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# Stacking of antimicrobial genes in potato transgenic plants confers increased resistance to bacterial and fungal pathogens

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ABSTRACT

*Solanum tuberosum* plants were transformed with three genetic constructions expressing the *Nicotiana tabacum* AP24 osmotine, *Phyllomedusa sauvagii* dermaseptin and *Gallus gallus* lysozyme, and with a double-transgene construction expressing the AP24 and lysozyme sequences. Re-transformation of dermaseptin-transformed plants with the AP24/lysozyme construction allowed selection of plants simultaneously expressing the three transgenes. Potato lines expressing individual transgenes or double- and triple-transgene combinations were assayed for resistance to *Erwinia carotovora* using whole-plant and tuber infection assays. Resistance levels for both infection tests compared consistently for most potato lines and allowed selection of highly resistant phenotypes. Higher resistance levels were found in lines carrying the dermaseptin and lysozyme sequences, indicating that theses proteins are the major contributors to antibacterial activity. Similar results were obtained in tuber infection tests conducted with *Streptomyces scabies*. Plant lines showing the higher resistance to bacterial infections were challenged with *Phytophthora infestans*, *Rhizoctonia solani* and *Fusarium solani*. Considerable levels of resistance to each of these pathogens were evidenced employing semi-quantitative tests based in detached-leaf inoculation, fungal growth inhibition and *in vitro* plant inoculation. On the basis of these results, we propose that stacking of these transgenes is a promising approach to achieve resistance to both bacterial and fungal pathogens.



## 1. Introduction

Potato is affected by many diseases producing substantial economic losses worldwide. Bacterial pathogens, like *Erwinia carotovora* and *Streptomyces scabies*, and fungal pathogens, like *Phytophthora infestans*, *Fusarium solani* and *Rhizoctonia solani*, are frequent in most production areas and require multiple control measures to mitigate their incidence (Stevenson et al., 2001; Termorshuizen, 2007). *Erwinia* is the causal agent of blackleg and soft-rot diseases, respectively involving severe symptoms in foliar and tuber tissues (Pérombelon, 2002). *Streptomyces*, though not seriously affecting yields, produces tuber scab lesions that significantly lessen potato marketability. Among fungal pathogens, *P. infestans* produces very destructive symptoms affecting leaves, stems and tubers (Erwin and Ribeiro, 1996), while *F. solani* and *R. solani* induce tuber and stolon diseases leading to sprout death, poor plant development, and reduced tuber number and size (Gerlach and Nirenberg, 1982; Sneh et al., 1991). While bacterial control is presently limited to prophylactic procedures, including use of certified seed tubers, minimizing of tuber bruising and disinfection previous to storage, fungal management relies mainly on cultural practices and extensive use of fungicides (Platt and Petters, 2006; Lebecka et al., 2006). Fungicides provide a considerable degree of protection, but their application introduces higher production costs, can induce fungal resistance, and involves increased environmental risks.

Genetic resistance to bacterial and fungal diseases has been difficult to implement due to the scarcity of natural R genes and the long periods involved in potato breeding. Strategies explored to generate resistance by means of genetic engineering include approaches affecting different steps of host-pathogen interactions, such as eliciting of plant defense responses, inhibition of bacterial or fungal virulence factors and expression of antimicrobial compounds of plant and non-plant origin (Shah, 1997; Mourgues et al., 1998; Melchers and Stuiver, 2000; Osusky et al., 2005). Though

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significant protection have been obtained in a number of cases, simultaneous resistance to a broad range of pathogens has been difficult to achieve. One possible way to attain this goal is pyramiding of transgenes encoding complementary antibacterial and antifungal activities (Campbell et al., 2002; Datta et al., 2002; Douglas and Halpin, 2009). Diverse transformation strategies have been employed for gene stacking, including conventional sexual crossing, re-transformation, co-transformation and transformation with linked transgenes (Qi et al., 2004; Halpin, 2005; Lee et al., 2007; Jha and Chattoo, 2009). Each of these approaches presents advantages and limitations that must be carefully considered according to the reproductive mode of the crop and the projected objectives.

Cationic antimicrobial peptides (CAPs) are widely present in nature and provide an early, non-specific defense barrier against bacteria, fungi and protozoa. Several hundred CAPs have been characterized in organisms including insects, crustaceans, mammals and plants (Zasloff, 2002). Models to explain their activity postulate an initial interaction between positive charges at CAPs' hydrophilic domains and negative charges at structural components of the bacterial membrane, followed by pore formation, physical and/or functional membrane disruption and subsequent cell lysis (Yeaman and Yount, 2003). CAPs are usually classified into those including α-helical peptides – such as dermaseptins, cecropins and magainins – and those including β-sheet peptides – such as defensins, protegrins and tachyplesins. Transformation of plant species with sequences encoding CAPs and their analogs conferred considerable levels of resistance to many phytopathogenic microorganisms (Arce et al., 1999; Chakrabarti et al., 2003; Alan et al., 2004). Dermaseptins are CAPs of 28–34 amino acids isolated from frogs of the *Phyllomedusa* genus exhibiting *in vitro* activity against bacteria, filamentous fungi protozoa and yeast. Potato plants transformed with the dermaseptin analog MsrA2 exhibit broad-range resistance to phytopathogenic fungi, including *Cercospora*, *Fusarium* and *Phytium* (Osusky et al., 2005).

Lysozymes hydrolyze the N-acetyl-D-muramic acid: N-acetyl-D-glucosamine linkage of peptidoglycans, resulting in partial degradation of bacterial cell walls and bacterial lysis (Höltje, 1996). Enzymes belonging to this family have been isolated from different sources and lysozyme-like activities are also present in the cellular vacuoles of several plant species. Constitutive accumulation of exogenous lysozyme in the apoplasmic space can be an effective barrier to arrest attack of both necrotrophic and biotrophic bacteria. Hen egg-white lysozyme accumulation in transgenic potato plants considerably inhibited growth of several bacterial species, including *E. carotovora* and *Pseudomonas syringae* (Kato et al., 1998). Similarly, expression of T4 and human lysozyme in tobacco and potato plants resulted in partial resistance to *E. carotovora* and *P. syringae* (Düring, 1993; Serrano et al., 2000), and expression of T4 lysozyme in apple cultivars provided significant resistance to infection by *E. amylovora* (Hanke et al., 1999). In addition, expression of human lysozyme in tobacco plants inhibited fungal growth, suggesting a possible utilization for the control other plant pathogens (Nakajima et al., 1997).

AP24 is a thaumatin-like pathogenesis-related protein belonging to the PR-5 family that was early characterized as an antifungal protein (Abad et al., 1996). AP24 induces cell lysis by a mechanism involving pore formation and dissipation of membrane potential (Selitrennikoff, 2001). Potato plants expressing a AP24 transgene showed increased resistance to *P. infestans* (Liu et al., 1994) and tomato plants transformed with tobacco AP24 and bean chitinase transgenes showed improved resistance to *Fusarium oxysporum* (Ouyang et al., 2005).

The three antimicrobial proteins included in this research work were selected on the basis of their low putative risk for human consumption. Hen egg-white lysozyme is one of the more extensively studied enzymes of this protein family and it is already present in many foodstuff components. Tobacco AP-24 is a defense response protein that has homologous counterparts in edible crops and shares significant sequence homology with thaumatin, a natural sweetener accepted as a safe flavoring agent in several countries (Singh et al., 1987). In addition, like other animal-derived antimicrobial peptides, dermaseptin derivatives have been proposed as potential food additives for the control of resistant microorganisms (Yaron et al., 2003).

In this paper, we report high levels of resistance to *E. carotovora* and *S. scabies* in several *Solanum tuberosum* lines transformed with different constructs expressing the dermaseptin, lysozyme and AP24 coding sequences. Lines exhibiting higher resistance to bacteria also showed increased resistance to infection with *P. infestans*, *R. solani* and *F. solani*, suggesting that the combined expression of these antimicrobial transgenes could be a suitable approach to obtain stable, broad-range protection against different kinds of potato phytopathogens.

## 2. Materials and methods

### 2.1. Genetic constructions

The dermaseptin coding sequence from *Phyllomedusa sauvagii* (GenBank AJ564794.1; http://www.ncbi.nlm.nih.gov/Genbank), including flanking sites for *Nco*I and *Xba*I, was synthesized using six partially overlapping oligonucleotides (Gao et al., 2003). The resulting DNA fragment was purified and cloned into plasmid pHES74, containing the *Cauliflower mosaic virus* (CaMV) 35S promoter, the *Tobacco mosaic virus* (TMV) Ω enhancer, the esporamine pre-peptide signal peptide and the nopaline synthase (Tnos) transcription termination sequence (López et al., 1996). The expression cassette was released from pHES74 by *Hind*III-digestion and subcloned into the pPZP200 binary vector carrying the *bar* gene as a selectable marker (Romano et al., 2003), thus originating plasmid pDe. The hen egg-white lysozyme sequence was obtained from plasmid pUC-lyso containing the lysozyme coding sequence fused to the barley α-amylase signal peptide and flanked by the double CaMV 35S promoter and the Tnos sequence (Serrano et al., 2000). The expression cassette was sub-cloned into another pZP200 version carrying the *hph* gene as selectable marker, originating plasmid pLy. Finally, a *Hind*III-fragment from plasmid pHAP17 (gently provided by Dr. Guido Jach, Max Planck Institute) including the long 35S promoter, the TMV Ω enhancer, the barley α-amylase signal peptide, the AP24 coding sequence and the Tnos sequence, was sub-cloned into the *Hind*III site of pLy to give plasmid pApLy. The same fragment was sub-cloned at the *HindIII* site of the pBIN19 binary vector carrying the *nptII* gene as selectable marker to generate plasmid pAp.

### 2.2. Plant material and potato transformation

Certified *S. tuberosum* (cv. Spunta) minitubers were used as starting material for plant propagation and transformation procedures. Transformation of minituber discs was carried out as described by Stiekema et al. (1988) using co-cultivation with *Agrobacterium tumefaciens* strain EHA101. According to the selector markers used in transformation, explants were transferred to regeneration medium supplemented with 10 mg/L hygromycin, 50 mg/L kanamycin and/or 1.5 mg/L glufosinate. Triple-transgenic plants were obtained by re-transformation of a dermaseptin-expressing line (De-8) with the pApLy construction. Plants developing in selective medium were multiplied in aseptic conditions for 2 months, transferred to soil and grown for 3–4 months in a growth chamber at 20 °C under a 16 h photoperiod. Minitubers of 2–4 cm in diameter were harvested and stored at 4 °C, 100%

relative humidity. Transgenic T0 plants used in infection assays were agamically multiplied and kept under selection for 3–4 consecutive years.

### 2.3. Bacterial and fungal strains

EcaT strain of *E. carotovora* spp. atroseptica was kindly provided by Dr. J. Kalazich (INIA-Remehue, Chile). *S. scabies* and *F. solani* were isolated from diseased potato tubers according standard procedures (Schaad et al., 2001) *R. solani* and *P. infestans* strains were obtained from Dr. C. Rovere (INTA-Castelar, Argentina).

### 2.4. Southern blot analysis

Genomic DNA from transgenic potato leaves was isolated using a CTAB-extraction protocol (Dellaporta et al., 1983). Five-μg aliquots were digested with restriction endonucleases *Xba*I, *Eco*RV and *Bam*HI, electrophoresed in 0.8% agarose gels and transferred to Nylon membranes. Fragments corresponding to the different transgenes were hybridized with specific cDNA probes labeled with $\alpha$-$^{32}$P dCTP and detected by autoradiography.

### 2.5. Preparation of leaf extracts and protein quantification

Five g of frozen leaf tissue were ground in liquid nitrogen and transferred to a tube containing chilled (4 °C) 10% TCA in acetone plus 0.07% β-mercaptoethanol. After vortex samples were precipitated at −20 °C for 45 min and then centrifuged at 14,000 *g* for 15 min at 4 °C. The supernatant was removed and the pellet was washed with pre-chilled 90% acetone containing 0.07% ß-mercaptoethanol. After a new centrifugation at 4 °C, 14,000 *g* for 15 min the pellet was washed 3 times and re-suspended in the acetone and spin at 14,000 *g* for 5 min at 4 °C. Lately the protein pellet was air dried in a laminar flow hood and stored at −20 °C. Determination of total soluble protein was done spectrophotometrically according to Bradford (1976) with BSA as the standard. AP24, dermaseptin and lysozyme concentration was extrapolated from standard curves in Western immunoblots. Samples from transgenic and control plants and standard dilutions were electrophoresed in SDS-polyacrylamide gels, transferred to nitrocellulose membranes and incubated with antibodies raised to BSA-conjugated dermaseptin (1:200), lysozyme (1:200) or AP24 (1:400) antibodies and a secondary antibody conjugated to horseradish peroxidase. Specific bands were detected by the Luminol A/$H_2O_2$ reaction. Chemiluminescence was detected on radiographic film and band intensities were quantified employing the ImageJ program and referred to total soluble protein. Antibodies to dermaseptin and lysozyme were obtained by rabbit immunization using BSA-conjugated dermaseptin (GL Biochem) and purified chicken lysozyme (Sigma) as antigens. Antibodies to AP24 were gently provided by Dr. Rovere (INTA, Castelar, Argentina).

### 2.6. Bacterial infection assays

*E. carotovora whole-plant infection assays:* Resistance was evaluated according to the procedure described by Arce et al. (1999). Each infection assay included 20 individuals clonally derived from a single transgenic event and an equivalent number of non-transformed controls. Inoculations were performed injecting 10 μL of bacterial suspension ($6 \times 10^7$ c.f.u.) in the axilar basal bud. Symptom development was monitored at 5, 10, 15 and 30 days post-infection (d.p.i.) and quantified according to an arbitrary disease index (DI; Fig. 5). Preliminary trials were repeated up to 5 times for most transgenic lines.

*E. carotovora tuber disc maceration assays:* Assays were conducted as described by Lapwood et al. (1984) with minor modifications. Each assay included 15 tuber discs derived from a single transgenic line and non-transformed controls. Maceration volume (MV) of inoculated tuber discs (10 μL of a bacterial suspension of $2 \times 10^5$ c.f.u.) was estimated by replacement of macerated tissue with an equal volume of water. Bacterial titers were estimated by suspending macerated tissue in 20 μL of sterile water and performing of serial dilution plating on agar nutrient medium.

*E. carotovora inhibition assays:* About 500 mg of fresh leaf tissue from axenically grown plantlets were ground in liquid $N_2$ and centrifuged at 9000 g for 15 min at 4 °C. Ninety five μL of the supernatant were then mixed with $1 \times 10^6$ cells of *E. carotovora* and incubated for 2 h at 22 °C. After 8–12 h for recovery at 28 °C in half strength LB medium, bacterial growth was quantified by OD measurements at 550 nm.

*E. carotovora sprouting inhibition assays:* Freshly harvested tubers were cut into small pieces containing a single tuber eye and pre-treated with 1 mg/L gibberellic acid to break tuber dormancy. Twenty pieces from each transgenic line and the non-transformed control were inoculated with fresh *E. carotovora* inoculum ($1 \times 10^6$ c.f.u.) and planted in sterile potting mix under controlled conditions. Tuber sprouting was evaluated after 3 weeks. Assays were repeated 3 times.

*S. scabies tuber infection assays:* Resistance was monitored according the procedure described by Labruyere (1971). Plants were grown till complete development (9–12 weeks) at 20 °C with minimal irrigation to promote bacterial infection. Newly formed tubers were harvested and evaluated for disease using an arbitrary scale (Fig. 5 and Supplementary Table 1). Assays comprised a single randomized block including 3 replications of 10–15 plants from each transgenic line.

*S. scabies inhibition assay:* Antibacterial activity of plant extracts was determined according the protocol reported by Keinath and Loria (1991). Bacterial growth was quantified by OD measurements at 600 nm after overnight recovery at 24 °C in LB medium.

### 2.7. Fungal infection assays

*P. infestans whole-plant assays: In vitro* whole-plant assays were conducted on aseptically grown 3–4 week-old plantlets. An agar block (1 $cm^2$) carrying *P. infestans* mycelium was placed 2 cm from the stem. Symptom development was recorded daily and plants were scored for resistance according to the development of either moderate or severe blight symptoms (leaf dark blotches, brown stem and plant collapse). Non-transformed control plants usually collapsed after 3–5 d.p.i.

*F. solani tuber infection assays:* Assays were carried out using a modification of the procedures described by Herrmann et al. (1996) and Osusky et al. (2005). Fresh weight of each minituber was determined at the beginning of the assay and at 72 h after inoculation with 20 μl of a $1 \times 10^8$ conidia/ml suspension. At this time, minitubers were longitudinally cut. The volume of necrotic tissue was estimated by calculating the volume of the cylindrical cavity created after removal of necrotic and dead tuber tissues caused by fungal infection. Assays included 25 tubers per line and were repeated three times.

*P. infestans* and *R. solani detached-leaf assays:* Detached-leaf infection assays were performed as described by Osusky et al. (2005). Plants used in these assays were grown for 3 weeks at 20 °C under a 16 h photoperiod. Each experimental block comprised 10 fully expanded leaves of similar size and physiological condition sampled from 15 to 20 individual plants representing a single transgenic line or the non-transformed control. Leaves were detached immediately before fungal inoculation. For *R. solani* inoculation, mycelium discs (0.5 cm in diameter) were placed at the upper left sector of each leaf. For *P. infestans* inoculation, a suspension containing $2 \times 10^4$ sporangia was spotted on each leaf after superficial

wounding with a sterile needle. Non-infected leaves from transgenic and control plants were included as negative controls. Leaves were disposed into sealed containers and incubated in the dark at 24 °C for 4–5 days. Leaves were scanned using a Hewlett Packard Scanjet G3110. Leaf images were outlined with the Photoshop 6.0 (Adobe Systems) and analyzed with Matrox Inspector 8.0 (Matrox Electronic Systems) to measure necrotic lesion areas. Total leaf area was determined by using the Region of Interest tool of the same program and performing a "blob" analysis. Individual leaf values were referred as the percentage of total leaf area (percentage of necrotic area; % NA) and used to calculate the mean values for each potato line.

*R. solani* and *F. solani growth inhibition assays:* Fungal growth inhibition was recorded using the diffusion assay reported by Roberts and Selitrennikoff (1988). A small agar plug (1 $cm^2$) containing mycelium from the respective fungal species was placed in the center of a Petri dish containing Potato Dextrose Agar medium and incubated for 48 h at 22–24 °C to allow spore germination and hyphal growth. Then, sterile filter discs were radially placed on the agar surface and loaded with 200 μL of plant extracts. Extracts from non-transformed plants were included as negative controls. Petri dishes were incubated for 5–7 days at 24 °C and fungal growth was recorded at this time.

### 2.8. Microscopic observations

Lesion pieces of about 25 $mm^2$ were sampled from tuber discs inoculated with *E. carotovora* at 24, 48 and 72 h post-inoculation. Specimens were fixed and dehydrated, critical point dried, mounted on aluminum stubs, metallized with gold by ion sputter-coating (SCD 030, Balzers) and examined by a JSMII scanning electron microscope (JEOL) at 15 kV. Tuber samples inoculated with *S. scabies* were observed under a stereoscopic microscope (Nikon, Optiphot-2). Mature hyphal cultures of *S. scabies* were incubated with 85 μL of transgenic and of non-transgenic plant extracts obtained as described for fungal grown inhibition assays. After a 24 h-incubation at 24 °C, effects on bacterial morphology were recorded by optic microscopy (Nikon).

### 2.9. Statistical analysis

Data obtained from the different bacterial and fungal infection assays were separately evaluated by analysis of variance (ANOVA). Mean procedures for randomized complete block experimental designs were carried out using Statistica 6.0 (StatSoft Inc., 1984–2001) and BIOMSTAT/STATISTICA. Fisher's least significant difference procedure (LSD) was used to compare means between DIs.

## 3. Results

### 3.1. Potato transformation and selection of Erwinia-resistant plants

Transformation of tuber explants with the pDe, pLy, pAp and pApLy genetic constructions (Fig. 1) resulted in single- and double-transgene plants expressing the dermaseptin, lysozyme, AP24 and AP24 plus lysozyme sequences, respectively. Triple-transgene plants were generated by re-transformation of a dermaseptin-expressing line (line De-8) with the pApLy construction. Transformation frequency for the individual constructions varied between 30 and 45%. In contrast, re-transformation frequency was much lower (0.75%) and only three ApLyDe lines could be obtained. A Southern blot analysis of lines included in most infection assays transgene copy numbers varying between 1 and 2 (Supplementary Fig. 1).

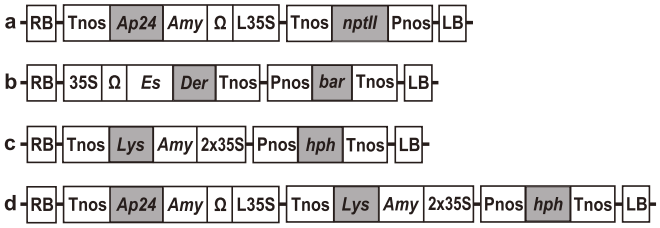



**Fig. 1.** Schematic representation of the genetic constructions used in this work. (a)–(d): Expression cassettes of the pAp, pDe, pLy and pApLy plasmids. 35S: CaMV 35S promoter; 2 × 35S: duplicated CaMV 35S promoter; L35S: "long" CaMV 35S promoter; Pnos: nopaline synthase promoter; Tnos: nopaline synthase transcription termination sequence; *Lys*, *Der* and *AP24*: *Gallus gallus* lysozyme, *Phyllomedusa sauvagii* dermaseptin and *Nicotiana tabacum* AP24 coding sequences, respectively; *bar*: phosphinothricin acetyl transferase; *hph*: hygromycin phosphotransferase; *nptII*: neomicyn phosphotransferase; Ω: TMV translational enhancer; *Es*: esporamine apoplastic signal peptide; *Amy*: barley α-amylase apoplastic signal peptide; RB and LB: right and left borders of *Agrobacterium* T region, respectively. Schemes are not to scale.

Candidate transformants were evaluated by PCR analysis. Seventy two R0 plants showing the expected diagnostic bands were micropropagated and preliminarily screened in whole-plant infection tests using an *Erwinia* inoculum (1 × $10^5$ c.f.u.). As a result, 19

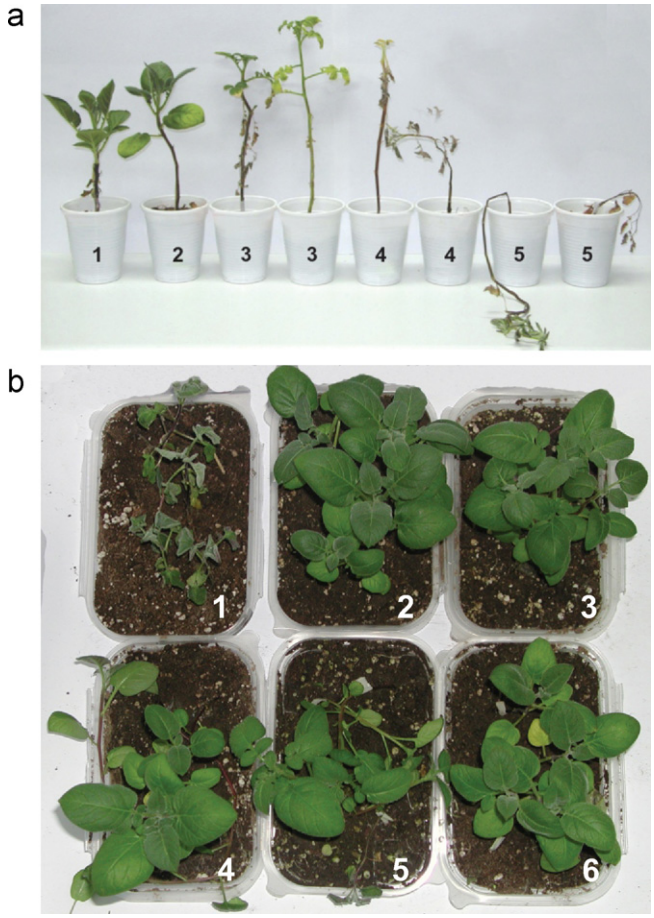



**Fig. 2.** (a) DI scale used in *Erwinia carotovora* infection assays. Numbers from left to right indicate increasing DIs. (1) healthy plant; (2) minor chlorotic symptoms; (3) chlorotic and necrotic mature leaves; (4) wilted and necrotic stem; (5) dead plant. (b) Enhanced resistance to *E. carotovora*. Whole-plant infection assays were performed as described in Section 2 using an inoculum of 6 × $10^7$ c.f.u. Representative 4-plant blocks for different transgenic lines are shown. Each assay included 20 plants per line. (1) Non-transformed; (2) ApLyDe-12; (3) ApLy-47; (4) De-8; (5) Ap-2; (6) Ly-55. Whole-plant assays were repeated at least 3 times for each transgenic line. Pictures were taken 4 weeks after bacterial inoculation.

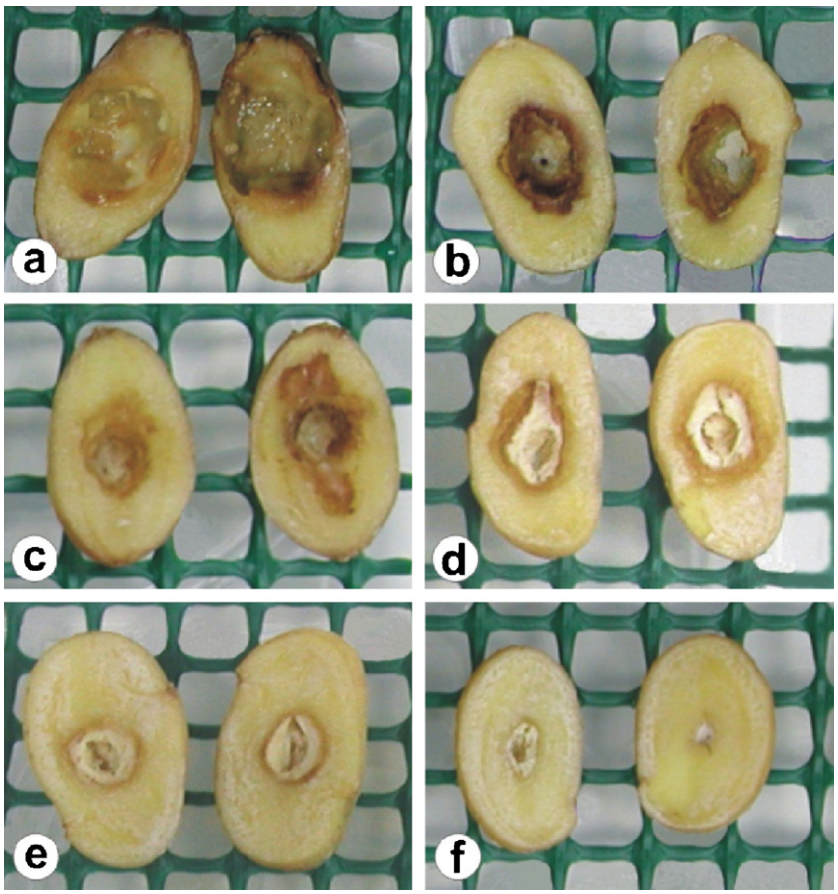



**Fig. 3.** Enhanced resistance to *Erwinia carotovora*. Tuber maceration assays were performed as described in Section 2 using an inoculum of $2 \times 10^5$ c.f.u. Representative symptoms from an assay including 15 tubers per line are shown. (a) Inoculated non-transformed; (b) inoculated Ly-55; (c) inoculated De-8; (d) inoculated ApLy-47; (e) inoculated ApLyDe-12; (f) non-inoculated non-transformed.

lines behaving as highly resistant were chosen for further analysis (Supplementary Table 1). General plant architecture, tuber size and number, and developmental time of these plants were consistent with the non-transformed phenotype. Statistical treatment showed no significant differences ($p = 0.08777$) between single transgenic Ap and Ly lines, but significant differences were found when comparing Ap and Ly lines with the dermaseptin expressing (De) lines ($p = 0.02777$ and $p = 0.02899$, respectively). Also, significant differences between these Ap and Ly lines were found when compared to double- (ApLy) and triple-transgenic lines (ApLyDe) ($p = 0.03399$ and $p = 0.04122$, respectively). Significant differences were found in all cases when comparing transgenic lines to non-transgenic control plants ($p \leq 0.05$).

In a secondary round of infection tests, the selected lines were ranked for resistance using a semi-quantitative disease index (DI; Fig. 2a). To better discriminate between highly resistant lines, bacterial inoculum was increased to $6 \times 10^7$ c.f.u. In these conditions, control plants died at about 15 d.p.i. In contrast, transgenic plants remained healthy or displayed only minor symptoms till 30–60 d.p.i. (Fig. 2b). Lines expressing the three transgenes (lines ApLyDe-7, ApLyDe-8 and ApLyDe-12; DIs: 1.0–1.4) performed better than lines expressing two transgenes (lines ApLy-35, ApLy-47, ApLy-48, ApLy-52 and ApLy-60; DIs: 1.7–2.1) or single transgenes (lines Ap-1, Ap-2, Ap-3, De-2, De-8, De-10, De-19, De-23, Ly-55, Ly-59 and Ly-80; DIs: 1.6–2.8) (Supplementary Table 1). Dermaseptin-transformed lines (lines De-2 and De-8; DIs: 1.6 and 1.9) displayed the strongest antibacterial resistance among single-transgene lines. To evaluate persistence of soft-rot symptoms, all transgenic lines were grown till maturity and their tubers collected. No visible signs of infection could be observed after 18 months of tuber storage; in turn, these tubers produce healthy plants and tubers when planted in sterile soil (results not shown).

The 19 lines tested in whole-plant assays were assayed in a tuber disc maceration assay (Fig. 3a–f). Nearly complete protection was found in tubers from lines expressing the three transgenes (ApLyDe-7, ApLyDe-8 and ApLyDe-12 lines; MVs: 0.13–0.23 mL). Tubers expressing two transgenes (ApLy-35, ApLy-47, ApLy-48, ApLy-52, ApLy-60; MVs: 0.24–0.29 mL) or single transgenes (De-2, De-8, De-10, De-19, De-23; MVs: 0.26–0.48 mL and Ly-55, Ly-59, Ly-80; MVs: 0.31–0.39 mL, respectively) showed also considerable levels of resistance. Conversely, non-transformed tubers showed extensive maceration (MV: 4 mL) (Supplementary Table 1). Lesions on triple-transgene tubers were about 10-fold smaller than those of controls, did not increase after initial infection and looked dry and corky. In contrast, lesions on control tuber discs comprised over 80–100% of the surface, increased continually during infection, and had watery aspect. Inhibition on *Erwinia* growth was also estimated by direct measurement of bacterial loads at 3 d.p.i. As compared to controls, a decrease of up to 6 orders in bacterial accumulation was found in triple-transgene tubers (lines ApLyDe-7 and ApLyDe-12; Supplementary Table 1). In addition, appearance of bacterial cells was examined by scanning electron microscopy. Samples from transgenic plants contained dispersed *Erwinia* cells showing wrinkled surfaces and diminished size. In contrast, samples from non-transformed plants contained cells exhibiting the normal phenotype (Supplementary Fig. 2a and b).

In addition, some selected lines were subjected to tuber sprouting assays to evaluate their performance in a context resembling

a

| | NT | Ap-2 | De-8 | Ly-55 | ApLy-47 | ApLyDe-12 |
|---|---|---|---|---|---|---|
| Ap24 | - | 0.14 (0.006%) | - | - | 0.12 (0.005%) | 0.12 (0.006%) |
| Dermaseptine | - | - | 0.21 (0.01%) | - | - | 0.37 (0.018%) |
| Lysozyme | - | - | - | 0.48 (0.022%) | 0.43 (0.02%) | 0.42 (0.018%) |

b

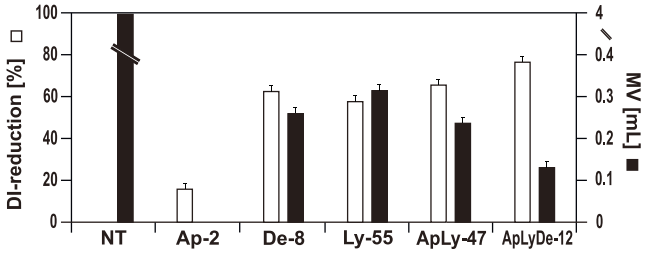



**Fig. 4.** (a) Protein accumulation in transgenic potato plants. Accumulation levels of dermaseptine, AP24 osmotine and lysozyme in transgenic lines were measured as described in Section 2. Protein accumulation values are expressed in μL/μg. Values in brackets express the respective protein amounts as the percentage of total leaf protein (%TLP). (b) DI-reduction values and MVs for transgenic and non-transformed lines. DI reduction values were calculated according to the equation DI × 100/5 in which the value 5 corresponds to the DI of infected controls (100% infection). Results represent the mean value of 5 independent assays ($n$ = 15–20). NT: non-transformed plants.

*Erwinia*-infected soils. In the stringent conditions used in this assay, only 0–10% of non-transformed tubers produced shoots after 3 weeks of planting. In contrast, sprouting of tubers in lines carrying two and three transgenes (lines ApLy-47 and ApLyDe-12) reached 75 and 90%, respectively. Lines transformed with single transgenes showed lower sprouting percentages (line De-8, 60%; line Ly-55, 28%; line Ap-2, 16%) (Supplementary Table 1). Consistent with these results, a leaf extract from line ApLyDe-12 inhibited bacterial growth by 85%, while extracts from lines ApLy-47, De-8, Ly-55, and Ap-2 induced lower growth inhibitions (72, 68, 60 and 52%, respectively) (data not shown).

### 3.2. Relative antibacterial activity of individual transgenes

Five transgenic lines showing comparable levels of the three antimicrobial proteins (lines Ap-2, De-8, Ly-55, ApLy-47 and ApLyDe-12; Fig. 4a) were chosen to evaluate the contribution of individual transgenes to antibacterial protection, as deduced from their respective DI reduction and MV values, in whole-plant and tuber infection assays. The triple-transgene line ApLyDe-12 behaved better (DI reduction: 76%; MV: 0.13 mL) than double- or single-transgene lines (Fig. 4b). However, the high resistance level shown by the single-transgene line De-8 (DI reduction: 62%; MV: 0.26 mL), expressing about half the dermaseptin level than line ApLyDe-12, indicated a strong antibacterial activity for this protein. Similarly, the resistance found in line Ly-55 (DI reduction: 58%; MV: 0.31 mL) pointed to lysozyme as the second major source of resistance. On the other hand, difference of resistance levels between line ApLy-47 (DI reduction: 64%; MV: 0.24 mL) and Ly-55 (DI reduction: 58%, MV: 0.31 mL), expressing similar lysozyme levels, suggested only a minor contribution of AP24 to bacterial resistance. This was confirmed in infection assays in which line Ap-2 (showing similar accumulation of AP24 than line ApLy-47) was unable to overcome the bacterial attack (DI reduction: 16%).

### 3.3. Streptomyces infection assays

To verify whether the selected lines were resistant to other pathogenic bacteria, additional tuber infection tests were conducted with *S. scabies* as infection agent. Only those lines showing high resistance to *E. carotovora* (lines De-8, Ly-55, ApLy-47 and ApLyDe-12) were included in this assay. While 100% of non-transformed tubers developed lesions, most ApLyDe-12 tubers remained unaffected and only 2% exhibited minor scab symptoms. Likewise, only 10% of ApLy-47 and 3–15% of De-8 and Ly-55 tubers developed lesions (Supplementary Table 2). Scabs of transgenic lines were only superficial and involved no more than 15% of tuber surface. In contrast, scabs in non-transformed controls comprised 70–80% of tuber surface and included lesions of up to 5 mm in deep (Fig. 5a). Resistance to *S. scabies* was also measured in a growth inhibition assay with transgenic leaf-extracts (Fig. 5b). Extracts from lines ApLyDe-12, ApLy-47, De-8 and Ly-55 produced inhibitions of 70, 55, 60 and 45%, respectively. No growth inhibition was observed extracts from non-transformed plants.

### 3.4. Fungal infection assays

It was expected that, in addition to their antibacterial effects, some of the transgene combinations could provide protection to fungal diseases. To evaluate this, some of the lines tested for antibacterial resistance were challenged with three fungal pathogens of high incidence in potato production. Different semi-quantitative tests were employed to evaluate the effects of each fungal species.

Two different assays were conducted using *P. infestans* as infecting agent. In the first test, plantlets from lines Ap-2, De-8, Ly-55, ApLy-47 and ApLyDe-12 and non-transformed controls were *in vitro* inoculated and evaluated for delay in symptom development and plant survival (Fig. 6a–e). The best performance was observed in lines Ap-2, Aply-47 and ApLyDe-12, in which most individuals remained unaffected or showed minor blight symptoms at 7–10 d.p.i. On the other hand, more than 50% of De-8, Ly-55 and control plantlets showed complete decay or exhibited severe symptoms. In the conditions of this assay, non-transformed plants died at about 3–5 d.p.i.

In a second *Phytophthora* infection test, detached leaves from the same lines were inoculated with a suspension containing $1 \times 10^5$ sporangia. Degree of fungal infection was estimated measuring lesion area percentages at 5 d.p.i. (Supplementary Table 3). Leaves from lines ApLy-47 and ApLyDe-12 showed the best performance displaying necrotic area percentages of 11.2 and 9.3, respectively, while leaves from line Ap-2, showed an intermediate degree of resistance (Figs. 6f and 8). With exception of Ly-55 line, which was severely affected, all other transgenic lines showed significant differences regarding non-transformed plants.

A similar leaf infection test was implemented for *R. solani* (Fig. 7a). At 5 d.p.i., the percentage of necrotic area encompassed almost 90% of non-transformed leaves. All transgenic lines showed significant differences regarding non-transformed plants. Line ApLyDe-12 performed as highly resistant showing only 10.5% of necrotic damage. Lines Ap-2, De-8, Ly-55 and ApLy-47 ranked in intermediate values exhibiting lesion areas of 27.2%, 29.5%, 41.1% and 23.7%, respectively (Supplementary Table 3). Lines De-8, Ly-55, ApLy-47 and ApLyDe-12 were also assayed in a growth-inhibition assay conducted with total leaf extracts. The highest inhibitory effect was observed with extracts of line ApLyDe-12. Extracts from lines Ly-55 and ApLy-47 induced intermediate inhibition levels, while extracts from line De-8 and non-transformed plants showed no signs of growth retardation (Supplementary Fig. 3a).

Resistance to *F. solani* was assayed using a semi-quantitative test measuring the necrotic volume induced by fungal infection in potato minitubers. Infection with this pathogen included lines transformed with single transgenes and double- and triple-transgene combinations (Supplementary Table 3 and Fig. 7b). Necrotic lesions in line ApLyDe-12 were mainly restricted to the inoculation site at 5 d.p.i., while they included

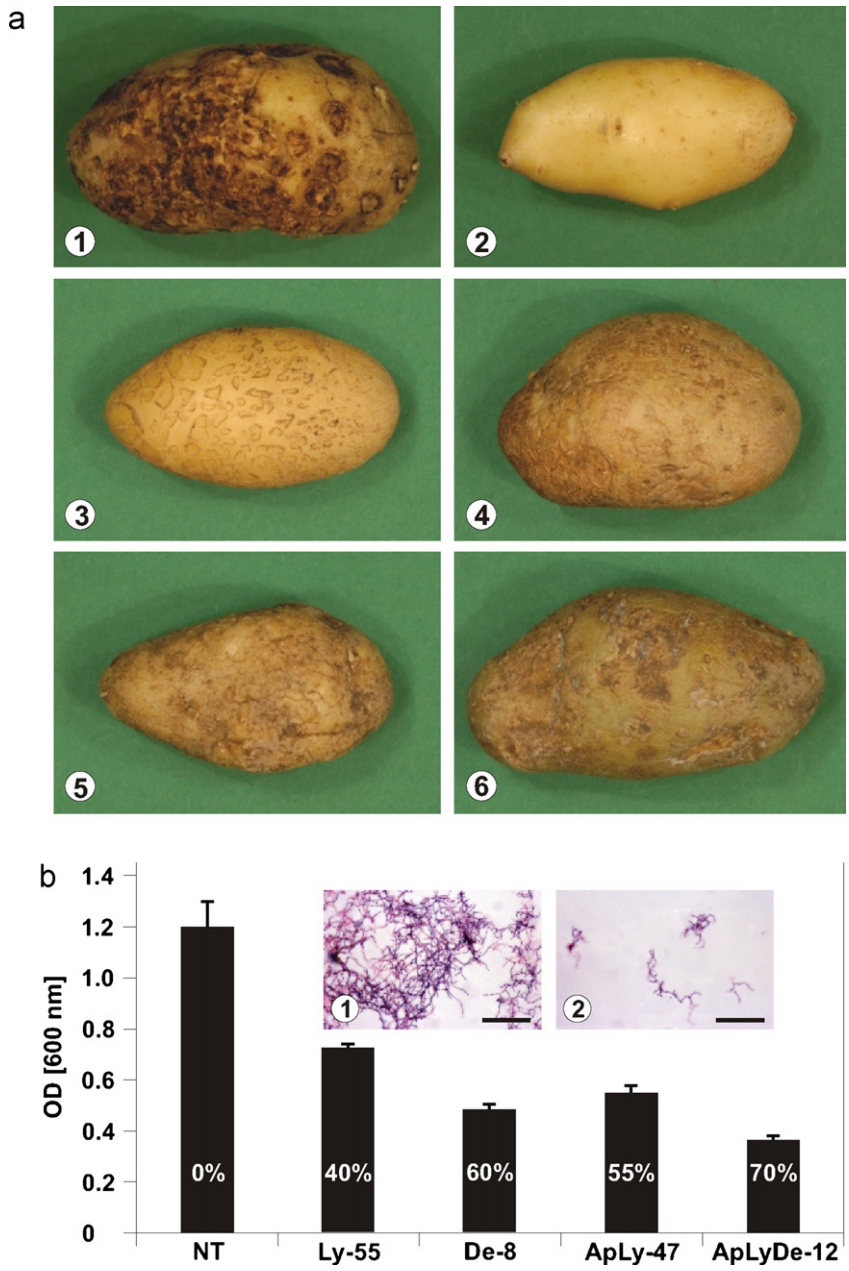



**Fig. 5.** (a) Enhanced resistance to *Streptomyces scabies*. The DI scale used to rank symptom development is detailed in the legend of Table III. Representative symptoms in transgenic and non-transformed tubers are shown. (1) Inoculated non-transformed tuber (DI = 3); (2) non-inoculated non-transformed tuber (DI = 0); (3) inoculated ApLyDe-12 tuber (DI = 1); (4) inoculated ApLy-47 tuber (DI = 1); (5) inoculated De-8 tuber (DI = 1); (6) inoculated Ly-55 tuber (DI = 2). (b) *S. scabies* growth inhibition assays by leaf extracts of different potato lines. Bacterial growth is expressed as OD values. Inhibition percentages are referred to the non-transformed extract. The bars represent the average of three independent assays. Inserts: Microscopical observations in a Neubauer chamber of *S. scabies* cultures treated with non-transformed leaf extract (1) and ApLyDe-12 leaf extract (2) after 48 h of incubation. Bacterial cells showed in the pictures correspond to a sample volume of 0.1 $mm^3$. Photomicrographs bars: 10 μm.

most of minituber volume in non-transformed controls. As observed in *Phytophthora* and *Rhizoctonia* assays, line ApLy-47 exhibited intermediate lesions, and lines Ap-2, De-8 and Ly-55 showed intermediate/severe damage. All transgenic lines assayed showed statistically significant differences as compared to non-transformed plants. In addition, leaf extracts from lines De-8, Ly-55, ApLy-47 and ApLyDe-12 were employed to conduct a *Fusarium* growth-inhibition test. Consistently with the results obtained in the minituber assays, extract from line ApLyDe-12 showed the highest degree of growth inhibition, followed by extracts from lines ApLy-47, Ly-55 and De-8 (Supplementary Fig. 3b). No inhibition of fungal growth was observed with non-transformed leaf extracts.

Values obtained for best-performing single-, double- and triple-transgenic lines (Ap-2, De-8, Ly-55, ApLy-47 and ApLyDe-12) in several of the infection assays described before were compared in terms of resistance levels to bacterial and fungal pathogens (Fig. 8a and b). After data normalization, a maximum resistance level of 1 was calculated ($1 - X_{new}$) for the triple-transgenic line ApLyDe-12 – exhibiting the best performance under stringent infection conditions – and a minimum resistance level of 0 was calculated for non-transgenic controls. As shown in Fig. 8b, single-transgenic lines De-8 and Ly-55 and double-transgenic line ApLy-47 exhibit remarkably high resistance levels when assayed against bacterial pathogens. Instead, resistance to fungal pathogens in single- and double-transgenic lines was variable and seems to be more

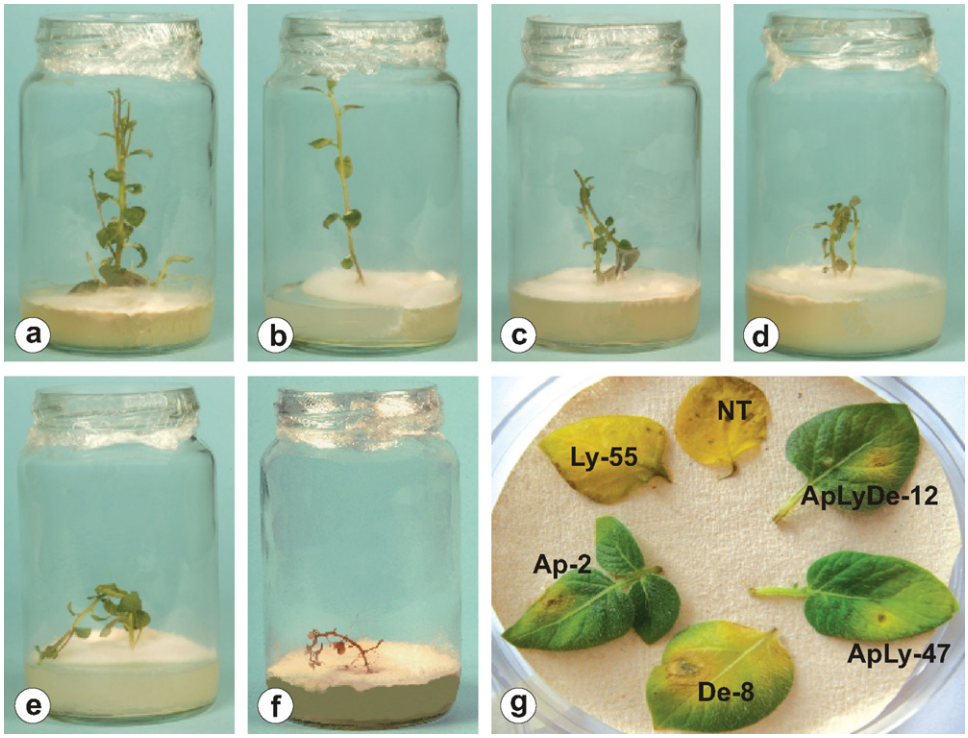



**Fig. 6.** Enhanced resistance to *Phytophthora infestans*. *In vitro* plantlet infections were performed as described in Section 2. (a) ApLyDe-12; (b) ApLy-47; (c) Ap-2; (d) De-8; (e) Ly-55; (f) NT, non-transgenic control. Plantlets shown in the figure are representative of blocks including 15 individuals. Symptom development was recorded at 10 d.p.i. All non-transgenic plants were completely necrotized at 3–5 d.p.i. (g) Detached-leaf assay in transgenic and non-transformed potato lines. Fully expanded leaves were inoculated with 60 μL of a suspension containing $2 \times 10^4$ sporangia. Lesions shown are representative of a block including 20 leaves from each potato line. The picture was taken at 5 d.p.i.

dependent on the presence of specific transgenes or transgene combinations.

## 4. Discussion

Attempts to introduce simultaneous resistance to bacterial and fungal diseases in *S. tuberosum* have been scarce and largely inconclusive. To attain this goal, we selected three proteins individually exhibiting broad antimicrobial activities. One of these proteins, chicken lysozyme, is a well studied enzyme affecting Gram-positive and Gram-negative bacteria (Serrano et al., 2000). Another of them, AP24 osmotine, provides strong protection to *P. infestans* and other pathogenic fungi (Liu et al., 1994; Ouyang et al., 2005). Complementing the activity of these two proteins,

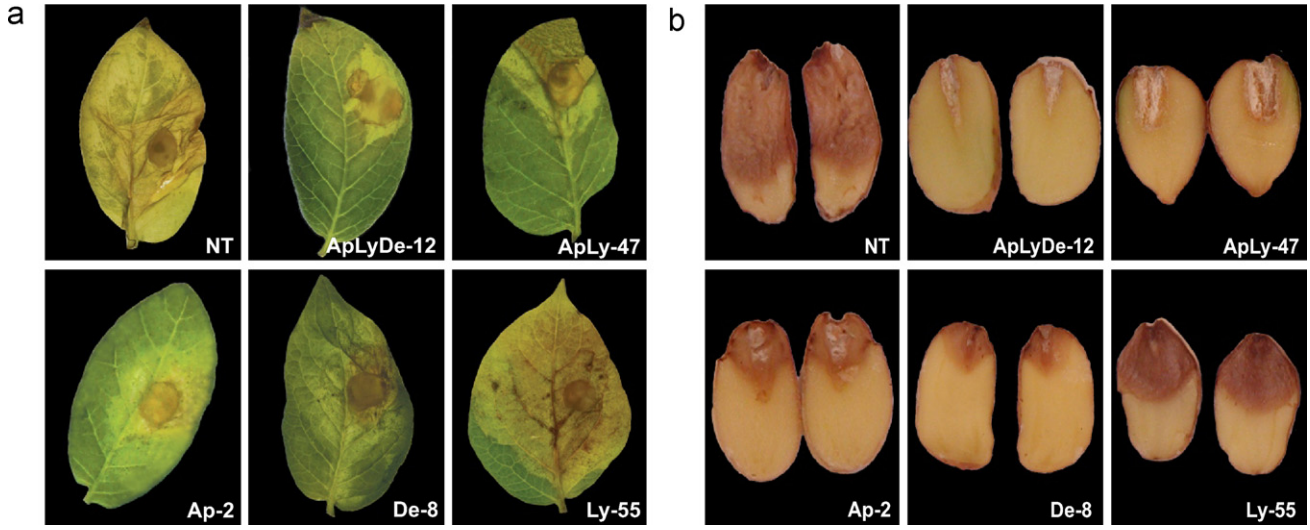
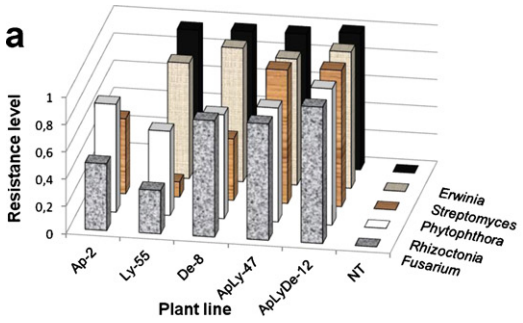



**Fig. 7.** Enhanced resistance to *Rhizoctonia solani* and *Fusarium solani*. (a) Detached-leaf assay with *Rhizoctonia solani*. Fully expanded leaves were inoculated at the upper right sector with hyphal plugs of 0.5-cm diameter. Lesions shown in the figure are representative of a block including 20 leaves from each potato line. (b) Tuber infection assay with *Fusarium solani*. Experimental blocks included 25–30 minitubers from each potato line. Each minituber was inoculated with a suspension containing $1 \times 10^8$ conidia/mL. Representative leaf and tuber lesions in non-transformed (NT), ApLyDe-12, ApLy-47, De-8, Ap-2 and Ly-55 lines are shown. Pictures were taken at 7 d.p.i. in both (a) and (b).

a




b

| | Ap-2 | Ly-55 | De-8 | ApLy-47 | ApLyDe-12 | NT |
|---|---|---|---|---|---|---|
| ***Erwinia*** | | 0,954 | 0,967 | 0,972 | 1 | 0 |
| ***Streptomyces*** | | 0,847 | 0,98 | 0,924 | 1 | 0 |
| ***Phytophthora*** | 0,55 | 0,11 | 0,45 | 0,979 | 1 | 0 |
| ***Rhizoctonia*** | 0,79 | 0,617 | 0,762 | 0,835 | 1 | 0 |
| ***Fusarium*** | 0,49 | 0,318 | 0,854 | 0,854 | 1 | 0 |

**Fig. 8.** (a) Data from Supplementary Tables 1–3 corresponding to transgenic lines Ap-2, Ly-55, De-8, ApLy-47 and ApLyDe-12 and NT, non-transgenic controls were normalized so as to compare values obtained in different units and scales. Normalization was achieved using the equation $X_{new} = X–X_{min}/X_{max}–X_{min}$. Resistance level was calculated as $1 – X_{new}$. According to this, a maximum resistance value of 1 was calculated for ApLyDe-12 transgenic plant line whereas a minimum value (0) was the result for NT control plants. (b) Normalized three-dimensional column chart representing the relative resistance level of the selected transgenic lines to the bacterial and fungal pathogens included in this work, as recorded in different infection assays. *Erwinia:* tuber disc maceration assay; *Streptomyces*: tuber infection assay; *Phytophthora*: detached leaf assay; *Rhizoctonia*: detached leaf assay; *Fusarium*: tuber infection assay. NT: non-transgenic controls.

dermaseptin supplies both antibacterial and antifungal protection to a broad range of plant pathogens (Osusky et al., 2005). Four genetic constructions, containing the AP24 osmotin, dermaseptin, lysozyme and AP24 plus the lysozyme coding sequences were used to transform tuber explants. Simultaneous expression of dermaseptin, lysozyme and AP24 was achieved by re-transformation of a dermaseptin-transformed line. A re-transformation strategy was chosen instead of co-transformation because it allowed direct comparison of dermaseptin levels between primary transformants and re-transformed lines.

The more effective combination of antimicrobial proteins, as well as their appropriate levels of accumulation, could not be predicted *a priori*. Since our ultimate goal was to isolate potato lines exhibiting simultaneous resistance to several pathogens, we adopted a selection scheme based on the performance of transformed plants under stringent infection conditions. Seventy two candidate plants carrying single transgenes or double- and triple-transgene combinations were screened for resistance to *E. carotovora* using high bacterial inoculums. Those exhibiting higher resistance levels to *Erwinia* infections and normal phenotype were selected for detailed characterization and additional inoculation with other potato pathogens. Thus, 19 lines showing strong resistance were chosen for additional examination (Supplementary Table 1). As expected from previous reports, lines showing the higher accumulation of recombinant proteins displayed the stronger levels of resistance. Lines transformed with double- and triple-transgene combinations (lines ApLy-47 and ApLyDe-12) performed better against bacterial infection, showing DI reductions of 65 and 75% in whole-plant infections (Fig. 4). However, some lines expressing individual transgenes (lines Ap-2, De-8 and Ly-55) also showed important resistance levels (DI reductions of 40, 62 and 57%, respectively). Results from other infection assays (tuber maceration, infected tuber sprouting, bacterial counts, bacterial growth inhibition with plant extracts) supported the tendencies observed in whole-plant infections. On the other hand, a series of infection assays conducted with *S. scabies*, showed reductions of tuber scab symptoms of up to 80% (Fig. 5a and Supplementary Table 2). Remarkably, while lysozyme activity affects mainly Gram-negative bacteria, it was also effective against *S. scabies* – a Gram-positive bacterium – as evidenced in the tuber-infection and growth inhibition assays conducted with line Ly-55.

The relative contribution of each antibacterial protein to *E. carotovora* resistance was estimated by comparing DI reductions and tuber MVs in lines expressing similar amounts of transgenic proteins. Thus, comparison between lines ApLyDe-12 and De-8 suggested that dermaseptin is the major individual contributor to antibacterial infection. A similar comparison between lines ApLy-47 and Ly-55 indicated a considerable contribution of lysozyme and a minor contribution of AP24 to the resistance phenotype. Supporting this, plants individually expressing the AP24 construction behaved poorly against *Erwinia* infections. These observations confirmed previous work showing high levels of protection in plants transformed with the dermaseptin or lysozyme sequences (Düring, 1993; Liu et al., 1994; Nakajima et al., 1997; Osusky et al., 2005).

The same transgenic lines tested with *E. carotovora* and *S. scabies* were inoculated with *P. infestans*, *R. solani* and *F. solani*, three phytophatogenic fungi provoking important potato losses. As found for bacterial infections, lines expressing double- and triple-transgene combinations performed better than those expressing individual proteins. Detached-leaf assays with lines expressing the AP24 sequences showed the higher levels of resistance to *P. infestans* and *R. solani*, reaching lesion reductions of up to 90% (Supplementary Table 2 and Fig. 6f and 7a). A comparable effect was apparent in tuber infection assays conducted with *F. solani* (Supplementary Table 2 and Fig. 7b). In particular, a strong antifungal activity was evidenced in line Ap-2. These results corroborate early reports describing the AP24 activity as a predominantly antifungal (Liu et al., 1994; Yun et al., 1998).

It is worth remarking that transgenic resistance to different pathogens could be greatly influenced by the genetic background of the parental cultivar (Arbogast et al., 1999; Ryan et al., 2004). In the case of Spunta, most reports categorize this cultivar as susceptible to *E. carotovora*, and *R. solani* and as moderately tolerant to *S. scabies*, *P. infestans* and *Fusarium* spp. Implementation of the transgenic strategy adopted here in other potato cultivars should contemplate their specific responses to most important potato pathogens.

Since the triple-transgene combination used in this work provides protection to five different potato pathogens, it is conceivable that the same transgene arrangement will be also effective against other bacterial and fungal pathogens affecting this crop. On the other hand, the results obtained in this work cannot be directly extrapolated to actual agricultural conditions, in which the combination of multiple biotic and abiotic effectors could result in a wide range of unexpected outcomes. Taking this into account, the evidences presented here must be further supported by conducting field trials in different agro-ecosystem and management contexts.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiotec.2011.11.005.

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