Plant Cell Tiss Organ Cult (2008) 92:73–84
DOI 10.1007/s11240-007-9306-4

ORIGINAL PAPER

Constitutive over-expression of two wheat pathogenesis-related genes enhances resistance of tobacco plants to *Phytophthora nicotianae*

Floriana Fiocchetti · Rosalinda D'Amore · Monica De Palma · Laura Bertini · Carla Caruso · Carlo Caporale · Antonino Testa · Gennaro Cristinzio · Francesco Saccardo · Marina Tucci

Received: 24 May 2007 / Accepted: 4 October 2007 / Published online: 17 October 2007

Abstract The potential role in plant defence of the two wheat pathogenesis-related proteins of class 4 Wheatwin1 and Wheatwin2, possessing high in vitro antimicrobial activity against several pathogens, was investigated through over-expression of their encoding genes *wPR4a* and *wPR4b* in transgenic tobacco plants. Several independent transformants were obtained, expressing high levels of either transgene when analysed by northern and western blotting. Accumulation of the *wPR4b*-encoded protein Wheatwin2 in the apoplast of transgenic plants was also demonstrated. When homozygous transgenic lines in the T4 generation were tested for increased tolerance to *Phytophthora nicotianae*, they were found to be significantly more resistant than both the wild type and their isogenic, non-*wPR4* transgenic lines. These results suggest that both Wheatwins might have in vivo antimicrobial activity, confirming earlier indications from in vitro assays.

Keywords Antimicrobial activity · Antimicrobial proteins · Plant defence · PR4 genes · Disease resistance

F. Fiocchetti · F. Saccardo
Department of Plant Production, University of Tuscia, Via S. Camillo De Lellis, Viterbo 01100, Italy

Present Address:
F. Fiocchetti
ENEA C.R. Casaccia, Department of Biotechnologies, Agroindustry and Health Protection, Via Anguillarese 301, Rome 00123, Italy

R. D'Amore · M. De Palma · M. Tucci (✉)
CNR – Institute of Plant Genetics, Via Università 133, Portici 80055, Italy
e-mail: mtucci@unina.it

L. Bertini · C. Caruso · C. Caporale
Department of Agrobiology and Agrochemistry, University of Tuscia, Via S. Camillo De Lellis, Viterbo 01100, Italy

A. Testa · G. Cristinzio
Department of Ar.Bo.Pa.Ve. - Plant Pathology, University of Naples, Via Università 100, Portici 80055, Italy

Introduction

Diseases have been one of the principal causes of crop losses ever since humans started to cultivate plants. Environmental and human health concerns have prompted much research on ecologically safe, non-chemical methods of disease control, including breeding of pathogen resistant varieties. The most important class of genes that has been used by breeders for disease control are the plant resistance (R) genes. Nevertheless, except for a few exceptions, the limited durability of single R-genes for many of the most important crop diseases makes it necessary to continue the discovery and introgression of new R-genes. In addition to conventional and advanced breeding techniques, over-expression of genes that encode proteins involved in the synthesis of toxic

compounds or proteins with direct inhibitory effect on the growth of pathogens has been proposed as an alternative approach in plant disease management (Cornelissen and Melchers 1993; Gao et al. 2000). Presently, much research is aimed at identifying and isolating genes that may confer long lasting resistance to target plants upon genetic transfer (Pelegrini and Franco 2005; Bhargava et al. 2007; Yang et al. 2007). A wide range of plant defence antimicrobial proteins has been identified and is being utilized in attempts to provide protection via overexpression in transgenic crops (Evans and Greenland 1998), since it has been shown that expression of antimicrobial proteins in transgenic plants is able to enhance their resistance against phytopathogenic fungi and bacteria (Gao et al. 2000; Vellicce et al. 2006; Jayaraj and Punja 2007).

The major class of antimicrobial proteins are collectively referred to as "pathogenesis-related proteins" (PR). These proteins, defined as proteins encoded by the host plant but induced specifically in pathological situations, not only accumulate locally at infection sites, but are also systemically induced concomitantly with the development of Systemic Acquired Resistance (SAR) against further infection by fungi, oomycetes, bacteria and viruses (Evans and Greenland 1998). It has been suggested that the collective set of PR proteins may be effective in inhibiting pathogen growth and multiplication and that they may, at least in part, be responsible for the state of SAR (Van Loon and Van Strien 1999). At present, a large number of PR-proteins have been characterised and grouped into 17 families based on their primary structure, serological relationships and biological activities (Christensen et al. 2002). Most of them show antimicrobial activity in vitro (Woloshuk et al. 1991), though only a few have been demonstrated to possess enzymatic or inhibitory activities such as chitinases (PR3, PR8, PR11), glucanases (PR2), peroxidases (PR9), ribonuclease-like (PR10) and proteinase inhibitors (PR6). A general characteristic of PR proteins is the presence of several isoforms localised in different cell compartments. Usually acidic proteins are secreted into the extracellular space while their basic counterparts are accumulated in the vacuoles due to the presence of a C-terminal extension of few amino acids which is essential for vacuolar targeting (Bednarek and Raikhel 1992; Nakamura and Matsuoka 1993). Since different isoforms of PR proteins have different pathogen specificity, it has been suggested that their localisation is important for their antimicrobial activity in relation to the predominant mode of pathogen invasion (Liu et al. 1996).

In the last years, we have extensively studied wheat PR proteins of class 4, one of the less known families of PRs. Four PR4 proteins from wheat kernels were isolated and sequenced, named Wheatwin1 to Wheatwin4 (Caruso et al. 1993; Caruso et al. 1996; Caruso et al. 2001a) and the cDNAs encoding Wheatwin1 and Wheatwin2 were cloned (Caruso et al. 1999b). The isolated Wheatwins were demonstrated to be specifically induced in wheat seedlings upon fungal infection (Caruso et al. 1999a) and to be effective in inhibiting hyphal growth and spore germination of pathogens in vitro (Caruso et al. 1996; Caruso et al. 2001b), although their action mechanism and interaction with pathogen molecular targets are still unclear. Caporale et al. (2004) reported that Wheatwin1 shows ribonuclease activity in in vitro experiments, which cannot be related to the action mechanism reported for other pathogen-induced ribonucleases grouped in the PR10 family, but rather to the classical acid-base mechanism of ribonuclease A and T1 involving two histidine residues.

Although some features of Wheatwins have started to be unravelled, their antimicrobial properties have never been tested in vivo. In this study we have investigated the properties of Wheatwin1 and Wheatwin2 by in vivo experiments with transgenic tobacco plants constitutively expressing either of the encoding genes. We have determined the subcellular localisation of Wheatwin2 and tested the susceptibility of transgenic plants to infections of the tobacco pathogen *Phytophthora nicotianae*. We demonstrate that the Wheatwin-expressing lines exhibit increased disease resistance, providing evidence for an actual in vivo role of these PR proteins in the protection against pathogens.

Materials and methods

Vectors construction

The genes used in the transformation experiments were *wPR4a* and *wPR4b*, two clones isolated from a

cDNA library of wheat seedlings (Caruso et al. 1999b) and cloned into the *Eco*RI site of pGEM4Z (Promega Corporation, Madison, WI, USA). After subcloning into the *Eco*RI site of pGEM7Z, the correct orientation of the inserts was verified by sequencing by the dideoxy chain termination method (Sanger et al. 1977) using an automated DNA sequencer (ABI Prism 310; Applied Biosystems, Foster City, CA, USA) and both M13 universal primers and sequence specific primers. Two randomly chosen plasmids showing the correct orientation of the fragment were named pW1G2 and pW2G5, carrying the cDNAs *wPR4a* and *wPR4b*, respectively.

For *Agrobacterium*-mediated transformation the two cDNAs were then cloned from pW1G2 and pW2G5 into the binary vector pKYLX71:35S^2, containing the constitutive 35S promoter with a duplicated enhancer region, the kanamycin resistance gene *nptII* for selection of plant transformants and the tetracycline resistance gene, used as bacterial selection gene. The w*PR4a* cDNA was excised from pW1G2 with *Xho*I and *Sst*I while the *wPR4b* insert was excised from pW2G5 as a *Hin*dIII-*Xho*I fragment prior to cloning into pKYLX71:35S^2 downstream of the CaMV 35S promoter. Ligation products were used to transform competent *E. coli* DH5α cells using the heat shock method (Sambrook et al. 1989) and bacterial cells were plated on Luria Bertani (LB) agar containing 50 μg ml^{-1} kanamycin and 12.5 μg ml^{-1} tetracycline and incubated at 37°C over night.

Plasmid DNA was isolated from putatively transformed *E. coli* colonies with the QIAprep Spin Miniprep kit (QIAGEN S.p.A, Milano, Italy) following the manufacturer's instructions and the insertion of genes inside the binary vectors was verified by digestion with restriction enzymes. Positive colonies were named DH5α/pW1KY and DH5α/pW2KY.

The above constructs were used to transform competent *Agrobacterium tumefaciens* LBA4404 or EHA105 cells by the freeze-thaw method (Zhang and Zeevaart 1999). 100 μl of competent cells were frozen in liquid nitrogen with 1 μg of plasmid DNA containing the appropriate insert. After a short incubation at 37°C for 5 min, cells were diluted in 1 ml of LB broth and incubated at 28°C, with shaking, for 2–4 h. Finally, cells were centrifuged, resuspended in 100 μl LB and plated on LB selective agar including 50 μg ml^{-1} streptomycin, 12.5 μg ml^{-1} tetracycline and 250 μg ml^{-1} rifampicin for LBA4404/pW2KY; 50 μg ml^{-1} kanamycin and 30 μg ml^{-1} rifampicin for EHA105/pW1KY and EHA105/pW2KY. Plates were incubated at 28°C and transformed colonies appeared within 2–3 days.

Tobacco leaf disk transformation

Stable tobacco (*Nicotiana tabacum* cv. Samsun NN) transformants were obtained by a modified leaf disk transformation method (Horsch et al. 1985). Two days before the co-culture one colony of each *A. tumefaciens* construct (taken from a fresh plate) was inoculated in 5 ml of LB broth including selective antibiotics and incubated overnight at 28°C with shaking at 200 rpm. The day after 1 ml of each bacterial solution was inoculated in 30 ml of LB broth including the same antibiotics and incubated overnight at 28°C with shaking at 200 rpm. After 24 h the bacterial cultures were centrifuged and resuspended in liquid RTE containing Murashige and Skoog (MS) salts (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968), 3% (w/v) sucrose, 2 mg l^{-1} 6-benzylaminopurine (BAP), 0.4 mg l^{-1} indoleacetic acid (IAA), pH 5.8, in order to obtain 0.2–0.3 O.D. at 600 nm. The obtained suspensions were used for the co-culture with tobacco explants.

Leaf disks of *N. tabacum*, obtained from leaves of young plants grown in vitro, were submersed in each *Agrobacterium* suspension for 10 min. Explants were blotted dry on Whatman 3MM paper to eliminate the excess of bacterial solution, transferred on RTE agar containing 100 mg l^{-1} acetosyringone (4′-hydroxy-3,5-dimethoxyacetophenone) and incubated in a controlled environment chamber at 23°C with a 16 h photoperiod and 40 μmol PAR m^{-2}s^{-1}. After two days the explants were transferred on RTE agar including 500 mg l^{-1} cefotaxime and 100 mg l^{-1} kanamycin, repeating the transfer every three weeks. Shoots regenerating from callus tissue were put on a medium containing MS salts, B5 vitamins, 3% (w/v) sucrose, 100 mg l^{-1} kanamycin and 200 mg l^{-1} cefotaxime. After rooting, plants were transferred to soil and grown in the greenhouse. Primary transgenics (T0) were self-fertilised to produce T1 seeds. Homozygosis of the transformed plants was verified by segregation of the kanamycin resistance character, which is strongly linked to the transgene. The assay

used to check the resistance was optimized by Weide et al. (1989).

Southern blot hybridisation

Total genomic DNA was extracted from transgenic and control tobacco leaves using the CTAB method described by Doyle and Doyle (1990) with slight modifications. Ten micrograms of genomic DNA digested with *Sst*I were size separated on a 0.8% (w/v) agarose gel and then transferred onto nylon Hybond^{TM}-N^{+} membrane (Amersham Pharmacia Biotech, Uppsala, Sweden) as described in Sambrook et al. (1989). The *wPR4b* cDNA was labelled with [α^{32}P]dCTP using the Prime-it II Random Primer Labeling Kit (Stratagene, La Jolla, CA, USA) and used as a probe. Membranes were prehybridised in 6X SSC, 5X Denhardt's reagent, 0.5% (w/v) SDS, 100 μg ml^{-1} salmon sperm DNA, for 2 h at 65°C. Then, the denatured probe was added to the prehybridisation solution (10^6 cpm ml^{-1}) and the membranes were incubated for 12–18 h at 65°C. After hybridisation, membranes were washed twice in 2X SSC, 0.1% (w/v) SDS; twice in 1X SSC 0.1% (w/v) SDS; once in 0.5X SSC, 0.1% (w/v) SDS and once 0.1X SSC, 0.1% (w/v) SDS; each washing step was conducted for 20 min at 65°C. Finally membranes were exposed to Kodak XAR-5 film for 36–48 h.

Northern blot hybridisation

Total RNA was isolated from transgenic and control tobacco leaves using the 'RNeasy' Plant Mini Kit (QIAGEN S.p.A., Milano, Italy) according to the manufacturer's instructions. Ten micrograms of RNA were separated on a denaturing 1.2% formaldehyde-agarose gel and blotted on a nylon Hybond^{TM}–N^{+} membrane (Amersham Pharmacia Biotech, Uppsala, Sweden) as described in Sambrook et al. (1989). The α^{32}P- labeled *wPR4b* cDNA probe prepared as in Southern blot analysis was used for hybridisation. Membrane prehybridisation and hybridisation were performed at 42°C in 5X SSC, containing 5X Denhardt's reagent, 10% (w/v) SDS, 40% (v/v) formammide. After hybridisation, membranes were washed twice in 2X SSC, 0.1% (w/v) SDS for 20 min at room temperature, then twice in 1X SSC, 0.1% (w/v) SDS for 20 min at room temperature, and finally once in 0.5X SSC, 0.1% (w/v) SDS and once in 0.1X SSC, 0.1% (w/v) SDS at 65°C for 20 min each time. Finally membranes were exposed to Kodak XAR-5 film for 48–72 h.

Western blot hybridisation

Leaves were harvested from in vitro grown tobacco plants and ground to a fine powder in a pre-chilled mortar with liquid nitrogen. The extraction buffer (Tris-HCl 20 mM, pH 7.00, containing 1% (w/v) insoluble polyvinylpoly pyrrolidone) was added to the powder (2.3 ml g^{-1} fresh tissue). After thorough vortexing, the buffer extracts were centrifuged at 14,000 rpm for 30 min and then supernatants were centrifuged again at the same speed for 10 min. Protein concentration of the clear supernatants was determined by Bradford assay (Bradford 1976) using bovine serum albumin as a standard. Equal protein concentrations were used for SDS-PAGE and immunoblotting following the procedures of Laemmli (1970) and Towbin et al. (1979), respectively. Recombinant Wheatwin2 purified from *E. coli* heterologously expressing the protein (Caruso et al. 2001b) was used as a western blot standard. Polyclonal antibodies raised in rabbit against purified Wheatwin1 expressed in the same heterologous system (Caruso et al. 2001b) were used as first antibody and goat anti-rabbit horseradish peroxidase-conjugated immunoglobulins (MP Biomedicals, Irvine, CA, USA) were used as second antibody. 4-Chloro-1-naphthol (Sigma-Aldrich) was used as a chromogen for detecting positive bands.

Separation of intracellular and extracellular fluids of tobacco transformants

Leaf extracellular fractions were isolated from tobacco transformants using a method described by Yan et al. (1997). A 4 g of young leaves were cut in strips with a razor blade and immersed in 5 ml of infiltration buffer (KH_2PO_4/K_2HPO_4 65 mM, pH 7.4, oxalacetic acid 0.15 mM). Infiltration of leaves was achieved under vacuum at 91 kPa on ice for 36 min, stopping and reactivating vacuum every 3–5 min.

Then the strips were transferred to a syringe closed with Miracloth (Calbiochem, Darmstadt, Germany) and centrifuged at 1500×*g* for 15 min at 4°C. The extracellular fluid was recovered as the flow-throw and stored at –20°C.

For the isolation of the soluble cytoplasmic proteins, the method described by Li et al. (1989) was followed. After collection of the extracellular fluid, leaves were homogenised in a mortar in 5 ml of 50 mM K-phosphate, pH 6.9, in the presence of sand, on ice. Crude extracts were centrifuged at 27000×*g* for 15 min at 4°C and the supernatants, containing soluble cytoplasmic proteins, were collected and stored at –20°C. Protein samples were used for SDS-PAGE and immunoblotting following the procedures described earlier.

Transgenic plants resistance

Transgenic tobacco plants were also assayed for susceptibility to *Phytophthora nicotianae*. In vivo susceptibility tests were performed on plants grown in pots, under standard greenhouse conditions till the stage of two true leaves and then inoculated with a pool of three *P. nicotianae* strains (Ph308, Ph348 and Ph398; collection of the Dept. of Ar.Bo.Pa.Ve. of the University of Naples "Federico II"). The strains were raised on V8-agar medium for 10 days at 21 ± 1°C, than homogenised in equal amounts. A 5 ml of suspension, containing 20,000 CFU ml^{-1}, were distributed around the stem base of each plantlet, inoculating at least ten plantlets per thesis. The above inoculum concentration was shown by preliminary tests to be the best in discriminating between lines. Negative control plantlets were treated with sterile V8-agar. The percentage of survived plants was evaluated at 10, 20 and 30 DPI. To validate the infections, samples from infected plants were regularly recovered to re-isolate the parasite in axenic cultures. Reported results are representative of at least two independent experiments and were analysed with the Tukey-Kramer Multiple Comparison Test ($P \leq 0.01$).

Results

Transformation of tobacco with wheat PR4 genes

In order to study the role of PR4 proteins in vivo, the wheat *wPR4a* and *wPR4b* genes, which encode Wheatwin1 and Wheatwin2 proteins, respectively (Caruso et al. 1999b), were expressed in transgenic plants of tobacco cv. Samsun NN. Wheatwin1 and Wheatwin2 are two highly homologous PR proteins of class 4 and are characterised by a strong activity against pathogenic fungi in in vitro assays (Caruso et al. 1996). To express *wPR4a* and *wPR4b* genes in tobacco, either *wPR4a* or *wPR4b* were cloned in pKYLX71:35S^2 downstream of the CaMV 35S promoter with double enhancer, giving the plasmids pW1KY and pW2KY, respectively, which also harboured the kanamycin resistance gene for selection of plant transformants (Fig. 1). Plasmids pW1KY and pW2KY were used for genetic transformation of tobacco cv. Samsun NN via *A. tumefaciens*. After selection on kanamycin-containing medium, 12 *wPR4a* and 54 *wPR4b* regenerated plants (T0) were obtained, which developed normally and showed

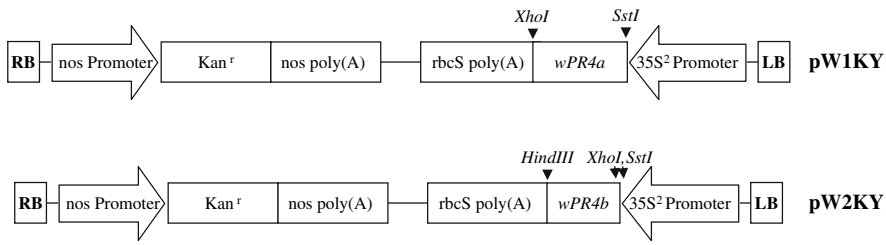

Fig. 1 Transformation cassettes containing either the *wPR4a* or the *wPR4b* cDNAs into the transformation vector pKYLX71:35S^2, giving the recombinant plasmids pW1KY and pW2KY, respectively. RB, right border; LB, left border; nos Promoter, nopaline synthase promoter; Kan^r, kanamycin resistance gene; nos poly(A), nopaline synthase polyadenylation signal; 35S^2 Promoter, double enhanced cauliflower mosaic virus (CaMV) 35S promoter; rbcS poly(A), ribulose-1,5-bisphosphate carboxylase small subunit gene polyadenylation signal. Relevant restriction sites are also indicated

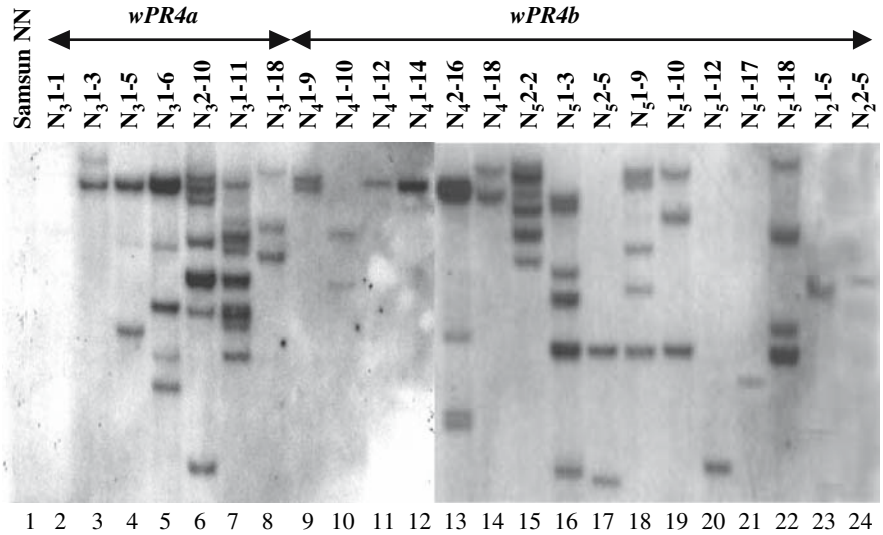

Fig. 2 Southern blot analysis of *wPR4a* and *wPR4b* tobacco regenerants. Genomic DNAs isolated from wild-type (Samsun NN) and from several independent tobacco regenerants were digested with *Sst*I and hybridised with a [α^{32}P]dCTP labeled *wPR4b* cDNA probe. Lane 1, Samsun NN; lanes 2–8, regenerants from transformation with *wPR4a*; lanes 9–24, regenerants from transformation with *wPR4b*

horticultural traits similar to the untransformed control (not shown).

In order to ascertain the insertion of the appropriate transgene into kanamycin-resistant regenerants, Southern blot analysis was performed. Twenty-three tobacco regenerants were randomly selected, 7 of which were transformed with the pW1KY construct and 16 with pW2KY. Genomic DNAs of these tobacco regenerants were digested with *Sst*I and subjected to Southern blot hybridization using *wPR4b* cDNA as the probe. Since *Sst*I cuts only once into the T-DNA, the number of restriction fragments complementary to the probe is coincident with the number of copies of the transgene inserted into the plant genome. The presence of either the *wPR4a* or the *wPR4b* transgenes was detected in all tested regenerants except $N_3$1-1 (Fig. 2) indicating that it probably escaped kanamycin selection. Most lines were found to carry multiple insertions (1–7 copies) of either transgene (Fig. 2).

Expression of *wPR4* transgenes in primary transformants

Some of the tobacco transformants showing a variable number of integrated copies of *wPR4a* or *wPR4b* in the Southern blot analysis were selected for expression studies. Namely, we selected genotypes with a low number of integrated copies of the transgene (1–2) and genotypes with a high number of inserted copies (3–9). Good expression levels for transgenic transcripts of the expected size were detected in all transgenic lines analysed by northern blot (Fig. 3a). The highest transgene expression was detected in $N_3$1-5 and $N_3$1-6 for *wPR4a* and in $N_5$1-3 for *wPR4b* and was not correlated with the number of inserted copies of the transgene, since elevated transcription is in some cases associated with low transgene copy number, as in $N_3$1-5, and in others with the presence of several copies of the transgene, as in $N_3$1-6 (Figs. 2 and 3a).

To verify expression of the transgenes at the protein level, western blot analysis was performed on the transformed lines analysed by northern blot assay. Total proteins were extracted from untransformed or primary transgenic tobacco leaves and the crude extracts were utilised for western blotting using a polyclonal antibody raised against recombinant Wheatwin1 which cross-reacts with Wheatwin2. A band of the expected size for Wheatwin1 and Wheatwin2 was detected in all tobacco transformants, revealing a good level of transgenic protein expression (Fig. 3b). No signal was detected in the untransformed control Samsun NN, confirming the specificity of the assay. It is worthwhile to highlight that signal intensity is not proportional to the actual concentration of either protein, since western blot analysis is not quantitative in our conditions.

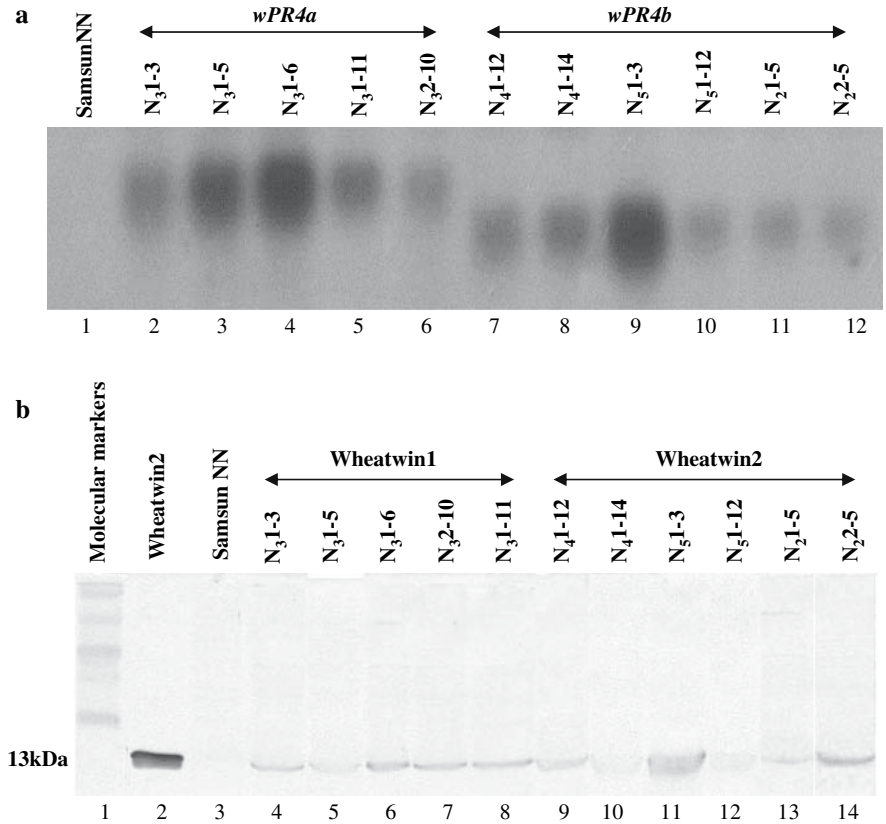

Fig. 3 Expression analysis of *wPR4a* and *wPR4b* in several independent tobacco transformants. (**a**) Northern blot of total RNAs isolated from wild-type (Samsun NN) and primary transgenic plants probed with the [α^{32}P]dCTP labeled *wPR4b* cDNA. Lane 1, Samsun NN; lanes 2–6, *wPR4a* transformants; lanes 7–12, *wPR4b* transformants. (**b**) Western blot of total proteins of wild-type (Samsun NN) and primary transgenic plants, processed with polyclonal anti-Wheatwin1 antibodies. Lane 1, molecular markers; lane 2, purified recombinant Wheatwin2; lane 3, Samsun NN; lanes 4–8, *wPR4a* transformants; lanes 9–14, *wPR4b* transformants

Characterisation of transgenic homozygous lines of tobacco and localisation of Wheatwin protein expression

The primary transgenic tobacco plants $N_3$1-5, $N_3$1-6 and $N_4$1-14 which in Southern blot analysis revealed one to a few stably inserted copies of the transgene were selected and selfed. Segregation of the transgene was indirectly followed by testing for the genetically linked kanamycin resistance. Selfing was repeated up to the T4 generation to obtain homozygous transgenic lines and their isogenic, non-transgenic lines in which all copies of the transgene had segregated away. The selected lines were analysed by western blotting (Fig. 4). The Wheatwin proteins were not expressed either in wild-type Samsun NN or in the kanamycin susceptible lines 315-2, 316-12 and 414-1, confirming that these lines did not carry Wheatwin1- or Wheatwin2-encoding transgenes. Good expression levels were detected in all kanamycin resistant lines, confirming the results obtained with western blotting of primary transformants, and demonstrating that neither *wPR4a* nor *wPR4b* transgenes had undergone gene silencing (Fig. 4).

Phenotypic analysis of the transgenic lines revealed that they were similar to wild-type plants both for morphological and for developmental traits

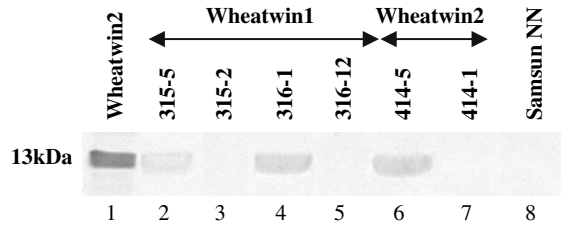

Fig. 4 Western blot analysis of selected T4 transgenic lines and of the relative controls. Total protein extracts of selected T4 homozygous *wPR4a* and *wPR4b* transgenic lines and of their isogenic lines in which the transgene segregated away, were analysed by western blotting with polyclonal anti-Wheatwin1 antibodies. Lane 1, purified recombinant Wheatwin2; lanes 2 and 4, *wPR4a* transgenic lines; lanes 3 and 5, their respective non-*wPR4a* transgenics; lane 6, *wPR4b* transgenic line; lane 7, its respective non-*wPR4b* transgenic; lane 8, wild-type (Samsun NN)

under regular greenhouse conditions (results not shown).

Subcellular localisation of native Wheatwin proteins in wheat is not known, although the absence of a vacuolar sorting signal peptide-encoding sequence in both *wPR4a* and *wPR4b* genes suggests secretion in the apoplast (Caruso et al. 1999b). In order to assess the actual localisation of Wheatwins, which is also important to understand their action mechanism against invading pathogens, extracellular and intracellular protein fractions, including soluble cytoplasmic proteins, were isolated from leaves of the homozygous transgenic line 414-5 and of its isogenic, non transgenic line 414-1. Both lines descend from the primary transformant $N_4$1-14, which revealed a single integrated copy of the *wPR4b* transgene in Southern blot assays, confirmed also by the 3:1 segregation of the kanamycin-resistant phenotype in its progeny. Protein fractions from leaves of the wild type Samsun NN were used as control. Protein extracts were analysed by western blotting using the anti-Wheatwin1 polyclonal antibody described above. The obtained results (Fig. 5) indicated that Wheatwin2 is secreted into the extracellular fluid in the transformed tobacco line 414-5. The trace amounts of Wheatwin2 found in the cell extracts might be due either to incomplete removal of the apoplastic fluid during preparation of the extract or to limited accumulation of the protein inside the cell or at the cell surface.

Sensitivity of transgenic plants to pathogens

The above-described *wPR4* transgenic lines selfed to homozygosity and their isogenic, non-transgenic lines were tested for pathogen resistance in the T4 generation together with the untransformed control Samsun NN. When tobacco plantlets were challenged with *P. nicotianae*, the percentage of survived plants of the transgenic lines ranged from 90 to 100%, which is slightly but significantly higher than the percentage found in both their isogenic, non-transgenic lines and the control (Fig. 6). This demonstrates that expression of either Wheatwin1 (lines 315 and 316) or Wheatwin2 (line 414) is able to increase resistance of transgenic tobacco to the pathogen.

Discussion

In recent years one of the solutions proposed to obtain a lasting resistance against different pathogens has been the over-expression of single genes encoding PR proteins with high antimicrobial activity in

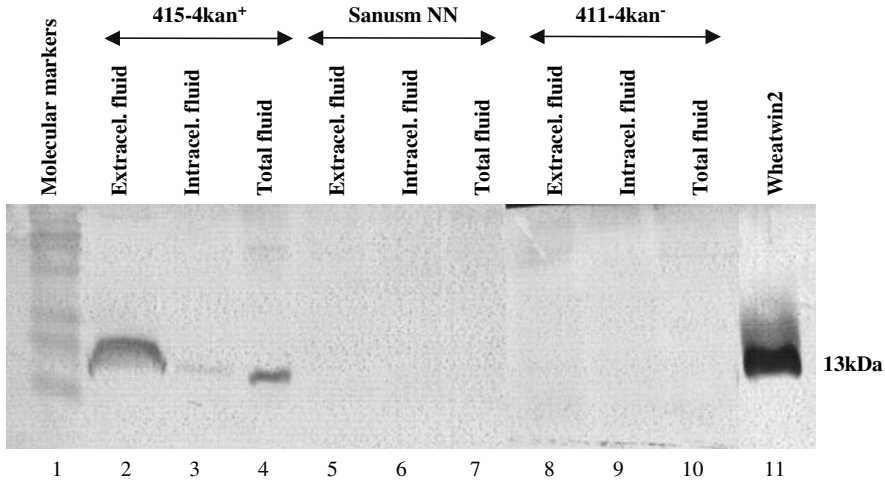

Fig. 5 Localisation of Wheatwin protein accumulation in transgenic tobacco. Total protein extracts of the *wPR4b*-transformed tobacco line 414–5 and of control lines were analysed by western blotting with polyclonal anti-Wheatwin1 antibodies. Lane 1, molecular markers; lanes 2, 5 and 8, extracellular fluid; lanes 3, 6 and 9, intracellular fluid; lanes 4, 7 and 10, total cellular fluid from the transgenic line 414-5 (lanes 2–4), the wild type Samsun NN (lanes 5–7) and from line 414-1, isogenic to 414-5 but not *wPR4*-transgenic (lanes 8–10), respectively. Lane 11, purified recombinant Wheatwin2

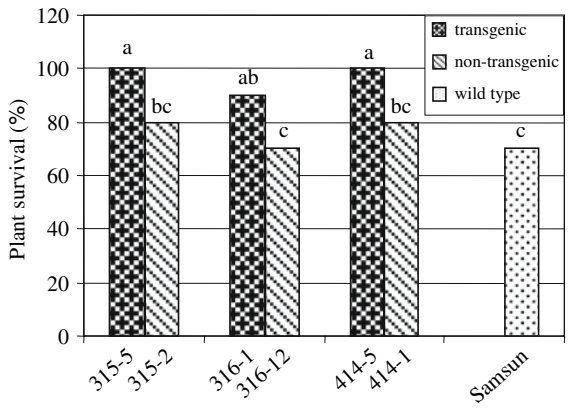

Fig. 6 Susceptibility of *wPR4* transgenic lines to *P. nicotianae* infection. At least ten tobacco plants of selected T4 homozygous *wPR4a* (315–5, 316-1) and *wPR4b* (414-5) transgenic lines were inoculated with spores from a pool of three *P. nicotianae* strains together with their respective non-*wPR4a* (315-2, 316-12) and non-*wPR4b* (414-1) lines and with the wild-type (Samsun NN). The percentage of survived plants at 30 DPI is reported. Values indicated with the same letter are not significantly different at the Tukey-Kramer Test ($P \leq 0.01$)

transgenic plants (Asao et al. 1997; Lan et al. 2000). Among the less studied PR proteins are those belonging to the PR4 family, although PR4 genes and their encoded proteins have been studied in several species (Stanford et al. 1989; Broekaert et al. 1990; Friedrich et al. 1991; Linthorst 1991; Hejgaard et al. 1992; Svensson et al. 1992; Potter et al. 1993; Ponstein et al. 1994). Besides, two highly homologous PR4 proteins (Wheatwin1 and Wheatwin2) were purified from wheat kernel and characterised thoroughly (Caruso et al. 1993; Caruso et al. 1996). Both Wheatwin1 and Wheatwin2 are induced by infection with *Fusarium culmorum* in wheat seedlings (Caruso et al. 1999a) and revealed a strong in vitro antimicrobial activity against both wide host-range pathogens such as *Botrytis cinerea* and wheat-specific pathogenic fungi like *F. culmorum* and *F. graminearum* (Caruso et al. 1996; Caruso et al. 2001b). The reported ribonuclease activity of Wheatwin1 (Caporale et al. 2004) may, at least in part, be responsible for its antimicrobial properties.

In the present study we described the expression of Wheatwin1 and Wheatwin2 in transgenic tobacco plants as well as the subcellular localisation of Wheatwin2 to provide evidence of an in vivo biological role for these proteins in plant defence system, confirming results obtained in in vitro assays. To this aim, we transformed the Wheatwin1 and Wheatwin2-encoding cDNAs *wPR4a* and *wPR4b* (Caruso et al. 1999b) into tobacco plants through *A. tumefaciens*-mediated transformation.

Northern blot analysis of selected primary transformants both with a low and with a high number of integrated copies of the transgene was performed in order to detect possible differences in the expression levels. As expected from several reports in the literature (Bhat and Srinivasan 2002; Butaye et al. 2005), no correlation was found between the transcription rate and the number of stably inserted copies of the transgenes. In fact, insertion of multiple copies of a transgene into the genome of a plant cell could increase the possibility of gene silencing and reduce gene expression (Stam et al. 1997; Butaye et al. 2005), while a transgene inserted in a unique copy is transcriptionally more active than those inserted in multiple copies (Hobbs et al. 1993; Bhat and Srinivasan 2002; Butaye et al. 2005).

In order to verify transgene expression at protein level western blot analysis was performed and all regenerants analysed were demonstrated to accumulate Wheatwin1 or Wheatwin2 at good levels, showing the absence of post-transcriptional gene silencing phenomena.

Basic isoforms of PR proteins are usually accumulated in the vacuoles, while acidic PRs are secreted to the extracellular space (Liu et al. 1996), and the different localisation was proposed to be crucial for their antimicrobial activity (Vierheilig et al. 1993; Liu et al. 1996; Kishimoto et al. 2002). In transgenic plants constitutively expressing some chitinases, subcellular localisation of the protein codified by the transgene was a key factor for the resistance against pathogenic fungi (Vierheilig et al. 1993; Kishimoto et al. 2002). As far as the localisation of the Wheatwins, we have demonstrated that Wheatwin2 is mainly secreted into the apoplastic fluid in tobacco transgenic plants. This result is consistent with the features of its deduced amino acid sequence (Caruso et al. 1999b), since it has a highly hydrophobic N-terminal domain with the characteristics of a signal peptide for translocation through the endoplasmic reticulum and lacks the C-terminal extension of few amino acids essential for vacuolar targeting, usually described in other basic PR proteins. Therefore, Wheatwin2 represents a rare example of a basic PR protein which is secreted in the

apoplast. Localisation of Wheatwin1 was not tested directly. However, since its sequence shares the same features as Wheatwin2 (presence of a N-terminal hydrophobic domain and absence of the C-terminal vacuolar-targeting peptide) we can speculate that Wheatwin1 might similarly accumulate extracellularly. PR1 proteins were found to be predominantly accumulated in intercellular spaces of *Phytophthora capsici*-infected pepper plants, where they may play a defensive role against the invading hyphae (Lee et al. 2000). Whether the localisation of the Wheatwins is relevant for their action mechanism against invading pathogens needs to be further investigated.

When homozygous (T4 generation) tobacco transgenic lines expressing *wPR4a* and *wPR4b* under the constitutive 35S promoter were tested for resistance to *P. nicotianae*, they were found more resistant compared to both their respective non-transgenic lines and the control Samsun NN, although to a limited extent. Indeed, several classes of PR proteins contribute to the complex battery of active defences recruited by the plant upon pathogen infection and overexpression of other PR proteins has been reported to increase resistance of transgenic plants to pathogens only partially (Asao et al. 1997; Lan et al. 2000).

We can conclude that constitutive over-expression of Wheatwin1 and Wheatwin2 in transgenic tobacco plants can confer decreased pathogen sensitivity, thus confirming their antimicrobial activity previously demonstrated in in vitro assays. Nevertheless, our results indicate that over-expression of a single component of the complex set of defences activated by the plant during pathogen infection is not sufficient to achieve a very high level of disease resistance.

Acknowledgements This research was partly supported by the Ministero dell'Istruzione, Università e Ricerca Scientifica (MIUR), project MIUR-FIRB (RBNE01KZE7) and by a grant from Consorzio Interuniversitario Biotecnologie (CIB). This is journal paper 101 of the CNR – Institute of Plant Genetics, Portici, Italy.

References

Asao H, Nishizawa Y, Arai S, Sato T, Hirai M, Yoshida K, Shinmyo A, Hibi T (1997) Enhanced resistance against a fungal pathogen *Sphaerotheca humuli* in transgenic strawberry expressing a rice chitinase gene. Plant Biotechnol 14:145–149

Bhargava A, Osusky M, Hancock RE, Forward BS, Kay WW, Misra S (2007) Antiviral indolicidin variant peptides: Evaluation for broad-spectrum disease resistance in transgenic *Nicotiana tabacum*. Plant Sci 172:515–523

Bednarek SY, Raikhel NV (1992) Intracellular trafficking of secretory proteins. Plant Mol Biol 20:133–150

Bhat SR, Srinivasan S (2002) Molecular and genetic analyses of transgenic plants: Considerations and approaches. Plant Sci 163:673–681

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Broekaert WF, Lee HI, Kush A, Chua NH, Raikhel NV (1990) Wound-induced accumulation of mRNA containing a hevein sequence in laticifers of rubber tree (*Hevea brasiliensis*. Proc Natl Acad Sci U S A 87:7633–7637

Butaye K, Cammue B, Delauré S, De Bolle M (2005) Approaches to minimize variation of transgene expression in plants. Mol Breed 16:79–91

Caporale C, Di Berardino I, Leonardi L, Bertini L, Cascone A, Buonocore V, Caruso C (2004) Wheat pathogenesis-related proteins of class 4 have ribonuclease activity. FEBS Lett 575:71–76

Caruso C, Caporale C, Poerio E, Facchiano A, Buonocore V (1993) The amino acid sequence of a protein from wheat kernel closely related to proteins involved in the mechanism of plant defence. J Protein Chem 12:379–386

Caruso C, Caporale C, Chilosi G, Vacca F, Bertini L, Magro P, Poerio E, Buonocore V (1996) Structural and antifungal properties of a pathogenesis-related protein from wheat kernel. J Protein Chem 15:35–44

Caruso C, Chilosi G, Caporale C, Leonardi L, Bertini L, Magro P, Buonocore V (1999a) Induction of pathogenesis-related proteins in germinating wheat seeds infected with *Fusarium culmorum*. Plant Sci 140:87–97

Caruso C, Bertini L, Tucci M, Caporale C, Leonardi L, Saccardo F, Bressan RA, Veronese P, Buonocore V (1999b) Isolation and characterization of wheat cDNA clones encoding PR4 proteins. DNA Seq 10:301–307

Caruso C, Nobile M, Leonardi L, Bertini L, Buonocore V, Caporale C (2001a) Isolation and amino acid sequence of two new PR-4 proteins from wheat. J Protein Chem 20:327–335

Caruso C, Bertini L, Tucci M, Caporale C, Nobile M, Leonardi L, Buonocore V (2001b) Recombinant wheat antifungal PR4 proteins expressed in *Escherichia coli*. Protein Expr Purif 23:380–388

Christensen AB, Cho BH, Næsby M, Gregersen PL, Brandt J, Madriz-Ordenana K, Collinge DB, Thordal-Christensen H (2002) The molecular characterization of two barley proteins establishes the novel PR-17 family of pathogenesis-related proteins. Mol Plant Pathol 3:135–144

Cornelissen B, Melchers L (1993) Strategies for control of fungal diseases with transgenic plants. Plant Physiol 101:709–712

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Evans IJ, Greenland AJ (1998) Transgenic approaches to disease protection: applications of antifungal proteins. Pestic Sci 54:353–359

Friedrich L, Moyer M, Ward E, Ryals J (1991) Pathogenesis-related protein 4 is structurally homologous to the carboxy-terminal domains of hevein, Win-1 and Win-2. Mol Gen Genet 230:113–119

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Gao AG, Hakimi SM, Mittanck CA, Wu Y, Woerner BM, Stark DM, Shah DM, Liang J, Rommens CMT (2000) Fungal pathogen protection in potato by expression of a plant defensin peptide. Nat Biotechnol 18:1307–1310

Hejgaard J, Jacobsen S, Bjorn SE, Kragh KM (1992) Antifungal activity of chitin-binding PR-4 type proteins from barley grain and stressed leaf. FEBS Lett 307:389–392

Hobbs SL, Warkentin TD, DeLong CM (1993) Transgene copy number can be positively or negatively associated with transgene expression. Plant Mol Biol 21:17–26

Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and General Method for Transferring Genes into Plants. Science 227:1229–1231

Jayaraj J, Punja ZK (2007) Combined expression of chitinase and lipid transfer protein genes in transgenic carrot plants enhances resistance to foliar fungal pathogens. Plant Cell Rep 26:1539–1546

Kishimoto K, Nishizawa Y, Tabei Y, Hibi T, Nakajima Mand Akutsu K (2002) Detailed analysis of rice chitinase gene expression in transgenic cucumber plants showing different levels of disease resistance to gray mold (*Botrytis cinerea*). Plant Sci 162:655–662

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lan HY, Tian YC, Wang CH, Liu GZ, Zhang LH, Wang LL, Chen ZH (2000) Studies of transgenic tobacco plants expressing β-1,3-glucanase and chitinase genes and their potential for fungal resistance. Yi Chuan Xue Bao 27:70–77

Lee YK, Hippe-Sanwald S, Lee SC, Hohenberg H, Hwang BK (2000) In situ localization of PR-1 mRNA and PR-1 protein in compatible and incompatible interactions of pepper stems with *Phytophthora capsici*. Protoplasma 211:64–75

Li ZC, McClure JW, Hagerman AE (1989) Soluble and bound apoplastic activity for peroxidase, ß-D-glucosidase, malate dehydrogenase and non-specific arylesterase, in barley (*Hordeum vulgare* L.) and oat (*Avena sativa* L.) primary leaves. Plant Physiol 90:185–190

Linthorst HJM (1991) Pathogenesis-related proteins of plants. Crit Rev Plant Sci 10:123–150

Liu D, Rhodes D, Paino D'Urzo M, Xu Y, Narasimhan ML, Hasegawa PM, Bressan RA, Abad L (1996) In vivo and in vitro activity of truncated osmotin that is secreted into the extracellular matrix. Plant Sci 121:123–131

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco cultures. Physiol Plant 15:473–497

Nakamura K, Matsuoka K (1993) Protein targeting to the vacuole in plant cells. Plant Physiol 101:1–5

Pelegrini PB, Franco OL (2005) Plant γ-thionins: Novel insights on the mechanism of action of a multi-functional class of defense proteins. Int J Biochem Cell Biol 37:2239–2253

Ponstein AS, Bres-Vloemans SA, Sela-Buurlage MB, van den Elzen PJM, Melchers LS, Cornelissen BJC (1994) A novel pathogen- and wound-inducible tobacco (*Nicotiana tabacum*) protein with antifungal activity. Plant Physiol 104:109–118

Potter S, Uknes S, Lawton K, Winter AM, Chandler D, Dimaio J, Novitzky R, Ward E, Ryals J (1993) Regulation of a hevein-like gene in *Arabidopsis*. Mol Plant Microbe Interact 6:680–685

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. A laboratory manual. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Sanger F, Micklen S, Coulson AR (1977) DNA sequencing and chain-terminating inhibitors. Proc Natl Acad Sci U S A 74:5463–5467

Stam M, Mol JNM, Kooter JM (1997) The silence of genes in transgenic plants. Ann Bot 79:3–12

Stanford A, Bevan M, Northcote D (1989) Differential expression within a family of novel wound-induced genes in potato. Mol Gen Genet 215:200–208

Svensson B, Svendsen I, Hojrup P, Roepstorff P, Ludvigsen S, Poulsen FM (1992) Prymary structure of barwin: a barley seed protein closely related to the C-terminal domain of proteins encoded by wound-induced plant genes. Biochemistry 31:8767–8770

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 76:4350–4354

Van Loon LC, Van Strien EA (1999) The current status of Pathogenesis-Related proteins In: Proceedings of the 9th International Congress on Molecular Plant-Microbe Interactions, Amsterdam, The Netherlands, 25–30 July 1999

Vellicce GR, Díaz Ricci JC, Hernández L, Castagnaro AP (2006) Enhanced resistance to *Botrytis cinerea* mediated by the transgenic expression of the chitinase gene *ch5 B* in strawberry. Transgenic Res 15:57–68

Vierheilig H, Alt M, Neuhaus JM, Boller T, Wiemken A (1993) Colonization of transgenic *Nicotiana sylvestris* plants, expressing different forms of *Nicotiana tabacum* chitinase, by the root pathogen *Rhizoctonia solani* and by the mycorrhizal symbiont *Glomus mosseae*. Mol Plant-Microbe Interac 6:261–264

Weide R, Koornneef M, Zabel P (1989) A simple, nondestructive spraying assay for the detection of an active kanamycin resistance gene in transgenic tomato plants. Theor Appl Genet 78:169–172

Woloshuk CP, Meulenhoff JS, Sela-Buurlage M, van den Elzen PJM, Cornelissen BJC (1991) Pathogen-induced proteins with inhibitory activity toward *Phytophthora infestans*. Plant Cell 3: 619–628

Yan X, Gonzales RA, Wagner GJ (1997) Gene fusions of signal sequences with a modified beta-glucuronidase gene results in retention of the beta-glucuronidase protein in the secretory pathway/plasma membrane. Plant Physiol 115:915–924

Yang X, Xiao Y, Wang X, Pei Y (2007) Expression of a novel small antimicrobial protein from the seeds of motherwort (*Leonurus japonicus*) confers disease resistance in tobacco. Appl Environ Microbiol 73:939–946

Zhang HX, Zeevaart JAD (1999) An efficient *Agrobacterium tumefaciens*-mediated transformation and regeneration system for cotyledons of spinach (*Spinacia oleracea* L.). Plant Cell Rep 18:640–645