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# Transgenic potatoes expressing a novel cationic peptide are resistant to late blight and pink rot

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**Abstract**

Potato is the world's largest non-cereal crop. Potato late blight is a pandemic, foliar wasting potato disease caused by *Phytophthora infestans*, which has become highly virulent, fungicide resistant, and widely disseminated. Similarly, fungicide resistant isolates of *Phytophthora erythroseptica*, which causes pink rot, have also become an economic scourge of potato tubers. Thus, an alternate, cost effective strategy for disease control has become an international imperative. Here we describe a strategy for engineering potato plants exhibiting strong protection against these exceptionally virulent pathogens without deleterious effects on plant yield or vigor. The small, naturally occurring antimicrobial cationic peptide, temporin A, was N-terminally modified (MsrA3) and expressed in potato plants. MsrA3 conveyed strong resistance to late blight and pink rot phytopathogens in addition to the bacterial pathogen *Erwinia carotovora*. Transgenic tubers remained disease-free during storage for more than 2 years. These results provide a timely, sustainable, effective, and environmentally friendly means of control of potato diseases while simultaneously preventing storage losses.

## Introduction

Worldwide crop losses due to microbial diseases are currently ∼$720B (US) annually, with an additional ∼$20B (US) spent on countervailing pesticides. These losses are particularly untenable considering the slated increase in human populations accompanied by the decline in available arable cropland and irrigation (Borlaug, 2000).

Bacterial and fungal diseases are currently one of the major factors impacting crop production. For example, nearly 20% of potatoes (*Solanum tuberosum* L.), the most important non-cereal crop in the world, are now lost due to diseases (James et al., 1990). The quality of potato tubers in storage or transit can be significantly influenced by bacterial soft rot. Although it primarily occurs in harvested potatoes, it has been reported on young tuber initials, tubers left in the soil, and even on plant stems (Powelson & Apple, 1984). The principal agent causing bacterial soft rot is the Gram-negative bacterium *Erwinia carotovora* ssp. *carotovora*. However, the most serious potato diseases are caused by fungi. In particular, potato late blight caused by the Oomycete *Phytophthora infestans*, has historically been responsible for devastating episodic losses such as the mid-nineteenth century Irish famine. Today, late blight is considered to be the most serious potato disease worldwide having reached pandemic proportions and costing upwards of $4B (US) annually. Containment has relied on increasing applications of highly toxic fungicides, which has inevitably resulted in increasing resistance against fungicides (Shattock, 2002).

DNA fingerprinting of preserved potato leaves infected with *P. infestans* indicates that the pathogen most likely originated in the Peruvian Andes and coevolved as the host species was propagated worldwide (Ristaino et al., 2001). In addition to resistance to fungicides a new mating type has emerged in Mexico and is now found in almost all countries where potatoes are grown. This raises the frightening possibility of

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additional variability through sexual recombination and genetic segregation, making this pathogen even more difficult to control.

Another *Phytophthora* species, *P. erythroseptica*, the causal agent of a tuber disease known as pink rot, is responsible for serious post-harvest losses. Unfortunately, resistance has now developed to the only effective fungicide available for disease control, mefanoxam (Dr H. Platt, AAFC, NB, Canada, personal communication). Consequently, new approaches to containment of pathogenic *Phytophthora* sp. are urgently required. Transgenic approaches provide effective means for disease resistance in crop plants (Dempsey et al., 1998; Mourgues et al., 1998; Salmeron & Vernooij, 1998; Punja, 2001). Most current approaches are based on the utilization of single dominant resistance (R) genes, mechanisms included in systemic acquired resistance (SAR) and transgenic expression of antifungal proteins. However, results often depend on the nature of the recipient plant, the specific pathogen, and the source of the gene (Punja & Raharjo, 1996) with narrowly defined benefits.

We sought to produce transgenic plants strongly resistant to a broad spectrum of phytopathogens through the expression of cationic antimicrobial peptides (CAPs) (*see* Hancock et al., 1995 for review). CAPs are important components of innate immunity and have been isolated from a wide variety of organisms (Zasloff, 2002). These widespread natural products vary greatly in their properties and spectrum of biological activities (Hancock & Lehrer, 1998). Most previous attempts to express CAPs in plants led to a very narrow spectrum or weak antibacterial activity and disease resistance. So far, very few CAP-expressing transgenic plants have shown any degree of resistance to fungal phytopathogens (Terras et al., 1995; Epple et al., 1997; Gao et al., 2000; Mitsuhara et al., 2000), and only expression of the gene encoding the synthetic CAP chimera, MsrA1, provided tobacco and potato plants with any degree of broad-spectrum antimicrobial resistance (Osusky et al., 2000). These studies indicated that the use of modestly active antimicrobial peptides in transgenic plants may be a better tactic than attempting to utilize the most active peptides. Here we describe use of a novel, small, naturally occurring CAP modified to be active against a broad range of pathogens including *P. infestans* and *P. erythroseptica*.

Temporins are a group of unusually small CAPs isolated from the skin secretions of the European red frog *Rana temporaria* (Simmaco et al., 1996). These 10–13 amino acid peptides along with indolicidin (Selsted et al., 1992) and bactenecin (Romeo et al., 1988) from bovine neutrophils, are among the smallest antimicrobial peptides found in nature. The most potent is temporin A, a non-cysteine-containing, linear α-helical peptide that possesses both antibacterial and antifungal activities (Harjunpaa et al., 1999). The lack of selectivity of temporins is thought to be related to a combination of positive charge distribution and hydrophobicity (Mangoni et al., 2000; Wade et al., 2000). Unlike structurally related CAPs from wasp venom (Argiolas & Pissano, 1984), the temporins are not hemolytic and hence unlikely to be cytotoxic.

Molecular modeling and engineering of such relatively small peptides permits the design of superior candidate CAPs with respect to biological activity and plant cytotoxicity. In this study, we attempted to increase the affinity of temporin A for negatively charged lipids by modifying the N terminus. To evaluate the antimicrobial activity *in vivo*, we expressed the newly created gene, *msrA3*, encoding a modified temporin A, MsrA3, in the potato cultivar Desiree. The results show that constitutive expression of *msrA3* in potato plants consistently and dramatically increased the resistance of growing plants and harvested tubers to *P. infestans*, *P. erythroseptica*, as well as the bacterial pathogen *E. carotovora*.

## Materials and methods

### *Construction of the* msrA3 *gene, and DNA manipulation*

Four oligonucleotides were prepared for construction of the *msrA3* gene: Oligo No. 1: 5′-ATGTTTCTGCC CCTAATCGGGAGGGTTCTCTCGGGAATCCTGT AA-3′; Oligo No. 2: 5′-TTACAGGATTCCCGAGAG AACCCTCCCGATTAGGGGCAAAACAT-3′; Oligo No. 3: 5′-GGTACCTCTAGACATATGTTTCTGCC CCTA-3′ (*Kpn*I, *Xba*I, and *Nde*I sites at the 5′-end of the gene); and Oligo No. 4: 5′-CTGCAGAGCTCTT ACAGGATTCCCGAGAG-3′ (*Pst*I and *Sst*I sites at the 3′-end of the gene). The *msrA3* gene was prepared using PCR with oligonucleotides 1 and 2 at a final concentration of 20 nM, and oligonucleotides 3 and 4 at a final concentration of 400 nM.

DNA manipulations were carried out as described (Sambrook et al., 1989). The enzymes were purchased from Amersham Pharmacia Biotech (England) or New England Biolabs (Beverly, MA, USA), and used according to manufacturer's instructions.

*Plant transformation and regeneration*

Transformation of *Agrobacterium tumefaciens* MP90 was done by the freeze–thaw method (Holsters et al., 1978). Transformation of potato plants was carried out as described by De Block (1988).

*Isolation of nucleic acids from plant tissue*

Genomic DNA was isolated from 100 mg of fresh leaves (Wang et al., 1993). Total RNA was isolated from 100 mg of leaf tissue using Trizol Reagent (GIBCO BRL, Rockville, MD, USA) according to manufacturer's instructions.

msrA3 *integration and transcription in transformed plants*

The *msrA3* coding sequence was PCR-amplified from total genomic DNA, and MsrA3 mRNA expression was determined by RT-PCR from total RNA isolated from control and transgenic Desiree. Primers used for PCR analysis were Oligonucleotide No. 3 (see above) and Oligonucleotide No. 5: 5′-CATCGCAAGACCGGCAACAGA-3′ (21-mer, 51 nucleotides downstream from *Sac*I site in pBI 121). The calculated size of the PCR product is 143 bp. The PCRs were performed using the *Taq* PCR Master Mix Kit from Qiagen (Mississauga, Ontario, Canada). Reverse transcription was performed using SuperScript II RNase $H^-$ Reverse Trancriptase from Gibco (Burlington, Ontario, Canada) with Random Hexamer Oligonucleotide [pd$(N)_6$] from Boehringer Mannheim (Mannheim, Germany) as a primer.

*Southern blot analysis*

Three micrograms of total genomic DNA from control and transgenic potato plants was digested with the restriction endonuclease *Eco*RI, electrophoresed and transferred to a Biodyne B nylon membrane (PALL Corporation, Ann Arbor, MI, USA) following the manufacturer's instructions for the alkaline transfer. The membrane-bound DNA was hybridized (O/N at 65 °C) using the *Hin*dIII–*Sac*I fragment from plasmid pDMSRA 3-1217 (containing the 2 × 35S promoter, AMV leader, and the *msrA3* gene) as a probe. The fragment was labeled with [$\alpha$-$^{32}$P] dCTP using the Random Primers DNA Labeling System (Gibco, Burlington, Ontario, Canada) according to manufacturer's instructions. The membrane was then washed twice with 2 × SSC, 0.1% SDS at 65 °C for 5 min each, once in 1 × SSC, 0.1% SDS at 65 °C for 10 min, and then exposed to a Phosphor Screen (Molecular Dynamics, USA). After exposure, the image was scanned using a PhosphorImager (Molecular Dynamics, USA).

*Antimicrobial assays*

The synthetic peptides temporin A and MsrA3 were synthesized by the University of Victoria Genome BC Proteomics Centre using a Model 430A Applied Biosystems peptide synthesizer (Foster City, CA, USA) with the 0.25 mmol scale FastMoc chemistry software. The reagents were from Applied Biosystems and Burdick and Jackson. Amino acids and Fmoc-Rink amide resins were supplied by Novabiochem (La Jolla, CA). Bactericidal activities of the peptides were determined against *Escherichia coli* and *E. carotovora* in microtiter plates in a final volume of 220 µl containing $\sim 1 \times 10^5$ bacteria/ml and desired amounts of peptides. The cell cultures were incubated at room temperature (RT) for 4 h, diluted, and spread on Luria–Bertani (LB) plates. After overnight incubation at 37 °C (*E. coli*) or 28 °C (*E. carotovora*), colonies were counted and the bactericidal activity was scored. Antimicrobial activities of protein extracts were determined as follows: approximately 200 mg of fresh tissue from potato plants growing on MS medium were ground under liquid $N_2$, centrifuged for 15 min at 4 °C in a microcentrifuge, and the supernatant was transferred to a new Eppendorf tube. 2.5 µl of *E. carotovora* cells from an overnight culture (diluted with LB to $A_{600} = 0.07$) were mixed with 97.5 µl of total protein extract. After a 2 h incubation at RT, 900 µl of LB were added and the samples were incubated overnight at 28 °C on the shaker. Then, absorbance at 600 nm was scored. Soft rot resistance of potato tubers was determined as described by Osusky et al. (2000).

Antifungal assays were performed with plant pathogen *P. infestans* US8 A2. The fungus was grown on rye agar (rye extract, 0.1% dextrose, 13.5 g/l agar) until the surface of the agar in the Petri dish was covered with the fungus. A sporangia suspension was prepared by flooding the Petri dish with 10 ml of sterile water, then incubating at RT for 60 min on the shaker. After subsequent incubation at 4 °C (2 h in dark), the sporangia were collected using a sterile Pasteur pipette and their concentration was adjusted to $1.5 \times 10^4$ sporangia/ml using sterile water. The plants in Magenta jars were then sprayed with this suspension. In the assays with detached leaves from mature potato plants

grown in soil for 2 months, three drops (20 µl each) of sporangia suspension were spotted on the leaf and the leaves were incubated at RT in high humidity for 10 days and photographed.

The pink rot tuber assay was performed using *P. erythroseptica* 367. The tubers were soaked in tap water for 30 min, hand washed, and dried at room temperature for 24 h. Then, a small well (using a sterile cork borer) was made into tubers and this was filled with a culture agar plug prepared from the culture of *P. erythroseptica* grown on V8 agar (20% Campbell's V8 juice, 0.3% $CaCO_3$, 15 g/l agar). The infected tubers were incubated at RT and high humidity in the dark. After 7 days, the tubers were cut longitudinally through the inoculation point, exposed to the air for 45 min and photographed using a Nikon Coolpix 990 digital camera. Images were opened in Adobe Photoshop v5.5 and total area of the tuber half was selected using the magnetic lasso tool. Total selected pixel area was recorded from the histogram function under the Image menu. The infected area was measured in the same manner and total infected area was calculated and represented as a percentage of the total area.

## Results

### *Modified temporin A*

The 13 amino acid CAP, temporin A (Figure 1(A)), is one of the smallest, natural antimicrobial peptides. We modified temporin A thereby creating MsrA3 by the addition of a positively charged hexapeptide that minimally altered the predicted structure of the modified molecule (Figure 1(B)). We assumed that the addition of a positive charge (the net charge of MsrA3 at pH 7 is +2) would increase the selectivity for negatively charged lipids and hence Gram-negative bactericidal activity. When temporin A and MsrA3 were assayed *in vitro* with both *E. coli* and the bacterial plant pathogen *E. carotovora* the bactericidal activity of MsrA3 was approximately twice as high as temporin A, killing 50% of *E. coli* at 70 µg/ml, compared to 150 µg/ml for temporin A. More importantly, MsrA3 killed *E. carotovora* with 3–4-fold higher efficiency (50% killing at 20 µg/ml) than temporin A (50% killing at 70 µg/ml); all *E. carotovora* were killed at 40 µg/ml MsrA3.

### *CAP expression vector pDMSRA 3-1217*

The modified temporin A gene (*msrA3*) flanked by appropriate restriction sites was constructed using the polymerase chain reaction (PCR), and cloned as an *Xba*I–*Sac*I fragment into the *Xba*I + *Sac*I-digested vector pBI 121 (Clontech, Palo Alto, CA, USA), thus replacing the β-glucuronidase (*gusA*) gene. The resulting plasmid was named pMSRA 3-1217. The *Hin*dIII–*Xba*I fragment containing the cauliflower mosaic virus (CaMV) 35S promoter was replaced by a *Hin*dIII–*Xba*I fragment from plasmid pBI 525 containing the duplicated enhancer CaMV 35S (2 × 35S) promoter, and an untranslated leader sequence from alfalfa mosaic virus (AMV) RNA4 that acts as a *cis*-active translational activator (Datla et al., 1993). The resulting expression vector was designated pDMSRA 3-1217 (Figure 1(C)).

### msrA3 *is integrated and transcribed in transformed plants*

The presence of *msrA3* in the potato genome was confirmed by PCR amplification of *msrA3* from genomic DNA isolated from transgenic plants. A DNA fragment of the expected size was present in all transgenic Desiree plants tested (Figure 2(A), lanes 2–9), whereas no band was present in the control (lane 1). The integration of *msrA3* into the genome of selected transgenic plants was confirmed by Southern blot analysis (Figure 2(B)). A single copy of *msrA3* was detected in transgenic plants TP3 (lane 2) and TP26 (lane 5), two copies were detected in TP12 (lane 3), while TP24 (lane 4) was found to contain four copies of the transgene in genomic DNA. As expected, *msrA3* was not detected in non-transformed control Desiree plants (lane 1).

Expression of *msrA3* was detected at the m-RNA level by RT-PCR (Figure 3(A)). Expression was confirmed in all transgenic lines tested (lanes 4–10); no PCR product was present in the control plant (lane 3). When DNase-treated RNA samples (without reverse transcriptase) from control and transgenic plants were used as a PCR substrate, absence of the product confirmed that the RNA samples were not contaminated by genomic DNA (data not shown).

Protein extracts from transgenic potato plants expressing MsrA3 (TP3, TP12, TP24) showed clear antimicrobial activities in liquid culture assays using *E. carotovora*, (Figure 3(B)), indicating the successful expression of the MsrA3 peptide. However, we were unable to directly detect MsrA3 in leaf or tuber tissue of transgenic Desiree potatoes. Also, repeated attempts to prepare rabbit antibody against temporin A or temporin A conjugated to Keyhole Limpet Hemocyanin were unsuccessful, reflecting general difficulties

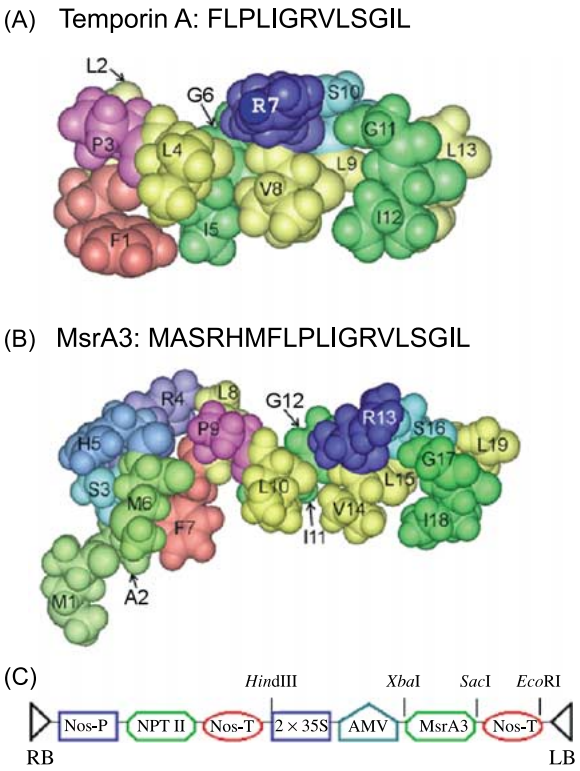


*Figure 1.* The structure and expression construct for MsrA3. (A) Amino acid sequence and predicted model of the conformation of the cationic peptide temporin A. Using the Insight II (version 97.2) molecular modeling program Homology (Molecular Simulations Inc., San Diego, USA), the temporin structure was drawn as an α-helix, based on the known α-helicity of temporin (Mangoni et al., 2000). The structure was then energy minimized using the Discover Program of Insight II. (B) Amino acid sequence and predicted model of the conformation of MsrA3 peptide. The MsrA3 structure was drawn as in (A). (C) Schematic diagram of expression vector pDM-SRA 3-1217. The abbreviations in the figure are as follows: RB and LB, the right and left border regions of the Ti plasmid; Nos-P and Nos-T, promoter and terminator, respectively, of the nopaline synthase gene; NPTII, neomycin phosphotransferase II; 2 × 35S, duplicated enhancer CaMV 35S promoter; AMV, leader sequence from alfalfa mosaic virus RNA4; MsrA3, the coding sequence for MsrA3.

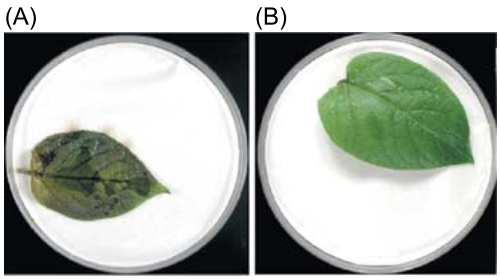


*Figure 4.* Control and transgenic Desiree potato challenged with the bacterial pathogen *E. carotovora*. Detached leaves from 4 to 6-weeks-old control (A) and *msrA3* transgenic (B) Desiree plants were wounded and infected with ~$5 \times 10^5$ cfu of *E. carotovora* and incubated at room temperature. Pictures were taken 3 days after infection (DAI).

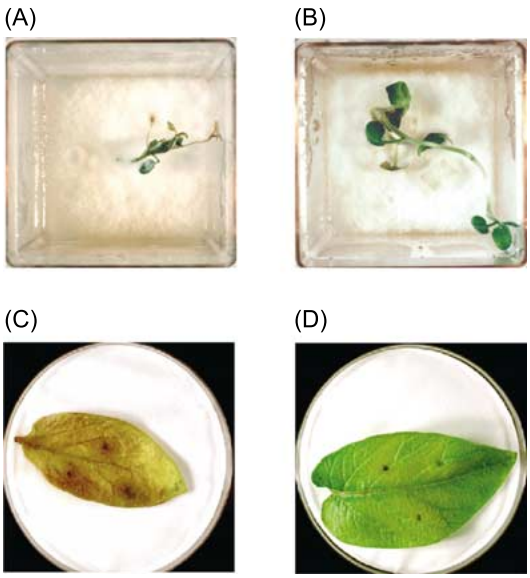


*Figure 6.* Potato plants challenged with *P. infestans*. After rooting in MS medium, the control (A) and *msrA3* transgenic (B) Desiree plants were challenged with *P. infestans*. Pictures were taken 17 DAI. Detached leaf from mature control (C) and *msrA3* transgenic (D) plants were infected with the sporangia of *P. infestans* and incubated at RT. Pictures were taken 10 DAI.

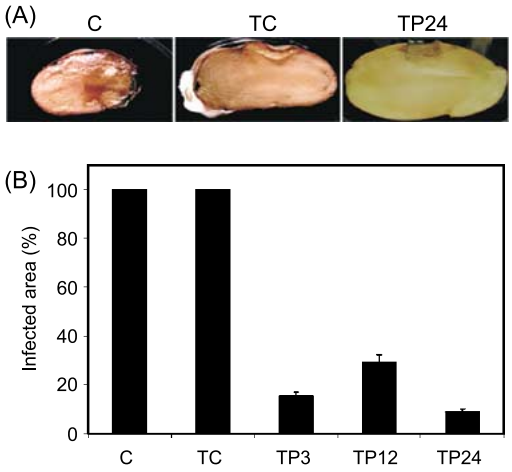


*Figure 7.* Resistance of potato tubers to pink rot. (A) Tubers from control (C), *GUS*-expressing transgenic (TC), and MsrA3-expressing transgenic (TP24) Desiree plants were infected with *P. erythroseptica*. (B) The infected areas of cut tubers were measured and represented as a percentage of the total area. The results represent the average and standard deviation of three experiments.

in raising antibodies against short, basic hydrophobic peptides, thus effectively precluding immunochemical analysis. Furthermore, MsrA3 in either tissue samples or chemically synthesized versions thereof, even at 20 μg/ml, were undetectable by chemical staining with Coomassie brilliant blue or silver.

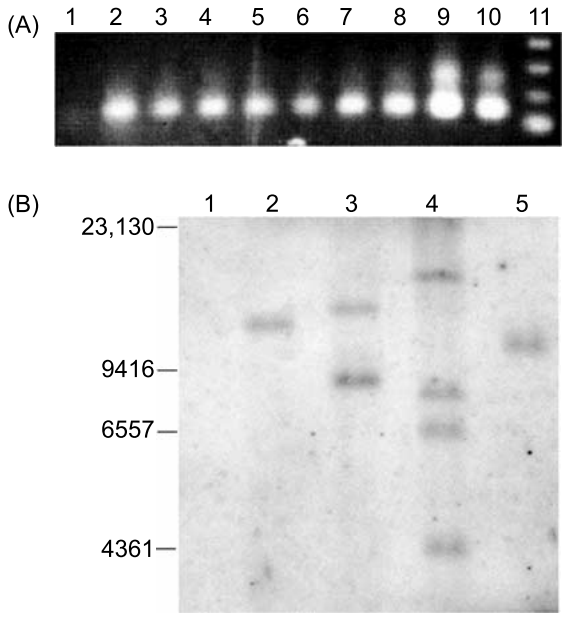


*Figure 2. msrA3* gene integration. (A) *msrA3* coding sequence was PCR-amplified from total genomic DNA isolated from control (non-transformed, lane 1) and transgenic (lanes 2–9) Desiree potato plants. Lane 10, PCR product from plasmid pDMSRA 3-1217 (positive control); lane 11, 100 bp ladder (Amersham Pharmacia Biotech). Bands represent 100, 200, 300, and 400 bp. (B) Southern blot analysis of selected transgenic potato plants. Genomic DNA isolated from nontransformed (lane1) and transgenic potato plants TP3 (lane 2), TP12 (lane3), TP24 (lane 4), and TP26 (lane 5) was digested with the restriction endonuclease *Eco*RI and analyzed as described in 'Materials and methods'. The numbers on the left indicate the positions of molecular weight marker (in bp).

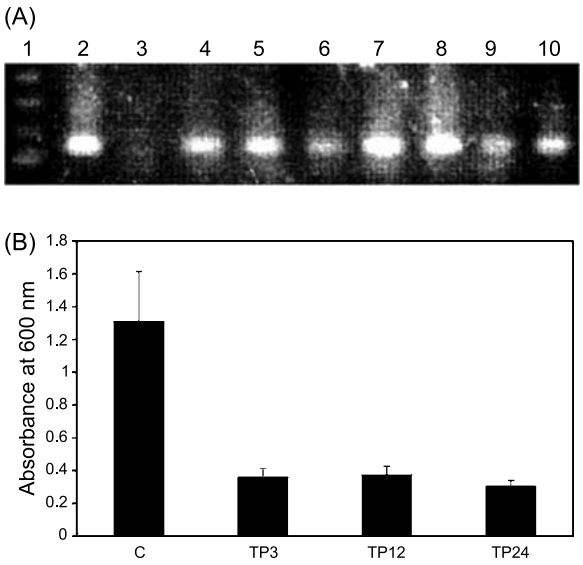


*Figure 3. msrA3* gene expression. (A) MsrA3 mRNA expression was determined by RT-PCR from total RNA isolated from control and transgenic Desiree. RT-PCR products from RNA isolated from control (lane 3) and transgenic (lanes 4–10) plants were analyzed. Lane 1, 100 bp ladder (Amersham Pharmacia Biotech), bands represent 100, 200, 300, and 400 bp; lane 2, PCR product from plasmid pDMSRA 3-1217 (positive control). (B) *In vitro* antibacterial activity of transgenic plants expressing MsrA3. Protein extracts prepared from Desiree control (C) and transgenic plants expressing MsrA3 (TP3, TP12, TP24) were incubated with *E. carotovora* and their inhibitory effect on the bacterial growth was evaluated. The results represent the average and standard deviation of three experiments.

### *Resistance of MsrA3-expressing plants to bacterial infection*

To investigate the ability of transgenic potato plants to resist bacterial infection, detached leaves from soil-grown, 4–6-week old transgenic and control potato plants were placed in Petri dishes with wet filter paper, wounded with a pipette tip, and inoculated with 50 µl ($\sim 5 \times 10^5$ cfu) of an *E. carotovora* suspension. After 1 day at RT, water-soaked areas were visible around the inoculated point of the control plant and the size of these areas increased daily. After 3 days, almost half of the leaf from the control plant had decayed (Figure 4(A)), whereas with the MsrA3-expressing transgenic plant (TP24) no decay was seen, and the leaf remained green (Figure 4(B)).

Because soft rot primarily attacks harvested tubers, we also tested tuber discs derived from control and transgenic plants for resistance to *E. carotovora*. After incubation for 3 days at RT with $\sim 2 \times 10^7$ cfu of *E. carotovora*, tuber discs from the control (C) plant lost almost 50% of their fresh weight. Similar losses (almost 45%) of fresh weight were observed with tubers from the transgenic control (TC) plants expressing

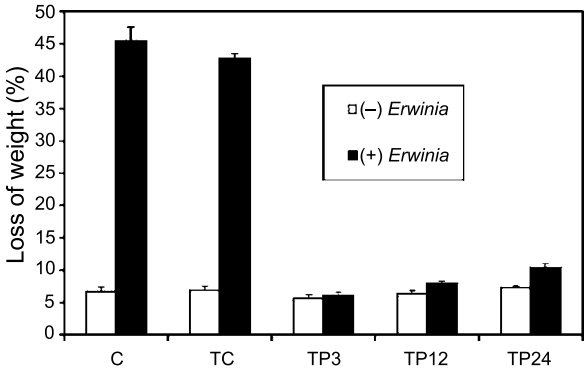


*Figure 5.* Soft rot resistance of transgenic potato tubers. Discs from control (nontransformed, C), transgenic control (*GUS*-expressing, TC) and MsrA3-expressing transgenic (TP3, TP12, TP24) potato tubers were infected with *E. carotovora*. The sensitivity/resistance to *E. carotovora* was expressed as the loss of weight of tuber tissue. The results represent the average and standard deviation of three experiments.

only β-glucuronidase. However, there was virtually no apparent loss of material from tuber discs which originated from MsrA3-expressing transgenic Desiree plants (TP3, TP12, TP24) and this was essentially comparable to uninfected discs (Figure 5), the small loss being mostly due to dehydration. Very similar results (not shown) were obtained when the same experiment was repeated using tubers stored for several months at 4 °C. Furthermore, when the tubers from transgenic line TP24, previously stored for over 26 months at 4 °C with no visible signs of deterioration, were planted into soil they produced healthy, morphologically normal plants with a normal yield (in size and number) of tubers when compared to control nontransgenic Desiree potato plants.

*Transgenic plants expressing MsrA3 resist late blight and pink rot*

To evaluate the fungal disease resistance potential, control (non-transformed) and transgenic (expressing MsrA3) plants were challenged with a strain of the fungal pathogen *P. infestans*, currently responsible for reducing global potato production by more than 15% (Khurama, 1998). These experiments were performed with potato plants growing on Murashige–Skoog (MS) medium using the sporangia of the highly virulent *P. infestans* US8 isolate A2. In control plants, the fungus readily penetrated both stems and leaves causing dehydration, pigment loss, and stem softening; sixteen days post-infection the control plant was dead (Figure 6(A)). Following an identical infectious challenge, the transgenic plant expressing MsrA3 (TP24) remained green and continued to grow normally through the fungal hyphae with no evidence of disease (Figure 6(B)).

In another set of experiments, detached leaf from 8-week-old control and transgenic (TP24) potato plants growing in soil were placed in Petri dishes with wet filter paper, wounded, and inoculated with a *P. infestans* US8A2 spore suspension (three inoculation spots per leaf, 300 spores per spot). After 4 days of incubation at RT, the first dramatic changes were observed on the leaf from the control plant, and 10 days after infection the leaf was completely brown and decomposed (Figure 6(C)). In contrast, the leaf from the transgenic plants expressing MsrA3 was still green with small necrotic spots only at the sites of inoculation (Figure 6(D)).

Another distantly related *Phytophthora* sp., *P. erythroseptica*, causes the economically serious loss of potato tubers during storage. Its name describes the characteristic pink color of infected tissue when sectioned tubers are exposed to air for 30–60 min (Salas et al., 2000). To evaluate resistance to pink rot, tubers of a non-transformed control, transgenic control (expressing *gusA*), and transgenic Desiree potato plants expressing MsrA3 were infected with agar plugs containing flourishing *P. erythroseptica* sporangia and incubated in the dark at RT and 100% humidity for 7 days. When sectioned longitudinally through the inoculation point and exposed to air for 45 min, the characteristic pink color developed on both the control (C), and transgenic control (TC) plants. However, the color of sectioned tubers from plants expressing MsrA3 (TP24) did not change and the small area of infection was restricted to the point of inoculation, thereby demonstrating resistance to pink rot (Figure 7(A)). Subsequently, several lines of transgenic potato tubers were similarly tested for pink rot resistance and assessed semi-quantitatively (Figure 7(B)). With control (C) non-transformed, and transgenic control (TC) tubers 100% of the cut area was clearly pink. The infected areas of tubers from transgenic plants expressing MsrA3 were considerably smaller, ranging from 9% (TP24) to 29% (TP12) depending on the plant. When we tested the tubers from the second generation, the results were very similar (data not shown). These results clearly demonstrate that expression of MsrA3 in potato protects tubers from pink rot and that resistance was stable through one generation of tuber propagation.

## Discussion

The antibacterial, antifungal, and non-hemolytic activities make the temporin A analogue, MsrA3, an excellent candidate for expression in transgenic plants. Weak Gram-negative bactericidal activity against normally sensitive, LPS-deficient strains of *E. coli* suggested this was due primarily to the low net charge of temporin A (Mangoni et al., 2000). Molecular modeling and engineering of peptides provide a powerful tool to generate peptides with desirable properties. In this case, the modeling was restricted to comparisons of temporin A and the N-terminally modified form to ensure that no drastic alterations to the α-helical structure were made. We have not fully tested other amino acid combinations at the N-terminus of temporin A, nor have we attempted to rationally design these by computer modeling. We merely tried the

simple modification of the N-terminus, based on intuitive reasoning as well as the previous success with peptide MsrA1 (Osusky et al., 2000). By increasing the charge of the modified temporin A (MsrA3), the bactericidal activity against *E. coli* and *E. carotovora* actually increased 2–4 times. High efficiency killing of *E. carotovora* (50% killing at 20 μg/ml) indicated that MsrA3 might have better potential for plant protection than the previously described, unrelated chimeric CAP, MsrA1 (Osusky et al., 2000) (50% killing at 36 μg/ml).

Experiments with protein extracts prepared from transgenic potato plants demonstrated powerful bactericidal activities when tested with *E. carotovora* – the primary cause of bacterial soft rot. When detached leaves from mature potato plants were artificially infected, the results clearly showed that the concentration of MsrA3 in the leaves of transgenic Desiree potatoes was high enough to effectively combat these *E. carotovora* challenges.

Tests with potato tubers harvested from *msrA3* transgenic plants revealed strong resistance against *Erwinia*-induced soft rot. When the same experiments were later repeated using tubers stored for several months at 4 °C, the results were very similar (not shown). Thus, the harvested tubers retained their antimicrobial characteristics during storage at 4 °C and remained resistant to soft rot, thereby potentially diminishing the losses of potato tubers during prolonged storage. Furthermore, when whole tubers were visually examined after 26 month storage at 4 °C, there were virtually no visible signs of natural deterioration, unlike their non-transgenic counterparts, which had wizened and blackened considerably. Thus, MsrA3-expressing transgenic tubers exhibited a profound extension of storage life.

Although the losses of potato crops due to bacterial diseases can be substantial, a far greater danger is imposed by fungal diseases. While bacteria cause less than 10% of diseases of potato, almost one third of potato diseases are attributed to fungi (Rich, 1991), in particular *Phytophthora* sp. (late blight, pink rot) and *Fusarium* sp. (*Fusarium* wilt, *Fusarium* dry rot). Late blight disease of potato is perceived as a serious threat to food security throughout the world. Resistance to fungicides has arisen and the liberal application and increasing reliance on fungicides and other pesticides are believed to contribute to chemical contamination of the environment and food supply. Combining yield losses and the cost of chemical control, late blight alone remains one of the most costly crop diseases. As a result, development of disease-resistant potatoes has been given a high priority by the international community (GILB – Global Initiative on Late Blight) (Niederhauser, 1999). Ongoing research programs include the breeding and selection of blight-resistant cultivars, understanding host–pathogen interactions and integrated pest management. One method of crop protection is through transgenic approaches and the use of judiciously selected and engineered CAPs as agents of disease protection. *In vitro* experiments showed that the Oomycetes appear to be insensitive to mixtures of chitinase and β-1,3-glucanase (Mauch et al., 1988) that have been effective with other types of fungi. Increased resistance of transgenic plants against *P. infestans* was achieved by increasing levels of $H_2O_2$ (Wu et al., 1995), expression of phytoalexins (Thomzik et al., 1997), pathogenesis-related proteins (Woloshuk et al., 1991; Liu et al., 1994; Zhu et al., 1996), enzymes involved in salicylic acid metabolism (Yu et al., 1999), or introgressing resistance genes from wild potato species (Song et al., 2003). However, in each case protection was limited and fairly narrow with respect to the pathogen spectrum. Recently, the use of signal transduction 'master switches' to engineer broad-spectrum disease resistance has been attempted with some success. Overexpression of NIM1/NPR1 in *Arabidopsis* led to resistance against several pathogens (Cao et al., 1998). In addition, other disease-resistant mutants have been described (Frye et al., 2001; Yang et al., 2001; Asai et al., 2002). However, it has been shown that engineering resistance through the use of these master switches has drawbacks. For example, constitutive expression of a defense pathway in mutants resulted in reduced plant vigor or yield. More importantly, there appears to be an antagonism between different defense pathways which leads to increased susceptibility to other pathogens (Hoffman et al., 1999).

Our results clearly demonstrate that the use of the structurally altered temporin analogue, MsrA3, is a promising tool in controlling the most significant, commercially relevant fungal pathogens – *P. infestans* and *P. erythroseptica*, as well as other fungal and bacterial pathogens, with no deleterious effects on plant morphology or productivity. The transgenic tubers expressing MsrA3 exhibited a profound extension of storage life. Our work describes a possible approach to addressing these problems and to providing a more effective, environmentally friendly, and economical alternative toward higher productivity.

Although concerns have been expressed by some regarding genetically-modified plants, those described here express only a small, apparently non-immunogenic peptide that would be readily degraded by alimentary tract proteases during consumption. Many cationic peptides are naturally produced by plants as part of their innate defenses against infection. Thus, the technology described here could have broad implications with respect to improved crop yields and increased storage life for a wide variety of food and non-food crops and perhaps help reduce the environmental impact associated with intensive agriculture. Studies to demonstrate the safety of this peptide for use in transgenic crop plants are in progress.

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