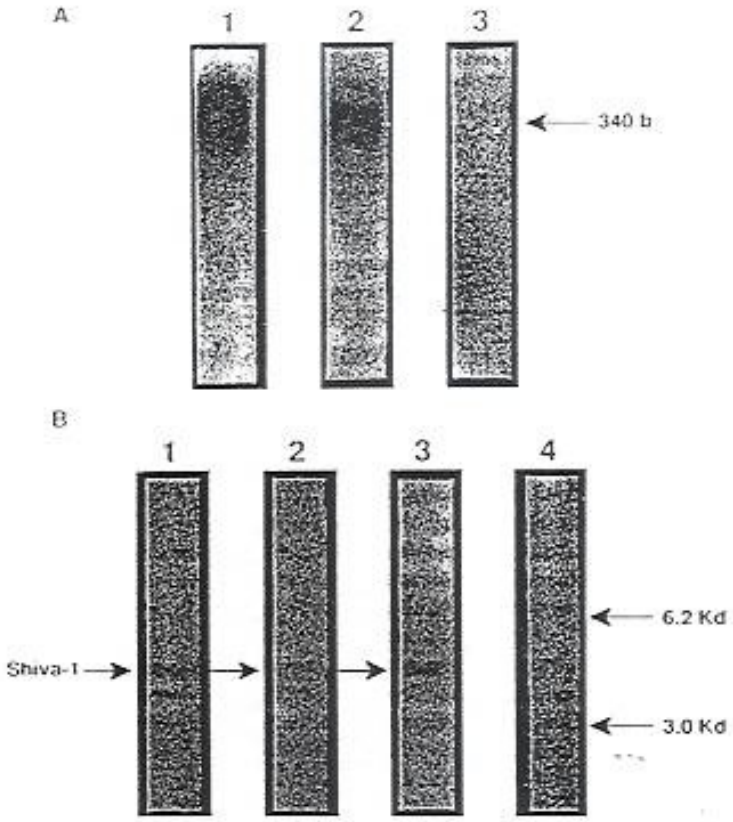

Fig. 3. Analysis of transformed Shiva-1 and control plants. (A) The expression of the Shiva-1 gene was tested at the RNA level by northern analysis. Lanes 1 and 2 were samples derived from Shiva-1 plants numbers 3 and 4, respectively. Lane 3 extract was derived from transgenic control plants. (B) Western analysis was conducted on total protein extracted from tobacco leaf tissue in order to confirm fidelity of translation. Lanes 1 and 2 were samples derived from Shiva-1 plants numbers 3 and 4, respectively. Lane 3 contained a few micrograms of Shiva-1 peptide which had been chemically synthesized as described in Materials and Methods. Lane 4 extract was derived from transgenic control plants.

The enhanced resistance of Shiva-1 plants when tested via stem inoculation is consistent with previous studies of the wound-inducible expression of PiII in potato plants. Kiel et al. [32] reported that after wounding a single leaf there is a systemic activation of the PiII gene in the upper part of the stem, but not in the lower part of the stem and roots. Assuming the expression of Shiva-1 is regulated in the same way at the cellular level, there will be a higher expression of the peptide in the cells surrounding the vascular tissue of the stem, which could explain the observed better overall response of the plants to the infection when plants were stem inoculated rather than root inoculated. Observations have shown in many systems tested [34 and unpublished data] a cell-proliferative effect of the peptides at very low concentrations. We can expect this effect at a high ratio of cells to peptide. It is tempting to speculate that in the root assay, at high inoculum, because of a very low level of expression in the roots in comparison to leaves and stems, a proliferative

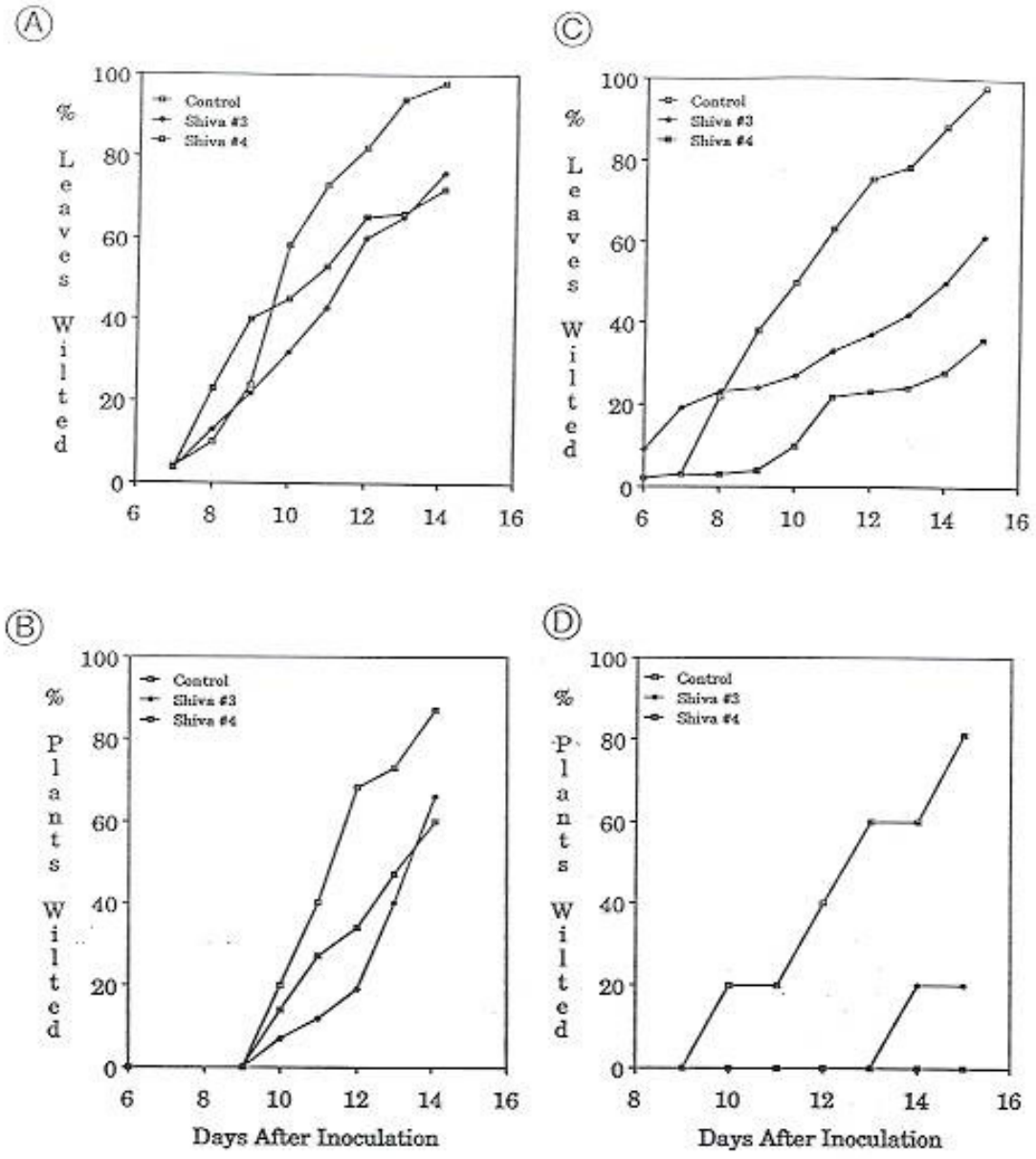

Fig. 4. Disease susceptibility. (A) From days 9 to 14 both Shiva-1 no. 3 and no. 4 were significantly slower to wilt at $\alpha = 0.1$. (B) From days 10 to 14 Shiva-1 no. 3 was slower to wilt at $\alpha = 0.1$ but Shiva-1 no. 4 was not significantly slower than the control. (C) From days 10 to 15 Shiva-1 no. 3 was slower to wilt at $\alpha = 0.001$ while Shiva-1 no. 4 was slower to wilt at $\alpha < 0.001$. (D) From days 8 to 15 Shiva-1 no. 3 was slower to wilt at $0.05 > \alpha > 0.025$ and Shiva-1 no. 4 was slower to wilt at $\alpha < 0.005$.

effect of the peptide on the bacteria takes place during the first days following infection. As the bacterial invasion proceeds upward, a systemic activation of the gene occurs with the strongest expression taking place in the vicinity of the vascular tissue which allows the plants to 'catch up'. By the end of the experiment, Shiva-1 plants clearly show less susceptibility than the control plants. Similarly, plants expressing the less active peptide, SB-37, may have provoked a proliferative rather than a lytic response on the pathogen, possibly accounting for the apparent inability of this peptide to control the infection.

Our in vitro data consistently showed *P. solanacearum* to be the bacterial plant pathogen with one of the highest LC_{50} values for all our peptides. Most other pathogens are more sensitive, with LC_{50} values in some cases in the submicromolar range (Table I). These results are encouraging, since introduction of the genes for SB-37, Shiva-1, and other lytic peptides into plants, where the more sensitive types of bacteria cause significant crop loss, may result in an enhanced resistance similar to that found for the tobacco plants in this study. Indeed, several groups have reported an enhanced level of resistance in plants expressing our cecropia attacin A gene construct in transgenic apple and *Anthurium* (J. Norelli and H. Kuehnle, pers. commun.).

Recently, there has been a report of increased resistance to infection by *P. syringae* pv. *tabaci* in transgenic tobacco plants [35]. In this study, the authors introduced a gene for a toxin-resistant acetyltransferase that detoxifies the phytotoxin produced by the bacterium resulting in enhanced plant resistance. Our approach is less specific and potentially more effective because the introduction of only one gene, Shiva-1 for example, may render plants less susceptible to bacterial diseases in general. Also, it may be more difficult for the pathogen to circumvent the lytic activity of the peptides, since a drastic modification of the bacterial membrane would seem to be necessary to permit pathogen resistance. Furthermore, we speculate that combination of several genes within the same line, perhaps, chicken lysozyme and attacin A with Shiva-1, may allow for potent synergy to develop and could permit an even higher level of plant resistance.

In summary, we have shown increased resistance in transgenic tobacco plants producing Shiva-1 peptide to the pathogenic action of *P. solanacearum* and resulted in a delay of symptoms and a significant reduction of plant mortality. Currently, these and other genes encoding newly designed, more active peptides, are being introduced into different plant species to test the generality of our proposed method. It is exciting to contemplate the future since some of these novel peptides also possess high in vitro cytotoxic activity against fungi and nematodes [36].

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References

1 C.J. Lamb, J.N. Bell, D.R. Corbin, M.A. Lawton, M.C. Mehdy, T.B. Ryder, N. Sauer and M.H. Walter, Activation of defense genes in response to elicitor and infection, in: C.J. Arntzen and C. Ryan (Eds.), UCLA Symposia on Molecular and Cellular Biology: Molecular Strategies for Crop Protection. Alan R. Liss, Inc., New York, 1987, no. 48, pp. 49–58.

2 R.I. Sawyer, Potatoes for the developing world, Lima, Peru. International Potato Center, 1984.

3 D.B. Collinge and A.J. Slusarenko, Plant gene expression in response to pathogens. Plant Mol. Biol., 9, (1987) 389–410.

4 P. Powell-Abel, R.S. Nelson, B. De, N. Hoffmann, S.G. Rogers, R.T. Fraley and R.N. Beachy, Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein. Science, 232 (1986) 738–743.

5 M. Vaeck, A. Reynaerts, H. Hofte, S. Jansens, M. De Beuckeleer and M. Van Montagu, Transgenic plants protected from insect attack. Nature, 328 (1987) 33–37.

6 J.M. Jaynes, K.G. Xanthopoulos, L. Destefano and J.H. Dodds, Increasing bacterial disease resistance in plants utilizing antibacterial genes from insects. BioEssays, 6 (1987) 263–270.

7 L. Destefano-Beltran, P. Nagpala, S. Cetiner, J.H. Dodds and J.M. Jaynes, Enhancing bacterial and fungal disease resistance in plants, in: M. Vayda and W. Park (Eds.), The Molecular and Cellular Biology of the Potato, C.A.B. International, Wallingford, UK, 1990, pp. 205–221.

8 P. Casteels, C. Ampe, F. Jacobs, M. Vaeck and P. Tempst, Apidaecins: antibacterial peptides from honeybees. EMBO J., 8 (1989) 2387–2391.

9 J.-Y. Lee, A. Boman, C. Sun, M. Andesson, H. Jornvall, V. Mutt and H.G. Boman, Antibacterial peptides from pig intestine: isolation of a mammalian cecropin. Proc. Natl. Acad. Sci. USA, 86 (1989) 9159–9162.

10 M.G. Rossmann and P. Argos, Protein folding. Annu. Rev. Biochem., 50 (1981) 497–532.

11 J. Lambert, E. Keppi, J.-L. Dimarcq, C. Wicker, J.-M. Reichart, B. Dunbar, P. Lepage, A.V. Dorsselaer, J. Hoffman, J. Fothergill and D. Hoffmann, Insect immunity: isolation from immune blood of the dipteran Phorma terranovae of two insect antibacterial peptides with sequence homology to rabbit lung macrophage bactericidal peptides. Proc. Natl. Acad. Sci. USA, 86 (1989) 262–266.

12 P. van Hofsten, I. Faye, K. Kockum, J.-Y. Lee, K.G. Xanthopoulos, I.A. Boman, H.G. Boman, A. Angstrom, D. Andreu and R.B. Merrifield, Molecular cloning, cDNA sequencing, and chemical synthesis of cecropin B from *Hyalophora cecropia*. Proc. Natl. Acad. Sci. USA, 82 (1985) 2240–2244.

13 M. Zasloff, Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms and partial cDNA sequence of a precursor. Proc. Natl. Acad. Sci. USA, 84 (1987)5449–5453.

4 E. Haberman, Bee and wasp venoms. Science, 177 (1972) 314–322.

5 L. Destefano-Beltran, P.G. Nagpala, J.H. Dodds and J.M. Jaynes, Use of genes encoding novel peptides and proteins to enhance disease resistance in plants. Appl. Biotechnol. Plant Pathol. (1992) in press.

6 M.J. Arrowood, J.M. Jaynes and M.C. Healy, In vitro activities of lytic peptides against the sporozoites of *Cryptosporidium parvum*. Antimicrob. Agents Chemother., 35 (1991) 224–227.

7 G.B. Fields and R.L. Noble, Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. Int. J. Pept. Protein Res., 35 (1990) 161–214.

8 L. Destefano-Beltran, P. Nagpala, J.H. Kim, J.H. Dodds and J.M. Jaynes, Genetic transformation of potato to enhance nutritional value and confer disease resistance, in: E.S. Dennis and D.T. Llewellyn (Eds.), Plant Gene Research: Molecular Approaches to Crop Improvement, Springer-Verlag, New York, 1991, pp. 17–32.

9 L. Willmitzer, G. Simons and J. Schell, The TL-DNA of octopine crown gall tumors form seven well-defined polyadenylated transcripts. EMBO J., 1 (1982) 139–146.

0 J. Sanchez-Serrano, M. Keil, A. O'Connor, J. Schell and L. Willmitzer, Wound-induced expression of a potato proteinase inhibitor II gene in transgenic tobacco plants. EMBO J., 6 (1987) 303–306.

1 H. Schagger and G. von Jagow, Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem., 166 (1987) 368–379.

2 M.S. Blake, K.H. Johnston, G.J. Russell-Jones and E.C. Gotschlich, A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on western blots. Anal. Biochem., 136 (1984) 175–179.

3 J.M Jaynes, C.A. Burton, S.B. Barr, G.W. Jeffers, G.R. Julian, K. White, F. M. Enright, T.R. Klei and R.A. Laine, In vitro effect of Novel Lytic Peptides on *Plasmodium falciparum* and *Trypanosoma cruzi*. FASEB, 2 (1988) 2878–2883.

24 J.M. Jaynes, G.R. Julian, G.W. Jeffers, K.L. White and F.M. Enright, In vitro effect of lytic peptides and normal and transformed mammalian cell lines. Peptide Res., 2 (1989) 157–160.

25 D. Kelly, W. Wolters and J.M. Jaynes, The effect of lytic peptides on fish pathogenic bacteria. J. Fish Dis., 13 (1990) 317–321.

26 J.M. Jaynes, Lytic peptides portend an innovative age in the management and treatment of human disease. Drug News Perspect., 3 (1990) 69–78.

27 M.J. Arrowood, J.M. Jaynes and M.C. Healy, Hemolytic properties of lytic peptides active against the sporozoites of *Cryptosporidium parvum*. J. Protozool., 38 (1991) 1615–1635.

28 R.G. Nordeen, S.L. Sinden, J.M. Jaynes and L.D. Owens, Activity of cecropin SB37 against protoplasts from several plant species and their bacterial pathogens. Plant Sci., 82 (1992) 101–107.

29 D. Hultmark, H. Steiner, T. Rasmusson and H.G. Boman, Insect immunity: purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of *Hyalophora cecropia*. Eur. J. Biochem., 106 (1980) 7–16.

30 S.G. Rogers, H.J. Klee, R.B. Horsch and R.T. Fraley, Improved vectors for plant transformation: expression cassette vectors and new selectable markers. Methods Enzymol., 123 (1987) 253–305.

31 H. Pena-Cortes, J. Sanchez-Serrano, M. Rocha-Sosa and L. Willmitzer, Systemic induction of proteinase inhibitor-II gene expression in potato plants by wounding. Planta, 174 (1988) 84–91.

32 M. Keil, J. Sanchez-Serrano and L. Willmitzer, Both wound-inducible and tuber-specific expression are mediated by the promoter of a single member of the potato proteinase inhibitor II gene family. EMBO J., 8 (1989) 1323–1330.

33 S.R. Jia, Y. Xie, T. Tang, L.-X. Feng, D-S. Cao, Y-L. Zhao, J. Yuan, Y-Y. Bai, C-X. Jiang, J.M. Jaynes and J.H. Dodds, Genetic engineering of chinese potato cultivars by introducing antibacterial polypeptide genes. Proc. Asia-Pacific Conf. Agr. Biotechnol. (1992) in press.

34 W.A. Reed, K.L. White, F.M. Enright, J. Holck, J.M. Jaynes and G.W. Jeffers, Enhanced in vitro growth of murine fibroblast cells and preimplantation embryos cultured in medium supplemented with an amphipathic peptide. Mol. Reprod. Dev., 31 (1992) 106–113.

35 H. Anzai, K. Yoneyama and I. Yamaguchi, Transgenic tobacco resistance to a bacterial disease by the detoxification of a pathogenic toxin. Mol. Gen. Genet., 219 (1989) 492–494.

36 J.M. Jaynes, Lytic peptides and their potential for enhancing disease resistance in plants. Acta Horticult. (1992) in press.