Transgenic plants expressing cationic peptide chimeras exhibit broad-spectrum resistance to phytopathogens

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Here we describe a strategy for engineering transgenic plants with broad-spectrum resistance to bacterial and fungal phytopathogens. We expressed a synthetic gene encoding a N terminus-modified, cecropin–melittin cationic peptide chimera (MsrA1), with broad-spectrum antimicrobial activity. The synthetic gene was introduced into two potato (*Solanum tuberosum* L.) cultivars, Desiree and Russet Burbank, stable incorporation was confirmed by PCR and DNA sequencing, and expression confirmed by reverse transcription (RT)-PCR and recovery of the biologically active peptide. The morphology and yield of transgenic Desiree plants and tubers was unaffected. Highly stringent challenges with bacterial or fungal phytopathogens demonstrated powerful resistance. Tubers retained their resistance to infectious challenge for more than a year, and did not appear to be harmful when fed to mice. Expression of *msrA1* in the cultivar Russet Burbank caused a striking lesion-mimic phenotype during leaf and tuber development, indicating its utility may be cultivar specific. Given the ubiquity of antimicrobial cationic peptides as well as their inherent capacity for recombinant and combinatorial variants, this approach may potentially be used to engineer a range of disease-resistant plants.

Keywords: cationic peptides, transgenic potato, phytopathogens, disease resistance

Phytopathogens are responsible for enormous losses ($30–$50 billion annually) in cultivated and stored crops and are a major impediment to effective food distribution worldwide[1]. Spoilage can also increase the incidence of carcinogens and affect human and animal health. Countervailing measures in the form of antimicrobials and pesticides not only significantly increase production costs and are regarded as serious environmental contaminants, they contribute to the increase in antimicrobial-resistant species. Plant genetic engineering is regarded as an effective means for providing resistance to phytopathogens[2]. Consequently, there has been considerable activity in developing disease resistance strategies; however, most of these have been fairly narrow with respect to the microbial spectrum of protection[2,3]. Only recently have alternative strategies afforded some degree of broad-spectrum resistance[4,5]. We therefore sought to produce transgenic plants strongly resistant to a broader spectrum of microbial phytopathogens through the expression of cationic antimicrobial peptides (CAPs)[6].

A large number of diverse natural antimicrobial peptides have been discovered over the last two decades (for reviews see refs 3,6,7). Although CAPs are structurally diverse, most of them fall into two general classes of structure: α-helical peptides, such as the cecropins and magainins, and β-sheet peptides, such as the defensins, protegrins, and tachyplesins[3,8–10]. These natural products vary greatly in their biological activity spectrum, killing bacteria (Gram-positive and -negative), fungi, protozoa, and even viruses at concentrations from 0.25 to 4 μg/ml (ref. 3). Thus the expression of CAPs in plants may introduce broad-spectrum resistance to phytopathogenic microorganisms[11,12].

Previous approaches to the expression of CAPs in plants have focused on antibacterial natural products of limited spectrum such as barley α- or β-hordothionin[13], barley α-thionin[14], horseshoe-crab tachyplesin[15], cecropin B, or even a cecropin A/B chimera[16–18], but the results have been either narrowly or weakly antibacterial. So far only transgenic tobacco expressing the plant defensin Rs-AFP2 from radish showed some resistance to the fungus *Alternaria longipes*[19].

Insect cecropins represent a family of small, highly basic α-helical antimicrobial peptides that form an important component in the immune response of insects[20]. Cecropins isolated from the giant silk moth, *Hyalophora cecropia*, are ~35–amino acid residues with amphipathic N termini and hydrophobic C termini[21]. All cecropins are potent antibacterials in vitro. In addition, several are effective against a number of plant pathogenic bacteria[22–24] in vivo. The 26–amino acid antibacterial peptide, melittin, is the major component of bee venom and has a predominantly hydrophobic N terminus with an amphipathic C terminus[25]. However, its powerful hemolytic activity[26] makes it therapeutically unsuitable and likely a poor candidate for transgenic applications.

Molecular modeling and engineering of peptides provides a powerful tool to generate chimeric peptides with potentially superior properties. An example of this approach is the strongly antibacterial chimeric peptide, CEMA, which contains eight amino acid residues from the antimicrobial peptides cecropin A and a modified melittin sequence at the C-terminus[27]. CEMA retains its powerful antimicrobial activity but exhibits a considerably reduced hemolytic activity (with a minimal hemolytic concentration of >100 μg/ml and an LD_{50} of >16 mg/kg intravenously and 100 mg/kg intraperitoneally in mice; R.E.W. Hancock, unpublished results), making it a better candidate for transgenic applications. However, preliminary studies suggested that CEMA was toxic to plants(Osuska et al., unpublished results).

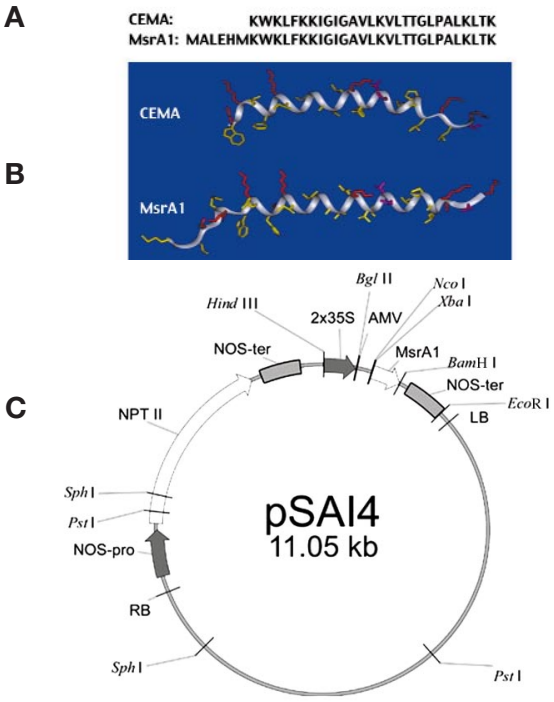

Figure 1. The structure and expression constructs for MsrA1. (A) The amino acid sequence of the cationic peptides CEMA and the N-terminally extended version, MsrA1. (B) The predicted models of the conformations of CEMA and MsrA1 peptides after associating with bacterial or fungal membranes. These peptides were modeled using Insight II software on a Silicon Graphics Indy computer (Mountain View, CA). The peptide structures are predicted to form amphipathic α-helices[43]. Cationic residues are indicated in red and hydrophobic residues in yellow. (C) The pSAI4 plasmid expression vector for MsrA1. The abbreviations in the figure are as follows: RB and LB, the right and left border regions of the Ti plasmid; NOS-pro and NOS-ter, promoter and terminator, respectively, of the nopaline synthase gene; NPT II, neomycin phosphotransferase II; 2×35S, duplicated enhancer CaMV 35S promoter; AMV, leader sequence from alfalfa mosaic virus RNA4; MsrA1, protein coding sequence of MsrA1.

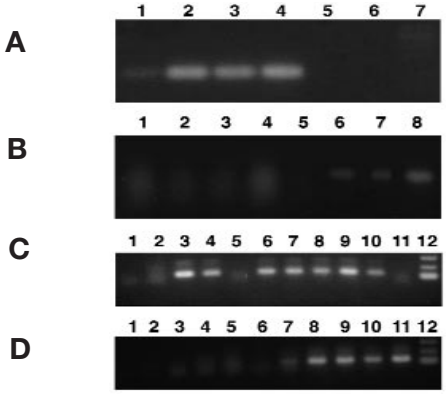

Figure 2. *msrA1* gene integration and mRNA expression. (A) *msrA1* coding sequence was PCR amplified from plasmid pSAI4 (positive control, lane 1); total genomic DNA was isolated from control (nontransformed, lane 5) and transgenic (lanes 2–4) Russet Burbank potato plants. Lane 6, no template in PCR (negative control); lane 7, molecular size standards (ϕX174 RF DNA/*HaeIII*). (B) MsrA1 mRNA expression was determined by RT-PCR from total RNA from control and transgenic Russet Burbank. PCR products from RNA isolated from control (lane 1) and transgenic (lanes 2–4) Russet Burbank, without reverse transcription (control), and RT-PCR products from control (lane 5) and transgenic (lanes 6–8) Russet Burbank were analyzed. (C) *msrA1* coding sequence was PCR amplified from total genomic DNA isolated from control (nontransformed, lane 2) and transgenic (lanes 4–11) Desiree potato plants. Lane 1, no template (negative control); lane 3, PCR product from plasmid pSAI4 (positive control); lane 12, 100 bp ladder (Pharmacia Biotech). Bands represent 100, 200, and 300 bp. (D) MsrA1 mRNA expression was determined by RT-PCR from total RNA isolated from control and transgenic Desiree. PCR products from RNA isolated from control (lane 1) and transgenic (lanes 2–5) Desiree, without reverse transcription (quality control), and RT-PCR products from control (lane 6) and transgenic (lanes 7–10) Desiree were analyzed. Lane 11, PCR product from plasmid pSAI4 (positive control); lane 12, 100 bp ladder (Pharmacia Biotech). Bands represent 100, 200, and 300 bp. The primers for PCR (A–D) were the same as used in pSAI4 construction.

Here we have attempted to reduce apparent toxicity by modifying the N terminus while retaining the original α-helical character. To evaluate its antimicrobial activity in plants, we constitutively expressed the synthetic gene *msrA1,* encoding the modified form of CEMA, in two potato cultivars, Desiree and Russet Burbank, which are normally susceptible to a number of phytopathogens. Our results show that expression of *msrA1* in potato plants consistently increased their resistance to both bacterial and fungal pathogens.

Results

Construction of expression vector, pSAI 4. The 28–amino acid, cecropin–melittin chimera, CEMA, contains a C-terminal 8–amino acid segment from cecropin A, an N-terminal 16–amino acid segment from melittin, with enhanced substitution of positive charges (2 additional lysine residues) on the C-terminal end. The six–amino acid extension at the N terminus of MsrA1 (Fig. 1A) only slightly altered the predicted structure of this cationic peptide (Fig. 1B). The net charge of the peptide MsrA1 was thus +6. Antibacterial activity was tested in vitro with both *E. coli* and *E. carotovora*. The antibacterial activity of MsrA1 was in fact eightfold lower than that of CEMA, with 50% killing at 36 μg/μl as compared to 4.5 μg/ml, respectively.

The plasmid vector, pBI524, contains a duplicated enhancer cauliflower mosaic virus (CaMV) 35S promoter and an untranslated leader sequence from alfalfa mosaic virus (AMV) RNA4 that acts as a *cis*-active "translational activator"[28]. After *msrA1* was cloned into pBI 524, the *Hin*dIII-*Eco*RI fragment, containing a 2 × 35S promoter with an AMV RNA4 translation-enhancing element and *msrA1* with a NOS terminator (NOS-ter), was ligated into *Hin*dIII + *Eco*RI digested vector pBI121 (thus replacing the 35S promoter and the β-glucuronidase (*GUS*) gene with the NOS-ter), yielding pSAI4 (Fig. 1C).

Gene *msrA1* is integrated and expressed in transformed plants. Two cultivars of potato, Russet Burbank and Desiree, were transformed with pSAI 4 by *Agrobacterium tumefaciens*–mediated transformation. The integration of *msrA1* into plant genomic DNA was confirmed by PCR amplification of the *msrA1* sequence from genomic DNA isolated from nontransformed (control), and transgenic plants. The appropriate DNA fragment was present in all three transgenic Russet Burbank plants tested (Fig. 2A, lanes 2–4), whereas no band was present in the control (lane 5). Similarly, in Desiree plants a band was detected in PCR products of genomic DNA (Fig. 2C) of transformed plants (lanes 4–11) but not in nontransformed plants (lane 2).

The expression of *msrA1* was tested at the RNA level using RT-PCR (Fig. 2B, D). Expression was confirmed in all transgenic lines of Russet Burbank (Fig. 2B, lanes 6–8), and Desiree (Fig. 2D, lanes 7–10) whereas no RNA product appeared from the control plants (Fig. 2B, lane 5 and Fig. 2D, lane 6).

The morphological characteristics and the yield of tubers of transgenic Desiree plants and tubers expressing MsrA1 were comparable to that of nontransformed plants (Fig. 3A–C). However, the expression of *msrA1* in cv. Russet Burbank caused morphological changes in transgenic potato plants when compared to the control plant (Fig. 3D–F). The most striking changes were observed on leaves that became curly, and on tubers that were much smaller and branched (Fig. 3F). This apparent lesion-mimic phenotype was observed in all Russet Burbank transgenic lines tested.

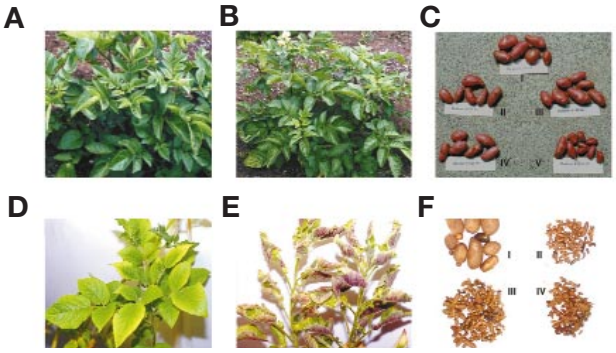

Figure 3. Morphological characteristics of transgenic potato plants and tubers. Desiree control (A) and *msrA1* transgenic plants (B) were photographed after transfer to soil in the greenhouse. Tubers from control (C I) and transgenic (C II, III, IV, V) plants were photographed after harvesting. Russet Burbank control (D) and *msrA1* transgenic plants (E) were photographed after transfer to soil. Tubers from control (F I) and transgenic (F II, III, IV) plants were photographed after harvesting.

Inactive MsrA1 variants and controls are biologically inactive. In a series of control experiments transgenic plants expressing GUS, NADPH:P450 reductase, or the cationic peptide CEMA, N-terminally fused to the human defensin prepro sequence[3,10], were selected in a similar fashion to the above constructs. None of these transgenic plants exhibited the disease-resistant trait (data not shown).

Phytopathogen resistance of *msrA1*-expressing plants. To evaluate the fungal disease resistance potential, control and transgenic Russet Burbank and Desiree plants were challenged with two different fungal pathogens under extreme conditions. The plants were challenged with the phytopathogen *Phytophthora cactorum*. After seven days, the fungus had grown over the surface of the Murashige–Skoog (MS) medium, and penetrated into both the roots and stems of control plants, causing dehydration, curling leaves, and softening stems. By 11 days post-infection, the control plants were infected from the roots to the tips, resulting in the yellowing of leaves and consequently the death of the plant. Remarkably, the transgenic plants were still green and continued to grow normally (Fig. 4) with no evidence of disease.

A similar set of experiments were performed with the fungus *Fusarium solani*. Six days post-infection, *Fusarium* had grown all over the surface of the MS medium, and the roots and stems of the control plant were severely damaged. Eleven days after infection, the control plant was dead while the transgenic plants were still growing normally with no evidence of infection (Fig. 5).

To investigate the ability of transgenic potato plants to confer resistance to *Erwinia carotovora*, causing bacterial soft rot of potato tubers, we incubated branches from each of Russet Burbank and Desiree control and transgenic cultivars with *Erwinia carotovora* in liquid MS medium. After one week, the control plants were seriously infected and growth inhibited, and subsequently they died. In contrast, the transgenic plants were unaffected and continued to grow demonstrating that expression of *msrA1* increased the resistance of potato plants to this bacterial pathogen (data not shown).

Tubers harvested from Desiree plants and stored at 4°C for several months were tested for resistance to *Erwinia*-induced soft rot. After six days of incubation with 2×10^7 colony-forming units of *E. carotovora*, the control (C) potato tubers had lost ~60% of their fresh weight because of soft rot. The loss of weight of tuber disks originated from transgenic Desiree plants (ME1, ME2, ME3) infected with *E. carotovora* was comparable to noninfected disks (Fig. 6A). In another experiment, the disks from nontransformed potato had largely decomposed, whereas the disks from MsrA1-expressing plants were unaffected (Fig. 6A, inset a). After six months of storage at 4°C, some tubers from nontransformed potato were infected and spoiled while transgenic lines remained healthy with no sign of disease (Fig. 6A, inset b).

Fast cation exchange chromatography was used to partially purify MsrA1 from transgenic potato tubers. The bactericidal activity (with *E. carotovora* as a target) was only observed in those fractions containing the peptide of the same size as MsrA1 (Fig. 6B). Both the peptide and bactericidal activity were completely absent from control, nontransgenic tubers of the same age. These results confirmed that tubers from transgenic plants contained biologically active MsrA1 in the concentrations sufficient to retain their antibacterial properties during prolonged storage at 4°C.

Safety of transgenic potatoes. To determine the toxic effect of transgenic potatoes Balb/C, six-week-old female mice were fed raw potato tubers. A control group of five mice were fed potato tubers from nontransformed plants, and three groups of five mice each were fed potato tubers from MsrA1-expressing transgenic plants (5 g of raw potato tuber per mouse per day). After one week, all mice lost almost 25% of their body weight (24.4 ± 1.5% in control group, 22.9 ± 0.9% in "transgenic" groups). This loss of weight was due to a nonbalanced diet, so it was supplemented with Rodent Laboratory Chow 5001 (Jamison Pet Food Ltd., Vancouver, BC, Canada; 3 g per mouse per day). After another week the loss of body weight was less apparent (8.4 ± 1.6% in control, 5.5 ± 1.2% in "transgenic" groups), and by the end of trial (21 days) the loss of weight was minimal (0.8 ± 0.6% in control, 1.3 ± 1.1% in "transgenic" groups). Mice whose diet was based on tubers from transgenic potatoes were as healthy and vital as those from the control group, and no differences in the behavior of animals were observed. Moreover, analysis of fecal pellets showed that the microflora (colony-forming units/mg of fecal sample) of control and experimental samples were comparable, indicating no adverse effects of peptides on gut microflora.

Discussion

The potato is a major food crop exceeded only by wheat, rice, and maize in world production for human consumption[29]. Considerable losses due to bacterial diseases such as blackleg and soft rot caused by *Erwinia* sp. are frequently encountered. Even greater losses occur from various fungal pathogens, especially *Fusarium* and *Phytophthora* sp. Recently, conceptually different approaches in plant genetics and molecular biology have shown promise with respect to enhancing broad-spectrum disease resistance, either through the upregulation of genes that enhance systemic acquired resistance[4], or through the enhancement of plant defense signaling[5]. Expression of CAPs in plants seems to be another promising approach.

In this study we made use of the broad-spectrum antimicrobial effect[30] as well as the reported higher tissue tolerance of the cecropin

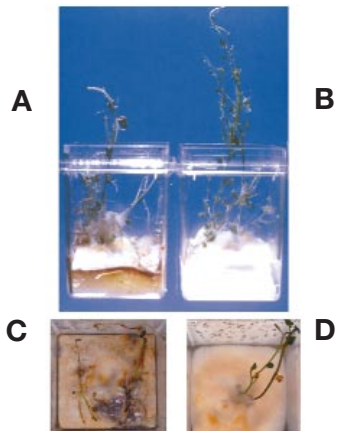

Figure 4. Transgenic potato challenged with the fungal pathogen *Phytophthora cactorum*. After rooting in MS medium, the control (A) and *msrA1* transgenic (B) Russet Burbank plants, and the control (C) and *msrA1* transgenic (D) Desiree plants were challenged with the fungus *P. cactorum*. Pictures were taken 11 days (A, B) or 19 days (C, D) after infection.

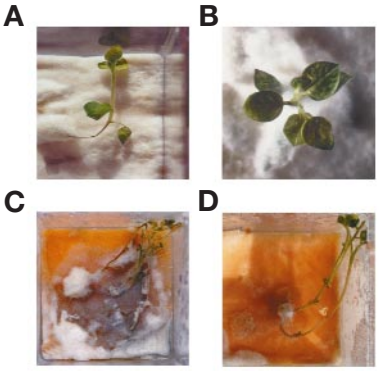

Figure 5. Transgenic potato challenged with the fungal pathogen *Fusarium solani.* After rooting in MS medium, the control (A) and *msrA1* transgenic (B) Russet Burbank plants, and the control (C) and *msrA1* transgenic (D) Desiree plants were challenged with *F. solani*. Pictures were taken 11 days (A, B) or 19 days (C, D) after infection.

A/melittin chimeric CAP, CEMA[27], as a candidate for transgenic expression. We further modified this candidate by the addition of a hexapeptide to dampen excellent antimicrobial activity, reasoning that too strong a biological activity might be counterproductive because toxicity for the host can often accompany strong biological activity[3]. Positive charges in the hydrophilic portion of the peptide are essential for antifungal activity[31]. By molecular modeling, MsrA1 was predicted to maintain a positively charged N terminus and overall amphipathic α-helical character.

Expression of *msrA1* strongly increased the resistance of these plants to bacterial (*Erwinia* sp.) as well as fungal (*Fusarium*, *Phytophthora* sp.) phytopathogens making MsrA1 an extremely promising tool in plant antimicrobial warfare. These results have been duplicated in tobacco though without the lesion-mimic effect (data not shown). However, in these and the studies of others it is difficult to compare the absolute degree of resistance. Here we invoked a highly stringent bioassay for disease resistance using cocultivation with high levels of aggressive phytopathogens with survival as the end point, whereas others have relied on less stringent assays such as the enhanced resistance toward lesion formation as an indicator of infectivity[4,5].

We suspect that this challenge model more closely represents a field situation where soil and infected plants provide a more constant reservoir of phytopathogens. In this regard the transgenic plants described here have been grown for more than two months in the presence of both bacterial and fungal pathogens with still no evidence of disease. Furthermore, the transgenic potato tubers produced here retain their antimicrobial characteristics for over a year in storage at 4°C and remain resistant to *Erwinia* soft rot. We have estimated the concentration of MsrA1 as ~3–4 μg/g of raw tuber tissue, which is sufficient to protect them from bacterial attack. Preliminary feeding trials for a month showed that the tubers from MsrA1-expressing transgenic potato plants were not toxic to mice (data not shown).

Constitutive expression of *msrA1* can also cause morphological changes, similar to the so-called "lesion-mimic" phenotype, but here only in the transgenic Russet Burbank potato. This phenomenon has been observed previously[32–35], where the expression of foreign genes in plants can trigger the activation of plant defense mechanisms normally activated only during pathogenesis in an attempt to curtail the pathogen[34–36]. However, as reported, this phenotype is only narrowly antimicrobial, thus differing from the response seen here with the Russet Burbank cultivar. In contradistinction, expression of *msrA1* in transgenic Desiree plants had virtually no deleterious effects on the morphology or yield of plants and tubers. Similarly, transgenic tobacco plants expressing MsrA1 also showed broad-spectrum disease resistance, normal morphology, and stable inheritance (data not shown). Thus the lesion-mimic phenotype appears to be specific to plant type.

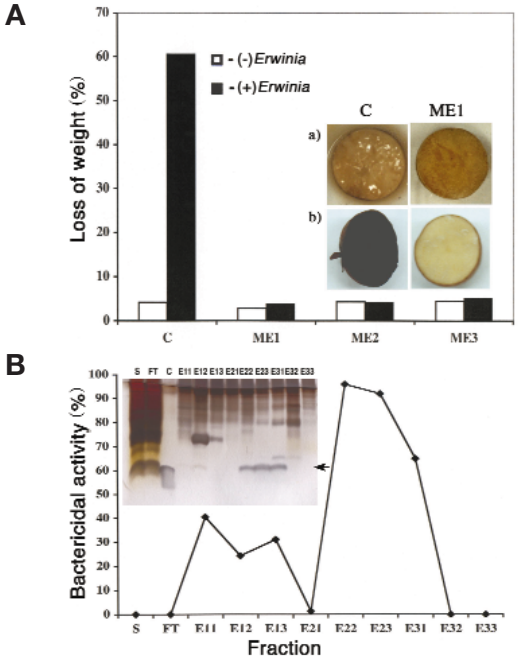

Figure 6. Transgenic potatoes resistant to *Erwinia carotovora.* (A) Soft rot resistance of MsrA1-expressing transgenic Desiree potato tubers. Disks prepared from tubers of control (C) and transgenic plants ME1, ME2, and ME3 were infected with *E. carotovora*. After six days at room temperature, rotted tissue was gently removed from the disks and the sensitivity/resistance to *E. carotovora* was expressed as the loss of weight of tuber tissue. Inset (a): Disks prepared from tubers of control (C) and *msrA1* transgenic (ME1) Desiree plants were infected with *E. carotovora*. The control disks were readily liquefied, while ME1 retained its integrity. Pictures were taken after six days incubation at room temperature. Inset (b): Tubers of control (C) and *msrA1* transgenic (ME1) Desiree potatoes, stored for six months at 4°C, were cut to halves and incubated another 24 h at 4°C. Rotted control tuber became black while ME1 remained healthy. (B) The bactericidal effect of fractions from ion exchange chromatography on *E. carotovora* were determined. S, filtered supernatant applied to the column; FT, flowthrough; E11–E13, fractions obtained by elution with 0.2 M NaCl; E21–E23, fractions obtained by elution with 0.3 M NaCl; E31–E33, fractions obtained by elution with 0.5 M NaCl. Inset: Proteins from ion exchange chromatography were separated on Tricine-SDS polyacrylamide gel and silver stained. Description of fractions is the same as above (C, 200 ng of chemically synthesized MsrA1). The arrow indicates the 3.8 kDa MsrA1 peptide band.

With regard to the mechanism of broad-spectrum resistance, members of the CAP family are powerful membrane antagonists, normally exhibiting a greater degree of specificity for microbial membranes. Presumably, invading pathogens encounter the host-derived CAP and invasion is halted through interference, such as by membrane depolarization. With Russet Burbank transgenics some interference with host membrane function perhaps triggers the expression of other host plant defense systems. This suggests that the structure of CAPs may need to be finely tuned to suit different plants. Nevertheless, the effective consequences are transgenic plant species exhibiting a profound resistance to phytopathogens.

Given the variety of naturally occurring CAPs and the relative ease of creating recombinant or combinatorial forms, engineering a wealth of highly disease-resistant plants may be possible. These results could have broad implications with respect to a reduction in the reliance on antimicrobials, improved crop yields, decreased disease incidence associated with monoculture, and increased storage times.

Experimental protocol

Nucleic acid manipulation. DNA manipulations were carried out as described[37]. The enzymes were purchased from Pharmacia Biotech (Uppsala,

Sweden) or New England Biolabs (Beverly, MA), and used according to manufacturer's instructions.

ECEMA expression vector construction. Primers used for PCR amplification of *msrA1* were as follows: 5′-primer (36-mer) 5′-CAAGGAAAAACGG**TCTAGA**GCATATGAAATGGAAAC-3′; 3′-primer (35-mer) 5′-GAACTCGAGCAGC**GGATTC**TTACTTAGTTAGCTTC-3′. The primers were designed to have *Xba*I recognition site at the 5′ end, and *Bam*HI recognition site at the 3′ end of the amplified fragment, respectively (bold). Plasmid pR78hproCEMA was used as a template for PCR. An amplified DNA fragment containing the CEMA sequence was purified using a NucleoSpin column (Clontech, Palo Alto, CA), digested with *Xba*I and *Bam*HI, and inserted into vector pBI524[28] digested with *Xba*I and *Bam*HI. The resulting vector was named pSAI1. Then, the *Hin*dIII-*Eco*RI fragment in pBI121 (Clontech) containing the CaMV 35S promoter, *GUS* gene, and NOS-ter sequences, was replaced by a *Hin*dIII-*Eco*RI fragment from pSAI1 (containing an enhanced 35S promoter with an AMV RNA4 translation-enhancing element, *msrA1*, and NOS-ter sequences). The resulting vector was specified as pSAI4 (Fig. 1C).

Plant transformation and regeneration. Transformation of *A. tumefaciens LBA4404* or *MP90* was done by the freeze-thaw method[38]. Transformation of potato cultivars Desiree and Russet Burbank was as described[39].

Isolation of nucleic acids from plant tissue. Genomic DNA was isolated from 10 g of fresh leaves by the method described[40]. Total RNA from plants was isolated as decribed[41].

Partial peptide purification. Ten grams of tissue from potato tuber were ground to a fine powder under liquid N_2 and extracted for 30 min at 4°C with 10 ml of extraction buffer (EB; 50 mM Bicine-NaOH pH 9.0, 1 mM EDTA, 20 mM NaCl, 1% Triton X-100) containing a protease and phosphatase inhibitor cocktail (P 9599, Sigma, St. Louis, MO). The homogenate was centrifuged for 30 min at 12,000 r.p.m. (Beckman J2-21; Beckman Instruments, Fullerton, CA) at 4°C and the supernatant filtered through a 0.45 μm filter to remove particulate material. The resulting homogenate was applied to the cation exchange column HiTrap SP (0.7 × 2.5 cm; Pharmacia Biotech) equilibrated with EB. After washing with six column volumes of EB containing protease inhibitors, bound proteins and peptides were eluted with a stepwise gradient of NaCl (0.2 M, 0.3 M, and 0.5 M) in EB with protease inhibitors. The fractions were then analyzed on Tricine-SDS polyacrylamide gels[42] and used for in vitro antibacterial assays.

Antimicrobial assays. Antifungal assays with plant *F. solani* and *P. cactorum* were performed in Magenta jars with two-week-old rooted plants growing in MS medium. Two slices (1 cm^2 each) of V8 agar (10% Campbell's V8 juice, 15 g/L agar) containing the fungus (*Fusarium* or *Phytophthora*) were applied to both sides of the tested plant, and incubated at room temperature for several weeks to assess plant survival.

Tubers harvested from control and MsrA1-expressing Desiree plants were tested for tuber tissue resistance to *E. carotovora*. For qualitative tests, 20 μl of a 10^{-2} diluted fresh bacterial culture (~2×10^7 colony-forming units) were pipetted onto the surface of the potato disks (1 × 2 cm) prepared from tubers using a sterile cork borer. Tuber disks were then incubated in Petri dishes at room temperature for six days. For more quantitative tests, a small well was made into tuber disks (2 × 3 cm). A 20 μl portion of a 10^{-2} diluted fresh bacterial culture was pipetted into the well and disks incubated at room temperature for six days. Soft rot tissue was gently removed from the tuber disks and the loss of weight of the tissue determined. Bactericidal effects of fractions from ion exchange chromatography were determined against *E. carotovora* in microtiter plates in a final volume of 220 μl containing ~1×10^5 bacteria/ml and 15 μl of each fraction. The cell cultures were incubated at room temperature for 4 h, diluted, and spread on Luria–Bertani plates. After overnight incubation at 28°C, colonies were counted and the bactericidal activity was scored (a sample containing 15 μl of extraction buffer containing protease inhibitors was used as a control).

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