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ORIGINAL PAPER

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Genetic modification of potato against microbial diseases: in vitro and in planta activity of a dermaseptin B1 derivative, MsrA2

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Abstract Dermaseptin B1 is a potent cationic antimicrobial peptide found in skin secretions of the arboreal frog *Phyllomedusa bicolor*. A synthetic derivative of dermaseptin B1, MsrA2 (*N*-Met-dermaseptin B1), elicited strong antimicrobial activities against various phytopathogenic fungi and bacteria in vitro. To assess its potential for plant protection, MsrA2 was expressed at low levels (1–5 μg/g of fresh tissue) in the transgenic potato (*Solanum tuberosum* L.) cv. Desiree. Stringent challenges of these transgenic potato plants with a variety of highly virulent fungal phytopathogens—*Alternaria*, *Cercospora*, *Fusarium*, *Phytophthora*, *Pythium*, *Rhizoctonia* and *Verticillium* species—and with the bacterial pathogen *Erwinia carotovora* demonstrated that the plants had an unusually broad-spectrum and powerful resistance to infection. MsrA2 profoundly protected both plants and tubers from diseases such as late blight, dry rot and pink rot and markedly extended the storage life of tubers. Due to these properties in planta, MsrA2 is proposed as an ideal antimicrobial peptide candidate to significantly increase resistance to phytopathogens and improve quality in a variety of crops worldwide with the potential to obviate fungicides and facilitate storage under difficult conditions.

Introduction

Cationic antimicrobial peptides (CAPs) are major constituents of innate cell-free defense mechanisms widely distributed in nature. Such peptides provide their hosts with a rapid non-specific defense against invading microorganisms. In the last 20 years, several hundred CAPs have been characterized from wide variety of organisms (Zasloff 2002). One of their major antimicrobial activities involves the disruption of bacterial membranes (Hancock and Scott 2000), resulting in the rapid killing of bacterial pathogens, including anaerobes (Oh et al. 2000) and Mycoplasmas (Nir-Paz et al. 2002). Furthermore, a few versions have the ability to inhibit other disease-causing microbes—fungi, protozoa and even plant and animal viruses—at concentrations not toxic to eukaryotic cells (Marcos et al. 1995; Baghian et al. 1997; Wachinger et al. 1998). Some CAPs reportedly even exhibit antitumorogenic activities (Chernysh et al. 2002). The potential of CAPs to combat microbial diseases provides a wealth of potentially new anti-infective modalities in an era when antibiotic resistance has emerged as a major medical issue (Guillemot 1999).

A considerable variety of CAPs have been isolated from the skin of various frogs that are known to live in environments infested with disease-causing microorganisms. Different frog species usually possess different sets of peptides adapted to specific climates, vegetation and habitats. These peptide sets probably evolved from an ancestral precursor with a hypermutable antimicrobial domain (Vanhoye et al. 2003). Dermaseptins (S and B) are a family of 28–32 amino acid CAPs expressed in the skin of the arboreal frogs *Phyllomedusa sauvagii* (Mor et al. 1991; Mor and Nicolas 1994a) and *P. bicolor* (Mor et al. 1994a; Fleury et al. 1998). Dermaseptins usually contain three to six lysine residues and, characteristically, a tryptophan residue at position 3. Different structural features likely modulate their activities against specific target organisms. The dermaseptins also show a dramatic synergy, resulting in as much as a 100-fold increase in antimicrobial activity of peptide combinations over single peptides (Mor et al. 1994b). The molecular elements residing in the N-terminal α-helical amphipathic segment (spanning residues 1–18) are principally responsible for the exceptional antimicrobial potency of dermaseptins (Mor and Nicolas 1994b). A

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particular advantage of dermaseptins is their broad-spectrum activities against both Gram-negative and Gram-positive bacteria (Nano-Venezia et al. 2002; Yaron et al. 2003), yeast (Coote et al. 1998), fungi (De Lucca et al. 1998), protozoa (Hernandez et al. 1992) and even herpes simplex virus type 1 (Belaid et al. 2002). Dermaseptins have also been shown to inhibit relatively inaccessible, unusually recalcitrant parasitic pathogens (Krugliak et al. 2000; Dagan et al. 2002). In contradistinction to many antibacterial peptides of animal and insect origin, dermaseptins do not lyse erythrocytes or other mammalian cells (Mor and Nicolas 1994b; Strahilevitz et al. 1994), thereby enhancing their selectivity, tolerance and hence their therapeutic potential.

One of the most potent dermaseptins is dermaseptin b, a 27-amino acid peptide that exhibits broad-spectrum antimicrobial activity although its potency is inferior, particularly against yeasts and fungi. However, when secreted as a 31-residue precursor with an N-terminus tetrapeptide extension (AMWK) (referred to as dermaseptin B1), the antimicrobial activity is far more potent than that of dermaseptin b (Strahilevitz et al. 1994).

Although conventional breeding has been relatively successful in developing disease-resistant plant cultivars, it has become an increasingly difficult task because of the limitations of resistance genes within usable gene pools. As a strategy to combat plant pathogens, transgenic approaches have become widely accepted for crop improvement (Melchers and Stuiver 2000; Rommens and Kishore 2000; Punja 2001; van der Biezen 2001; Campbell et al. 2002). Due to their wide spectrum of antimicrobial activity at low concentrations that are relatively non-toxic to eukaryotic cells, the CAPs—in particular, dermaseptins—represent promising candidates for transgenic application in plants. CAPs of plant (Epple et al. 1997; Gao et al. 2000; Fagoaga et al. 2001; Kanzaki et al. 2002; Ok et al. 2003), insect (Mitsuhara et al. 2000; Osusky et al. 2000; Sharma et al. 2000; Banzet et al. 2002) and amphibian (De Gray et al. 2001; Chakrabarti et al. 2003; Ponti et al. 2003; Osusky et al. 2004) origin have been expressed in transgenic plants, but only few of them have provided the target plants with any degree of broad-spectrum antimicrobial resistance.

Despite their unique broad-spectrum antimicrobial and, in particular, antifungal properties, dermaseptins have not yet been used for transgenic application. The investigation reported here was carried out to evaluate the suitability of the CAP, MsrA2 (dermaseptin B1 with an additional L-methionine at its N-terminus), as an inhibitor of plant microbial pathogens, particularly fungi. The in vitro results showed that MsrA2 was indeed able to inhibit the growth of an exceptionally broad range of phytopathogenic fungi, including *Alternaria*, *Cercospora*, *Fusarium*, *Phytophthora*, *Pythium*, *Rhizoctonia* and *Verticillium* sp. The gene for MsrA2 was then constructed, cloned and expressed in transgenic potato plants. The antibodies raised against the synthetic peptide detected MsrA2 in leaf extracts of transgenic lines at levels varying from 1 μg/g to 5 μg/g of fresh tissue. At this level, the constitutive expression of MsrA2 consistently and dramatically increased the resistance of transgenic potato plants and harvested tubers against bacterial and, more notably, against a variety of fungal pathogens without apparent consequences to the host.

Materials and methods

Peptide synthesis

MsrA2 was synthesized by the University of Victoria Genome BC Proteomics Center using a Model 430A Applied Biosystems peptide synthesizer (Foster City, Calif.) with the 0.25-mmol scale FASTMOC chemistry software. The reagents were from Applied Biosystems and Burdick and Jackson (Honeywell; Muskegon, Mich.). Amino acids and Fmoc-Rink amide resins were supplied by Novabiochem (La Jolla, Calif.).

Construction and cloning of the *msrA2* gene

For the construction of the *msrA2* gene, four oligonucleotides were prepared using Applied Biosystems 391 DNA Synthesizer. The sequences of Oligo no. 1 (5′-ATGGCCATGTGGAAAGACGTTCTGAAAAAGA-TCGGTACTGTCGCCCTCCATGCAGGG-3′) and Oligo no. 2 (5′- TTACTGCGAGATGGTGTCGGCT-ACTGCTCCAAGCGCGGCCTTCCCTGCATGGAG-GGCGACAGT- 3′) were derived from the amino acid sequence of dermaseptin B1 (with an additional methionine at its N-terminus); oligo no. 3 (5′-TCTAG-AGGTACCATGGCCATGTGGAAAGACG-3′) and Oligo no. 4 (5′-CAAGCTTCTGCAGAGCTCTTACT-GCGAGATGGTGTCGG-3′) were designed with restriction sites suitable for subsequent cloning. The *msrA2* gene was prepared using PCR with oligonucleotides 1 and 2 at a final concentration of 20 n *M* and oligonucleotides 3 and 4 at a final concentration of 400 n *M* and *Taq* DNA polymerase from Amersham Pharmacia Biotech (Baie d'Urfe, Quebec, Canada). The PCR product was purified using the NucleoSpin Extraction kit from Clontech (Palo Alto, Calif.), cut with *Xba*I and *Sac*I restriction endonucleases and cloned into the *Xba*I + *Sac*I-digested vector pBI221 (Clontech). Following sequencing, the *msrA2* gene, together with the nopaline synthase terminator (Nos-T), was cut using restriction endonucleases *Nco*I and *Eco*RI. The resulting fragment was cloned into the *Nco*I + *Eco*RI-digested pBI524 (Datla et al. 1993) creating the plasmid pDSB5242. The *Hin*dIII-*Eco*RI fragment was cloned into the *Hin*dIII + *Eco*RI-digested pBI121 (Clontech). The resulting expression vector was specified as pDDSB1212. DNA manipulations were carried out as described by Sambrook et al. (1989). The enzymes were purchased from Amersham Pharmacia Biotech or New

England Biolabs (Beverly, Mass.) 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). The transformation of potato plants was as described by De Block (1988).

msrA2 integration, transcription and expression in transformed plants

Genomic DNA was isolated from 100 mg of fresh leaves using the GenElute Plant Genomic DNA Miniprep kit (Sigma-Aldrich, Oakville, ON, Canada) according to the manufacturer's instructions. The integration of *msrA2* into genomic DNA was confirmed by Southern blot analysis. Three microgram-aliquots 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 Corp, Ann Arbor, Mich.) following the manufacturer's instructions for alkaline transfer. Membrane-bound DNA was hybridized [overnight (O/N) at 65°C] using a *Hin*dIII-*Sac*I fragment from plasmid pDDSB1212 (containing 2× 35S promoter, AMV and the *msrA2* gene) labeled with α-[^{32} P]dCTP by the Random Primer DNA Labeling System (Gibco, Burlington, ON, Canada) as a probe. The membrane was washed twice with 2× SSC, 0.1% sodium dodecyl sulfate (SDS) at 65°C for 5 min each, once with 1× SSC, 0.1% SDS at 65°C for 10 min, then exposed to a Phosphor Screen and the image scanned using PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). MsrA2 mRNA expression was determined by Northern blot analysis. Total RNA was isolated from 100 mg of leaf tissue using Trizol Reagent (GibcoBRL, Rockville, Md.) according to the manufacturer's instructions. Total RNA (40 μg) from control and transgenic potato plants was electrophoresed and transferred to a Biodyne B nylon membrane following the manufacturer's instructions for RNA transfer. The [^{32}P]-labeled (see above) *Xba*I-*Eco*RI fragment from pDDSB1212 (containing the *msrA2* gene and Nos-T) was used as a probe. After O/N hybridization at 65°C, the membrane was washed once with 2× SSC, 0.5% SDS at room temperature (RT) for 5 min, twice with 2× SSC, 0.1% SDS at RT for 10 min each, once with 1× SSC, 0.1% SDS at 60°C for 5 min, then exposed and scanned as described above.

Protein extracts for Western blotting were prepared from 200 mg of leaf tissue of control and transgenic potato plants. The plant material was ground in liquid nitrogen and homogenized in 0.4 ml of 0.5 *M* HCl. After a 10-min centrifugation (13,000 rpm, 4°C), the proteins from the supernatant were precipitated with 1.2 ml of acetone and incubated at −20°C for 20 h. The precipitated proteins were sedimented and the pellet was dried using a speed-vac (20 min) and dissolved in 8 *M* urea, 5% acetic acid (30 min at 65°C). The proteins were then fractionated using acid urea polyacrylamide gel electrophoresis (Jutglar et al. 1991) and electroblotted onto an Immobilon P^{SQ} membrane (Millipore, Bedford, Mass.). Immunodetection was performed according to the manufacturer's instruction using anti-MsrA2 polyclonal antibody raised in rabbit (dilution 1:2,000), anti-rabbit IgG with conjugated horseradish peroxidase (StressGen Biotechnologies, Victoria, BC, Canada; dilution 1:80,000) and SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, Ill.).

Antibacterial and antifungal assays

Bacterial strains *Escherichia coli* DH5α and *Erwinia carotovora* were grown on Luria-Bertani (LB) plates or in liquid medium. Bactericidal activities of dermaseptin B1 were determined in microtiter plates in a final volume of 100 μl containing approximately 5×10^6 bacteria/ml and desired amounts of peptides (prepared in sterile distilled H_2O). The cell cultures were incubated at RT for 4 h, diluted and spread on LB plates. After O/N incubation at 37°C (*Escherichia coli*) or 28°C (*Erwinia carotovora*), colonies were counted and the bactericidal activity scored. Antimicrobial activities of protein extracts were determined as follows: approximately 200 mg of fresh tissue from potato plantlets growing on MS medium was ground in liquid N_2, centrifuged for 15 min at 4°C in a microcentrifuge, and the supernatant transferred to a new Eppendorf tube. Then, 5 μl (approx. 1×10^4) of *E. carotovora* cells from the O/N culture was mixed with 95 μl of total protein extract. After a 2-h incubation at RT, 900 μl of LB was added and the samples incubated with shaking O/N at 28°C; absorbance at A_{550} was then scored. Soft rot resistance of potato tubers was determined as described by Osusky et al. (2000).

The cultures of *Fusarium solani*, *F. oxysporum*, *Alternaria alternata*, *Cercospora beticola*, *Botrytis cinerea*, *Rhizoctonia solani*, *Phytophthora cactorum*, *P. erythroseptica*, *P. infestans*, *Pythium irregulare* and *P. paroecandrum* were grown and maintained on potato dextrose agar (PDA; Difco, Detroit, Mich.). To determine the ability of peptides to inhibit the growth of fungi, we first placed a small agar plug containing the fungus at the center of each petri dish filled with PDA. When the circle of the fungus reached a diameter of 35–40 mm, sterile filter paper discs were placed on the medium, almost touching the fungus. Specific amounts of peptide were pipetted onto the paper discs in 15-μl volumes (sterile water was used as a control), and the plates were incubated for 18–48 h at RT, at which time the pictures were taken. Liquid assays to access antifungal activity of MsrA2 were performed in 96-well microtiter plates in a total volume of 200 μl of potato dextrose broth (PDB; Difco) containing 7×10^4 conidia/ml of *F. solani* (pre-germinated for 5 h at RT) and var-

ious concentrations of MsrA2. After an O/N incubation at RT, absorbance at 550 nm was scored and the samples photographed. Antifungal assays on potato leaves from mature plants were performed with plant pathogens *Phytophthora infestans* US8 A2, *R. solani*, *Pythium paroecandrum* and *P. splendens*. *P. infestans* US8 A2 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. The sporangia suspension was prepared by flooding the petri dish with 10 ml sterile water, then incubating with shaking at RT for 60 min. After subsequent incubation at 4°C (2 h in the dark), sporangia were collected using sterile Pasteur pipettes and the concentration adjusted to 1.5×10^4 sporangia/ml using sterile water. Three drops (20 µl each) of sporangia suspension were spotted on the leaf, and the leaves were incubated at RT under conditions of high humidity for 5–7 days and photographed. In the case of *R. solani*, *Pythium paroecandrum* and *P. splendens*, the leaves were inoculated with agar plugs containing the growing fungal culture, incubated for 5–7 days under conditions of high humidity at RT and photographed.

The resistance of potato tubers to *F. solani* was determined as follows: tuber slices for inoculation were prepared from healthy tubers stored for 3 months at 4°C. Before the experiment, the tubers were washed with sterile H_2O, blotted dry, cut into slices (approx. 5 mm thick), placed on wet sterile filter paper in petri dishes and then inoculated with 100 µl of *F. solani* conidia suspension (1.5×10^6 conidia/ml). After a 7-day incubation at RT in the dark, the tuber slices were photographed.

The pink rot tuber assay was performed using *P. erythroseptica* 367 as described previously (Osusky et al. 2004).

Results

Activity of dermaseptin B1 against plant pathogens in vitro

To evaluate the potential antimicrobial activity of MsrA2 (MAMWKDVLKKIGTVALHAGKAALGAVADTISQ) against various phytopathogens, the synthetic peptide was tested against both bacteria and fungi. In the initial studies, the laboratory strain *E. coli* DH5α was used as a model organism. As can be seen in Fig. 1a, the MIC (minimal inhibitory concentration—the concentration at which the peptide totally inhibits growth) for MsrA2 was 75 µg/ml. However, the bactericidal activity of MsrA2 was higher against the important plant bacterial pathogen *Erwinia carotovora*, killing all bacteria at 50 µg/ml (Fig. 1b). The activity of MsrA2 against *E. carotovora* was similar to that of a different peptide class, MsrA3 (100% effective at 40 µg/ml; Osusky et al. 2004), and approximately fourfold lower than that of yet another class, MsrA1 (100% effective at 12.5 µg/ml; Osusky et al. 2000).

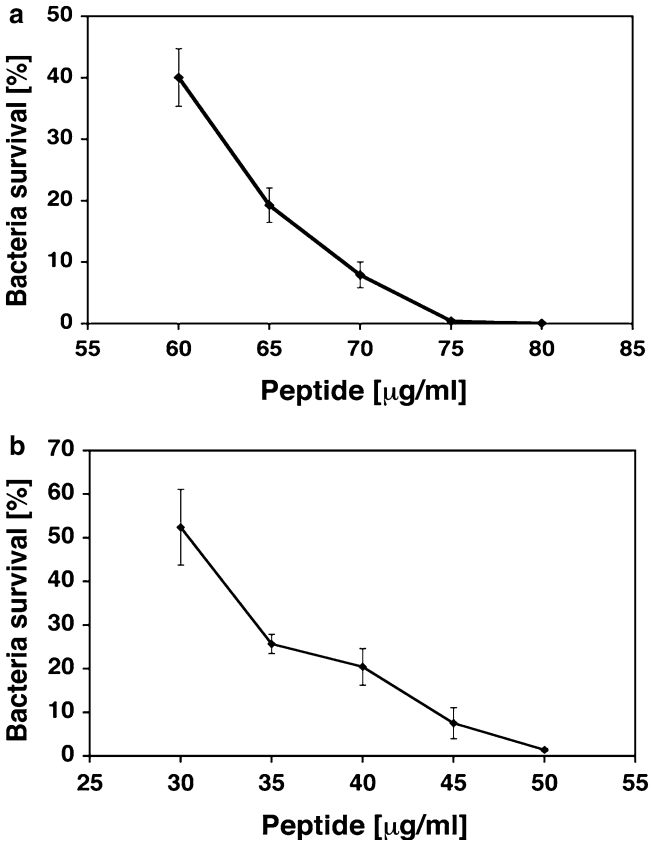

Fig. 1 Bactericidal activities of synthetic MsrA2. MsrA2 (at the indicated concentrations) was incubated with *Escherichia coli* D-H5α (**a**) or *Erwinia carotovora* (**b**) as described in the Materials and methods and the survival of bacteria scored. Results represent the average and standard deviation of three independent experiments

As fungi are responsible for the most devastating of plant diseases, MsrA2 was selected as a promising candidate for its potential in plant protection. Consequently, its antifungal activities against *Fusarium solani*, *F. oxysporum*, *Alternaria alternata*, *Cercospora beticola*, *Botrytis cinerea*, *Rhizoctonia solani*, *Phytophthora cactorum*, *P. erythroseptica*, *Pythium irregulare* and *P. paroecandrum* were used to test its ability to inhibit fungal growth. In these assays, a small agar plug containing the fungus to be tested was placed in the center of a petri dish containing solid growth medium. The mycelium grew outward as a ring until it contacted the peripherally arrayed assay discs containing MsrA2. The retardation in growth was then observed around the discs containing various amounts of the peptide sufficient to inhibit fungal growth. In most cases, 5 µg of MsrA2 was enough to significantly inhibit the growth of fungal mycelia (Figs. 2a–h), with 2.5 µg of the peptide clearly inhibiting the growth of *P. irregulare* and *P. paroecandrum* (Fig. 2i). When the conidia of *F. solani* were incubated in the presence of different concentrations of MsrA2 in liquid culture, the peptide was able to completely inhibit the growth of the fungus at 10 µg/ml (Fig. 3). At this concentration conidia were totally destroyed and only fragments were visible under the light

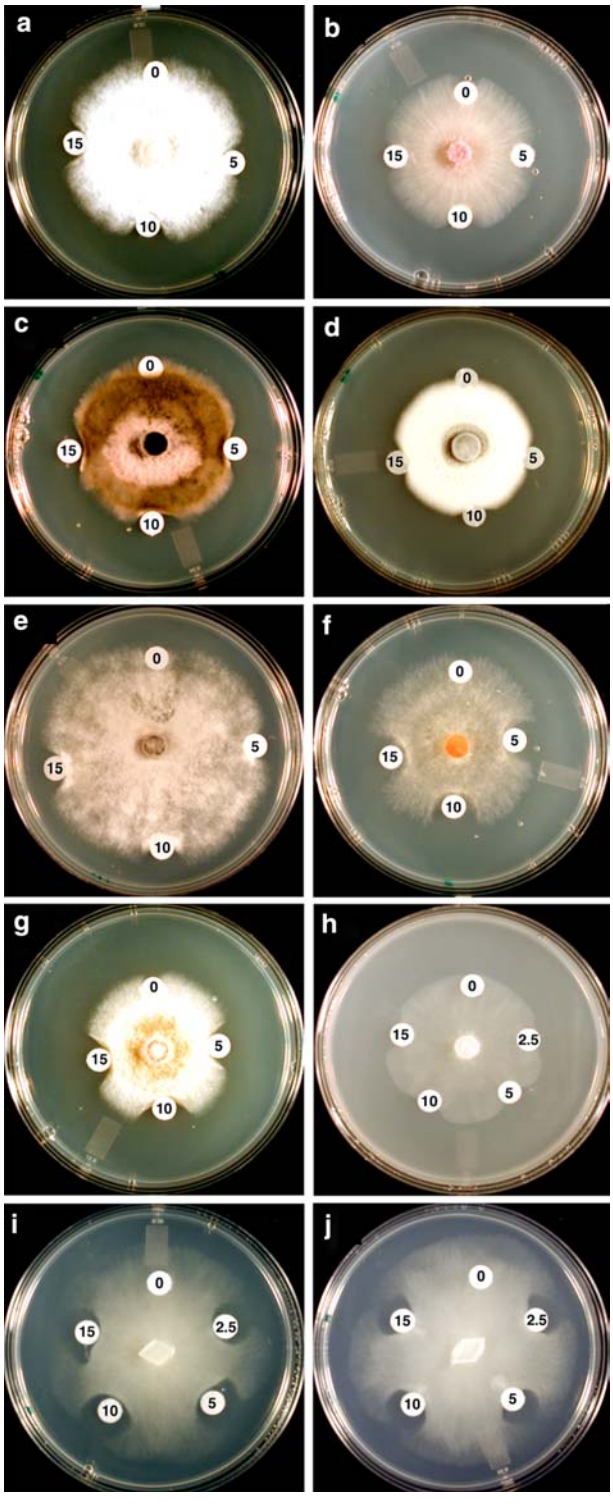

Fig. 2 Antifungal activities of MsrA2 in vitro. *Fusarium solani* (**a**), *F. oxysporum* (**b**), *Alternaria alternata* (**c**), *Cercospora beticola* (**d**), *Botrytis cinerea* (**e**), *Rhizoctonia solani* (**f**), *Phytophthora cactorum* (**g**), *P. erythroseptica* (**h**), *Pythium irregulare* (**i**) and *P. paroecandrum* (**j**) were incubated with the indicated amounts (in micrograms) of synthetic MsrA2 applied in a total volume of 15 µl on sterile paper discs. Pictures were taken after 48 h at RT (pictures **i** and **j** were taken 18 h following application of the peptide)

microscope (Fig. 3, inset b). In the control (without MsrA2), *F. solani* grew normally and the growing hyphae were clearly visible (Fig. 3, inset a).

Production of transgenic potato plants containing the *msrA2* gene

To achieve a high level of constitutional expression of MsrA2 in transgenic plants, the expression was driven by the duplicated enhancer cauliflower mosaic virus (CaMV) 35S (2× 35S) promoter with an untranslated leader sequence from alfalfa mosaic virus (AMV) RNA4 that acts as a *cis*-active translational activator (Datla et al. 1993). The gene for MsrA2 was assembled using the PCR, and the expression vector pDDSB1212 was constructed as described in the Materials and methods.

The potato cultivar Desiree was transformed with pDDSB1212 by means of *A. tumefaciens*-mediated transformation, and the transformants were selected for their resistance to kanamycin (50 mg/l). For further characterization, 20 plants originating from independent transformation events were selected for PCR analysis. The presence of the *msrA2* gene in the potato genome was confirmed by PCR amplification of the *msrA2* sequence from genomic DNA isolated from transgenic plants (data not shown). The integration of *msrA2* into the genome of selected transgenic plants was confirmed by Southern analysis (Fig. 4a). A single copy of *msrA2* was detected in transgenic plants D1-5 and D1-9, while D1-1 was found to contain two copies of the transgene in genomic DNA. As expected, *msrA2* was not detected in the non-transformed control Desiree plant.

The expression of *msrA2* was further examined at the RNA level using Northern analysis (Fig. 4b). The expression of mRNA was confirmed in all of the trans-

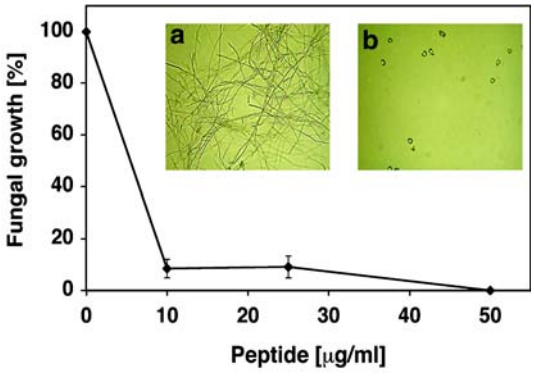

Fig. 3 Fungicidal activity of MsrA2. A conidial suspension of *F. solani* was incubated with the indicated concentrations of MsrA2 as described in the Materials and methods and fungal growth scored. The results represent the average and standard deviation of three independent experiments. *Inset*: Samples containing no MsrA2 (**a**) and 10 µg/ml MsrA2 (**b**) were viewed under light microscope and photographed

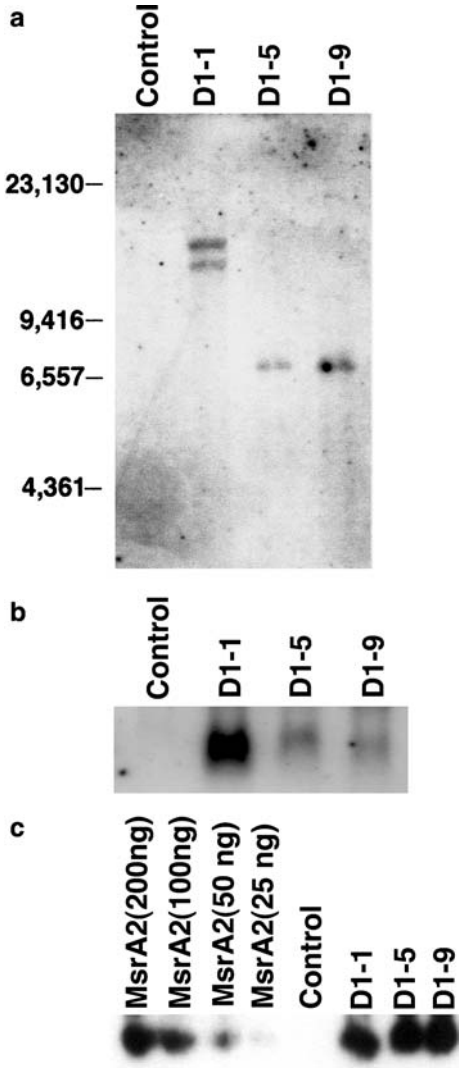

Fig. 4 *msrA2* gene integration and expression. **a** Southern blot analysis of selected potato transgenic plants. Genomic DNA isolated from nontransformed (*Control*) and transgenic potato plants (*D1-1*, *D1-5*, *D1-9*) was digested with the restriction endonuclease *Eco*RI and analyzed as described in the Materials and methods. The numbers on the left indicate the positions of the molecular-weight marker (in basepairs). **b** Northern blot analysis of selected potato transgenic plants. Total RNA isolated from nontransformed (*Control*) and transgenic potato plants (*D1-1*, *D1-5*, *D1-9*) was electrophoresed and analyzed as described in the Materials and methods. **c** Western blot analysis of MsrA2 in transgenic potato plants. Proteins isolated from non-transformed Desiree (*Control*) and transgenic potato plants (*D1-1*, *D1-5*, *D1-9*) were resolved using acid-urea-polacrylamide gel electrophoresis (AU-PAGE), transferred to an Immobilon P^{SQ} membrane and analyzed as described in the Materials and methods. Synthetic MsrA2 (200, 100, 50 and 25 ng) was used to estimate the level of expression

genic lines tested, with the highest expression in transgenic plant D1-1 that corresponded to two copies of the transgene. mRNA expression in plants D1-5 and D1-9 containing a single copy of *msrA2* gene was significantly lower. No signal was detected in the non-transformed control Desiree plant.

To determine the approximate level of the MsrA2 peptide in transgenic plants, we performed Western analysis using the polyclonal antibody raised against synthetic MsrA2 peptide (Fig. 4c). The MsrA2 peptide was detected in all three transgenic plants tested, while no MsrA2 peptide was detected in the control non-transformed Desiree plant. To estimate the level of expression of the MsrA2 peptide, we analyzed known amounts of synthetic MsrA2 alongside the plant extracts (25–200 ng). The level of expression of the MsrA2 peptide was estimated to be approximately 1–5 μg/g of fresh leaf tissue. No significant differences were observed between transgenic lines with one (D1-5, D1-9) or two copies (D1-1) of the *msrA2* gene.

Selected transgenic potato plants were grown in growth chambers and the tubers harvested. The morphological characteristics of the green parts and the morphology and yield of tubers of transgenic Desiree plants were not affected by the transgene expression and were comparable to that of control, non-transformed plants.

Antimicrobial activities in planta

The quality of potato tubers in storage and in transit can be significantly impacted by bacterial soft rot—the disease caused by the Gram-negative bacterium *Erwinia carotovora* ssp. *carotovora*. To select those plants with potential resistance to this disease while the tubers are still growing in soil, we devised a simple assay with leaf plant extracts. Fresh plant extracts isolated from control and transgenic plants were mixed with *E. carotovora*, and the growth inhibition was measured directly (Fig. 5a). The extract from the non-transgenic plant (Desiree) was used as a control. The extracts from transgenic plants inhibited the growth of *E. carotovora* by 55% (D1-9) to 70% (D1-1). When the extract from the transgenic control plant (D-GUS; Desiree expressing β-glucuronidase) was used, no growth inhibition was observed.

Tubers harvested from transgenic plants D1-1, D1-5 and D1-9 and non-transgenic control plants were used in the previously devised tuber soft rot resistance test (Osusky et al. 2000). Three tuber discs derived from these as well as from control (Desiree) and transgenic control (D-GUS) plants were infected with approximately 2×10^7 colony forming units (cfu) of *E. carotovora*. After 3 days of incubation at RT, approximately 50% of the fresh weight was lost from tuber discs originated from control and transgenic control plants as a result of soft rot. The tubers from transgenic plants expressing MsrA2 exhibited significantly less rot than the controls, losing merely 7% (D1-9) to 25% (D1-1) of their fresh weight (Fig. 5b).

Harvested tubers were then stored at 4°C and periodically visually examined. After 26 months of storage, most of the tubers from non-transgenic Desiree plants were fully decomposed (Fig. 5b, inset a), as were the

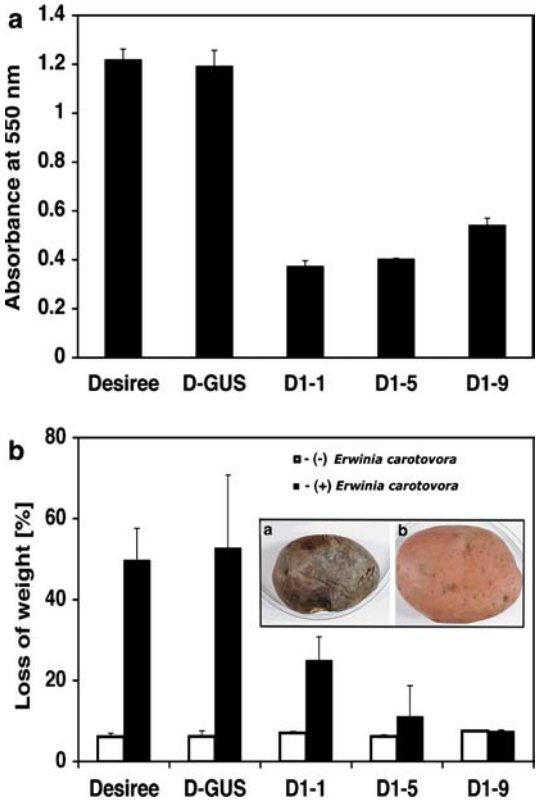

Fig. 5 Resistance of potatoes expressing MsrA2 to *Erwina carotovora*. **a** Protein extracts prepared from a nontransgenic control (*Desiree*), transgenic control (*D-GUS*) and transgenic plants expressing MsrA2 (*D1-1*, *D1-5* and *D1-9*) were incubated with *E. carotovora* and their inhibitory effects on the bacterial growth measured. Results represent the average and standard deviation of three independent experiments. **b** Soft rot resistance of potato tubers expressing MsrA2. Discs from nontransgenic control (*Desiree*), transgenic control (*D-GUS*) and MsrA2-expressing transgenic (*D1-1*, *D1-5* and *D1-9*) potato tubers were infected with *E. carotovora*. The sensitivity/resistance to *E. carotovora* was expressed as the loss of weight of tuber tissue. Results represent the average and standard deviation of three independent experiments. *Inset*: Tubers of nontransgenic Desiree (**a**) and transgenic D1-9 potato (**b**) were stored at 4°C for 26 months and photographed. Control tubers had totally blackened and decomposed, while tubers from the MsrA2-expressing plant remained healthy

tubers from transgenic control D-GUS. However, transgenic tubers were without visible signs of natural deterioration (Fig. 5b, inset b). When these latter tubers were planted in soil, they produced plants and tubers with normal morphology (not shown).

Fungal disease resistance of transgenic plants

To study the response of MsrA2-expressing potato plants to fungal infections, several experiments were performed. Detached leaves from 8- to 10-week-old plants (Desiree, D-GUS, D1-1, D1-5, D1-9) growing in growth chambers in soil were placed in petri dishes on wet filter paper and inoculated with the spore suspension or, simply, with agar plugs containing actively growing different strains of fungi. The best results were achieved with the plant D1-9 (Fig. 6).

When the leaves were wounded and inoculated with a *Phytophthora infestans* US8 A2 spore suspension (three spots per leaf, 300 spores per spot), the first dramatic changes were observed after 3 days of incubation at RT . Seven days after inoculation (DAI) the leaves from control (Fig. 6a) and transgenic control plants (Fig. 6c) had decomposed, while the leaf from the MsrA2-expressing plant D1-9 was still green with only small necrotic spots at the sites of inoculation (Fig. 6b).

A similar approach was used in assays with other fungal phytopathogens. Detached leaves from control and transgenic plants were inoculated with agar plugs containing growing cultures of *Rhizoctonia solani* (Fig. 6d–f), *Pythium paroecandrum* (Fig. 6g–i), or *Pythium splendens* (Fig. 6j–i). Five to seven DAI, the leaves from the non-transgenic Desiree control (Fig. 6d, g, j), and transgenic control D-GUS (Fig. 6f, i, l) were heavily infected. In contradistinction, the infected areas on the leaves originated from transgenic plants were much smaller and limited to the area around the site of contact with fungus-containing agar plugs (Fig. 6e, h, k).

Other fungi that considerably influence the quality of potato tubers in storage, are *Fusarium solani* and *Phytophthora erythroseptica*. *F. solani* is the common cause of dry rot of potatoes and is the most common fungus responsible for seed piece decay (Boyd 1972). To determine the resistance of potato tubers to this fungus, we infected slices of tubers from nontransgenic and

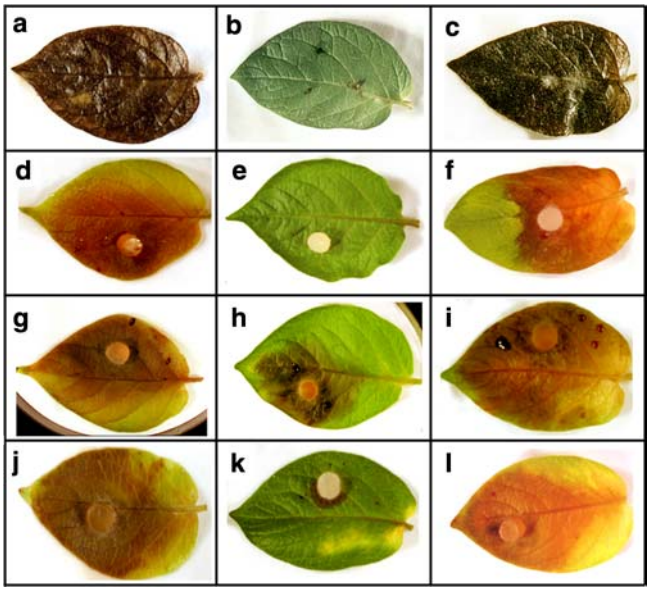

Fig. 6 Resistance of transgenic potatoes expressing MsrA2 to fungal infection. Detached leaves from mature nontransgenic Desiree control (**a**, **d**, **g**, **j**), transgenic D-GUS control (**c**, **f**, **i**, **l**) and MsrA2-expressing transgenic D1-9 (**b**, **e**, **h**, **k**) plants were infected with sporangia of *Phytophthora infestans* (**a**–**c**) or with an agar plug containing a growing culture of *Rhizoctonia solani* (**d**–**f**), *Pythium paroecandrum* (**g**–**i**) and *Pythium splendens* (**j**–**l**) and incubated at RT. Pictures were taken 7 days after infection (DAI)

transgenic plants with a conidial suspension (100 µl of 1.5×10^6 conidia/ml) of *F. solani*. At 7 DAI, tuber slices from nontransgenic Desiree and transgenic control D-GUS had clearly decomposed accompanied by heavy fungal growth, while the slice from transgenic MsrA2-expressing tuber D1-9 was without any trace of fungus (Fig. 7a). The disease caused by *P. erythroseptica*—pink rot—reflects the characteristic pink color of sectioned tubers when exposed to air for 30–60 min (Salas et al. 2000). To evaluate resistance to pink rot, we infected tubers harvested from control and transgenic plants with agar plugs from an actively growing *P. erythroseptica* culture and incubated them in the dark at RT and 100% humidity for 7 days. The tubers were then cut longitudinally through the inoculation point and exposed to air for 60 min. The characteristic pink color of *P. erythroseptica* infection developed on those tubers from both the nontransgenic Desiree and transgenic D-GUS control plants (Fig. 7b). However, the color of the tuber tissue from transgenic plant D1-9 expressing MsrA2 (Fig. 7b) had not changed, and the small decomposed area was restricted to the point of inoculation. After a semi-quantitative analysis of the cut area of several plant lines (Fig. 7c), we observed that virtually 100% of the affected cut area of tubers from control (Desiree) and transgenic control (D-GUS) plants were clearly pink. The effect of *P. erythroseptica* on tubers originated from transgenic plants expressing MsrA2 was greatly reduced and the area affected was between 6% (D1-9) and 9% (D1-5).

These results clearly demonstrate that the antifungal properties of MsrA2, when expressed at low levels in transgenic potato plants, effectively protect both plants and tubers from such devastating diseases as late blight, dry rot and pink rot.

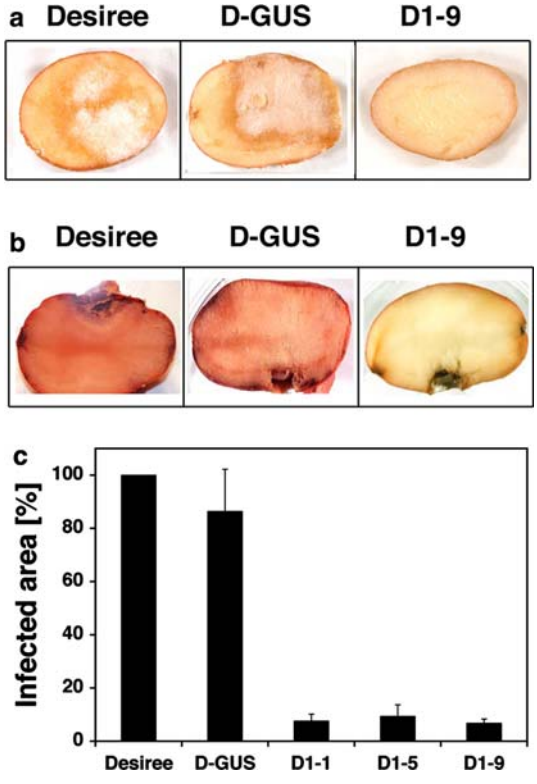

Fig. 7 Resistance of potato tubers to fungal diseases. **a** Tuber slices from nontransgenic control (Desiree), transgenic control (D-GUS) and transgenic MsrA2-expressing (D1-9) plants were infected with *Fusarium solani* and photographed after 7 days at RT in dark. **b** Tubers from nontransgenic control (Desiree), transgenic control (D-GUS) and transgenic MsrA2-expressing (D1-9) plants were infected with *Phytophthora erythroseptica*. After 7 days of incubation at RT and 100% humidity, the tubers were cut longitudinally through the inoculation point, exposed to the air for 60 min and photographed. **c** Tubers from nontransgenic control (*Desiree*), transgenic control (*D-GUS*) and transgenic MsrA2-expressing (*D1-9*) plants were infected with *P. erythroseptica* and treated as in (**b**). The infected areas of the cut tuber were then measured and used as an index of the percentage of the total area. Results represent the average and standard deviation of three independent experiments

Discussion

The production of food supplies needed to meet the nutritional requirements of a rapidly growing world population is the major and most important goal of modern-day agriculture. One prediction is that, by the year 2010, the total demand only for cereals in Asia will have increased by 35% (Fischer et al. 2000). The potato (*Solanum tuberosum* L.) is the fourth largest crop worldwide (after rice, wheat and maize) and is grown in more countries than any other crop except maize (Ortiz 2001).

The production and cultivation of the potato in general is commonly threatened by reoccurring bacterial and fungal infections. Nearly 20% of potato crops are currently lost as a result of disease (James et al. 1990). Over the last few decades, agrochemicals have been used to contain phytopathogens, however, the liberal application of fungicides and other pesticides for crop plant protection has contributed to chemical contamination of the environment as well as to the rise of fungicide-resistant phytopathogens (Staub 1991; Kadish and Cohen 1992; Shattock 2002) and has given rise to a powerful antichemical public sentiment.

Strategies to improve the health of crops through genetic engineering have included the transgenic expression of plant, fungal or bacterial hydrolytic enzymes, pathogenesis-related proteins, components of plant defense response pathways, antimicrobial proteins, peptides and many others (for a review, see Punja 2001). Within the framework of generating transgenic plants resistant to microbial infection(s), questions arise regarding the spectrum of activity of the product to be used in the transgenic plant as well as its cytotoxicity to plant or animal cells.

One of the most widely used defense systems employed against microbes involves membrane-active, positively charged antimicrobial peptides (Hancock and Scott 2000). Because the primary target of such CAPs is the cell membrane and not specific receptors or

substrates, these peptides usually confer their activity against a broader spectrum of pathogenic microorganisms. Consequently, there is a lesser probability of resistance arising by variation of its metabolic pathways.

In the search for the best candidate(s) for transgenic expression in plants, we concentrated on the peptides expressed in frog skin. The principal constituents of the skin secretion from the South American hylid frogs *Phyllobates sauvagii* and *P. bicolor* are the linear α-helical CAPs dermaseptin S and dermaseptin B. The most potent member of dermaseptin family is dermaseptin B1, which is about 20-fold more active than dermaseptin S against filamentous fungi (Strahilevitz et al. 1994). Its broad-spectrum antifungal properties, together with low cytotoxicity, make dermaseptin B1 an excellent candidate for expression in transgenic plants.

Although in vitro assays do not always reflect the actual activities and efficacies of CAPs in the host organism, they provide valuable data on their potential activities against pathogens. To analyze the suitability of the dermaseptin B1 derivative, MsrA2, to fight plant pathogens, we tested its ability to inhibit the growth of important bacterial and fungal phytopathogens. The bactericidal activity of MsrA2 against *Erwinia carotovora* was approximately the same as that of MsrA3 (Osusky et al. 2004) but was fourfold lower than that of MsrA1 (Osusky et al. 2000). However, MsrA2 showed a remarkable spectrum of activity against fungi. When MsrA2 was used to inhibit the growth of agronomically important fungal pathogens— *Fusarium, Alternaria*, *Rhizoctonia*, *Phytophthora* and *Pythium* sp.—its activity was far superior to those of other peptides previously tested (MsrA1, MsrA3, temporin A; data not shown). The growth of most of the fungi was inhibited by as little as 5 μg of MsrA2, and in some cases, 2.5 μg of MsrA2 was enough to inhibit fungal growth (*Pythium irregulare* and *P. paroecandrum*). When examined microscopically, the hyphae around those discs containing MsrA2 were visibly shortened and had ceased growing (data not shown). Although the disruption and permeabilization of target cell membranes is probably the most commonly explained mechanism of action ascribed to CAPs (Hwang and Vogel 1998), presumably they cause membrane depolarization at the growing tip of fungal hyphae and the loss of membrane electrochemical potential, thereby abolishing the transport of nutrients and perhaps other energy-driven functions. Nevertheless, our simple assay permitted the easy screening of large number of phytopathogens. However, we achieved a better picture of the peptide's antifungal activity using liquid assays in which the peptide can freely interact with spores or growing hyphae. Using *F. solani* as a model, we observed that MsrA2 at a concentration of 10 μg/ml completely inhibited growth and destroyed conidia, leaving only cell fragments visible by light microscopy. Thus, the in vitro antifungal activities of MsrA2 indicated the potential for improving disease resistance of plants via the transgenic approach.

The phytopathogenic bacterium *Erwinia carotovora* is the cause of blackleg in potato plants in the field and soft rot of tubers in storage. The protein extract prepared from the leaves of transgenic potato clearly inhibited the growth of *E. carotovora* in liquid culture. Assays with potato tubers harvested from transgenic plants expressing MsrA2 showed strong resistance against *Erwinia*-induced soft rot. However, when compared to transgenic potatoes expressing MsrA1 (Osusky et al. 2000), the protection against this bacterial pathogen was significant but less powerful. When tubers from control and transgenic plants were stored at 4°C and periodically examined, after 26 months those tubers from plants expressing MsrA2 were virtually without visible signs of natural deterioration, while their non-transgenic counterparts had wizened and blackened considerably. When the former were planted into soil, even after such a long storage, they readily produced normal plants and tubers. Thus, the expression of MsrA2 extended the storage life of tubers without damaging their main physiological functions.

A far greater danger for potato crops, and for many others, is imposed by fungal diseases. The presence of phytopathogenic fungi not only causes a decline in the quality and quantity of crops, they often cause acute cytotoxicity in animals and humans. Many genera of fungi produce highly toxic metabolites called mycotoxins that cannot be destroyed by normal processing and, consequently, infested produce has to be destroyed. Mycotoxins can only be avoided by preventing the growth of the fungus responsible. Fungal diseases represent almost one-third of the diseases common to potato (Rich 1991), with the most devastating caused by *Phytophthora* sp.—late blight and pink rot. As a means to control these diseases, fungicides have been increasingly relied upon, and their liberal application is thought to have contributed to chemical contamination of the environment and food supply.

One transgenic approach to increase the resistance against *P. infestans*, the greatest threat to potato growers worldwide, involved the expression of phytoalexins (Thomzik et al. 1997), pathogenesis-related proteins (Zhu et al. 1996), but protection proved to be limited and fairly narrow. Other disease-resistant mutants have been described in which the signal transduction "master switches" were used to engineer broad-spectrum resistance (Frye et al. 2001; Yang et al. 2001; Asai et al. 2002). The use of these master switches has unfortunately resulted in reduced plant vigor and yield or, occasionally, in increased susceptibility to other pathogens (Hoffmann et al. 1999). The estimated concentration of MsrA2 in leaf tissue was approximately 1–5 μg/g of fresh tissue which, although not high, seems to be sufficient to protect the plants from the attack of pathogen(s). Constitutive expression of MsrA2 at this level is apparently non-toxic to transgenic plants as no deleterious effects on the morphology or yield of plants and tubers could be seen.

Mounting evidence suggests that the innate immune system of plants shares many parallels with that of vertebrates and insects (Cohn et al. 2001; Dangl et al. 2001) and relies on similar downstream signaling components. The expression of some CAPs in animals enhanced the immune response (Hancock and Diamond 2000; Chernysh et al. 2002) so there is a strong probability that the same is possible in plants. The exact mechanisms or pathways for induced resistance remain unknown. However, to exclude the possibility that the increased resistance of our transgenic potatoes might by triggered simply by the transformation event, we included control plants transformed with the vector pBI 121 (expressing GUS) in all our assays. As the increased resistance was observed only with the samples prepared from plants expressing peptide MsrA2 our results strongly suggest that the resistance of transgenic potato plants is caused by the expression of the dermaseptin B1 derivative—the peptide MsrA2. Work is in progress to elucidate if any other components of the immune signaling pathways are activated in response to the expression of MsrA2 in transgenic plants.

In previous investigation (Osusky et al. 2004) we showed that a structurally modified temporin A analog, MsrA3, imbued transgenic potatoes with strong and broad-spectrum resistance traits to phytopathogens. However, plants expressing MsrA2 were resistant to a broader spectrum of fungal pathogens, including not only potato fungal pathogens but also the fungal strains causing the diseases in other crops as well. Furthermore, the transgene expression did not have any deleterious effects on plant morphology and productivity. Since MsrA2 showed in vitro antimicrobial activities against so many plant pathogens, its use to increase plant resistance against diverse microbial infections is not necessarily limited to potatoes but theoretically could be used in other crops (wheat, barley, corn, sugar and beet), trees and even flowers.

Although concerns have been often expressed regarding genetically modified plants, many cationic peptides are naturally produced by plants and animals, including humans, as part of their innate defenses against infection. Preliminary studies to show the safety of MsrA2 in crop plants have been positive and are continuing. The use of MsrA2 could feasibly improve the yields, storage life and quality of a variety of food and non-food plants and as such should be a boon, particularly in developing countries. The success of a transgenic approach in plant disease control will likely play an essential role in helping feed the world, reducing the environmental impact of intensive agriculture and improving the fragile quality of our health and environment.

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