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Increased tolerance to *Phytophthora citrophthora* in transgenic orange plants constitutively expressing a tomato pathogenesis related protein PR-5

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Abstract

Phytophthora citrophthora is the most widely spread oomycete plant pathogen over all the citrus growing areas and represents one of the major causes of crop losses. Constitutive over-expression of genes encoding proteins involved in plant defence mechanisms to disease is one of the strategies proposed to increase plant tolerance to oomycete and fungal pathogens. P23 (PR-5), a 23-kDa pathogenesis-related protein similar to osmotins, is induced in tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) plants when they are infected with citrus exocortis viroid, and its antifungal activity has been demonstrated in *in vitro* assays. We have successfully produced transgenic orange (*Citrus sinensis* L. Obs. cv. Pineapple) plants bearing a chimeric gene construct consisting of the cauliflower mosaic virus 35S promoter and the coding region of the tomato pathogenesis-related PR-5. Nine regenerated transgenic lines constitutively expressed the PR protein. They were challenged with *Phytophthora citrophthora* using a detached bark assay. A significant reduction in lesion development was consistently observed in one transgenic line in comparison to the control plants. This same line achieved plant survival rates higher than control plants when transgenic trees were inoculated with oomycete cultures. These results provide evidence for the *in vivo* activity of the tomato PR-5 protein against *Phytophthora citrophthora*, and suggest that this may be employed as a strategy aimed at engineering *Phytophthora* disease resistance in citrus.

Introduction

Phytophthora spp. cause foot rot and gummosis, the most serious soilborne diseases of citrus worldwide. Foot rot is an injury of bark on the trunk or roots near ground level. Gummosis is a rotting of bark anywhere on the tree. Nursery trees and young orchid trees can be rapidly girdled and killed. Large trees may be killed but usually they are only partially girdled, and the injury causes a decline of the canopy, with defoliation, twig dieback, and short growth flushes. The most common and important *Phytophthora* spp. that attack citrus are *P. parasitica* Dast. and *P. citrophthora* (Smith & Smith) Leonian. *P. citrophthora* attacks aerial plant parts more frequently than *P. parasitica* and also produces brown rot, a disease that affects fruits causing a firm light brown decay, and finally fruit fall. Affected fruits entering the packinghouse commonly create a postharvest decay problem [5].

Currently, control in the field against foot rot and gummosis diseases has been based on the use of tolerant rootstocks, maintaining the plants under the best cultural conditions, and by the application of costly agrochemical treatments. However, there are few high quality rootstocks tolerant to *Phytophthora*, which limitates the development of citriculture in many ar-

eas. Moreover, there are no effective ways to control the brown rot disease. Therefore, in the long term, the development of crops that possess durable genetic resistance provides the best prospect for effective, economical and environmentally safe control of the epidemic spread of notoriously difficult to manage *Phytophthora* diseases. Genetic transformation offers an attractive system for the introduction of genes conferring resistance or tolerance into specific citrus rootstocks and varieties.

A strategy proposed to increase fungal and oomycete protection has been the constitutive overexpression in plants of genes whose products have *in vitro* antifungal activity. A wide range of plant defensive antifungal proteins has been identified and is being utilised in attempts to provide protection via expression in transgenic crops [6]. The major class of these antimicrobial proteins are collectively referred to as 'pathogenesis-related proteins' (PRs). These PRs, defined as proteins coded for by the host plant but induced specifically in pathological situations, do not only accumulate locally in the infected site, but are also induced systemically associated with the development of Systemic Acquired Resistance (SAR) against further infection by fungi, oomycete, bacteria and viruses [6]. Induction of PRs has been found in most plants [34]. It has been suggested that the collective set of PRs may be effective in inhibiting pathogen growth, multiplication and/or spread and they may, at least in part, be responsible for the state of SAR [34].

Fourteen main classes of PRs (PR-1-14) have been classified based on primary structure, serology, and/or enzymatic or biological activity in providing protection against pathogens [34]. Among these PR proteins, osmotin and thaumatin-like proteins have been recognised as members of plant PR-5-type proteins [33, 34]. There are now many lines of evidence indicating that proteins of the PR-5 group from various plant species have *in vitro* antifungal activity against several classes of fungi and oomycetes [34, 35].

P23 is a 23-kDa PR-5 protein induced in tomato plants when they are infected with citrus exocortis viroid (CEVd) [24]. It is homologous to the salt-induced tomato NP24 protein and displays sequence identity with the *Phytophthora*-induced antifungal protein AP24 [35]. *In vitro* assays indicated that the purified P23 protein inhibits the growth of several phytopathogenic fungi and oomycetes, as *Trichothecium roseum*, *Fusarium oxysporum* f. sp. *lycopersici*, *Colletotrichum coccodes* and *Phytophthora citrophthora* [25].

In this study, we have investigated the antifungal properties of tomato P23 (PR-5) *in vivo* by introducing the tomato PR-5 coding sequence under the 35S promoter in sweet orange (*Citrus sinensis* L. Osb. cv. Pineapple) plants, and testing their tolerance to *Phytophthora citrophthora* infections. We demonstrate that transgenic plants constitutively expressing tomato PR-5 protein exhibited significant protection against this citrus pathogen, providing evidence of an *in vivo* role of this PR protein in disease resistance.

Materials and methods

Vector constructions

A PR-5 clone was isolated from a tomato VFN8 genomic library (Clontech), using a PR P23 cDNA [25] as a probe. The genomic PR-5 clone (EMBL accession number AJ277064) contained a complete ORF without introns. The coding sequence was amplified from the phage clone by PCR using the following oligonucleotides: 5′-CG GATCCTACAACTTCTTATAC-3′ (forward primer) and 5′-GGAGCTCCAAATGCACTCTTG-3′ (reverse primer), yielding a DNA sequence of 967 bp. A PR-5 expression cassette was constructed by placing the PR-5 coding sequence under the transcriptional control of an enhanced cauliflower mosaic virus (CaMV) 35S promoter (Figure 1), and a *nos* terminator was added to the PR-5 3′ end. All these operations were carried out in pBLUESCRIPT phagemid (Stratagene).

This construct was finally subcloned into the plant transformation binary vector pBI121 at the unique *Hin*dIII site. The resulting plasmid was named pBI121.*P23*. In this vector, the PR-5 expression cassette is flanked by the neomycin phosphotransferase II (*npt*II) gene driven by the nopaline synthase (*nos*) promoter and terminator sequences, which provides resistance to kanamycin, as a selectable marker, and the β-glucuronidase (*uidA*) gene driven by the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (*nos*) terminator, which was used as reporter and selectable marker gene (Figure 1).

Plant transformation and regeneration

PBI121 and pBI121.*P23* were introduced in the disarmed *Agrobacterium tumefaciens* strain EHA105 [10] by electroporation. Bacteria were cultured overnight in an orbital shaker at 28 °C and 200 rpm in LB medium [26] containing 25 mg/l kanamycin and

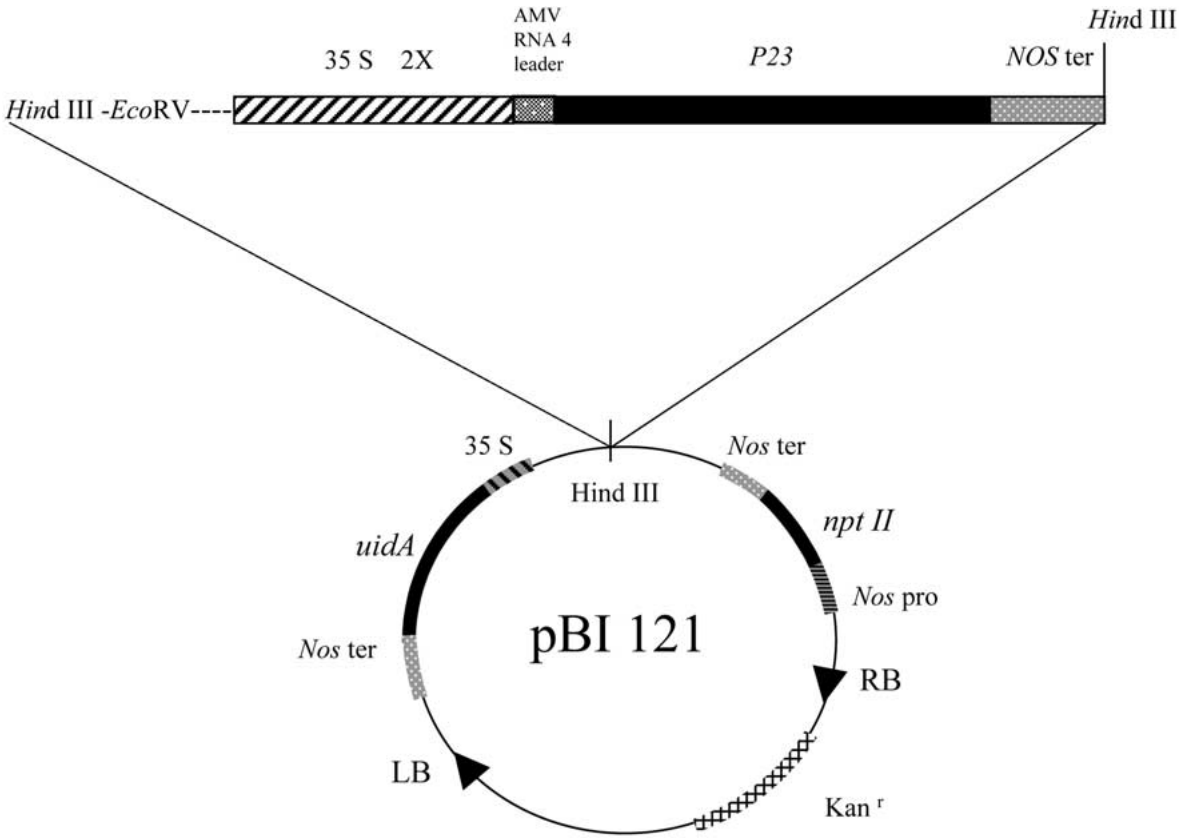

Figure 1. Schematic representation of the tomato *P23 (PR-5)* chimeric gene construct used in *Agrobacterium*-mediated transformation experiments. The tomato *PR-5* gene was inserted as a *Hin*dIII fragment into the binary plasmid pBI121. The location of important genetic elements within the binary vector pBI121.*P23* is indicated. RB: right border; LB: left border; Kan^r: kanamycin resistance gene; *nptII*: neomycin phosphotransferase II gene cassette; *uid*A: β-glucuronidase gene cassette. Translatable tomato *PR-5* gene is driven by double enhanced cauliflower mosaic virus (CaMV) 35S promoter, AMV RNA4 leader sequence and *nos* terminator.

25 mg/l nalidixic acid. Bacterial cells were pelleted at 3500 rpm for 10 min, resuspended and diluted to 4×10^7 cells/ml in liquid inoculation medium, which consisted of MS salt solution of Murashige and Skoog [19], 0.2 mg/l thiamine hydrochloride, 1 mg/l pyridoxine hydrochloride, 1 mg/l nicotinic acid and 3% (w/v) sucrose, pH 5.7.

Six-month-old greenhouse-grown Pineapple sweet orange seedlings were used as the source of tissue for transformation. Stem pieces (20 cm in length) were stripped of their leaves and thorns, disinfected for 10 min in a 2% (v/v) sodium hypochlorite solution and rinsed three times with sterile distilled water. Internodal stem segments (about 1 cm long) were incubated for 15 min in 10-cm-diameter plates containing 15 ml of the bacterial suspension in inoculation medium by gentle shaking. The infected explants were blotted dry on sterile filter paper and placed horizontally on plates with CM medium for a 3-days co-cultivation period. CM medium consisted of MS salts, 1 mg/l thiamine hydrochloride, 1 mg/l pyridoxine hydrochloride, 1 mg/l nicotinic acid, 3% (w/v) sucrose, 2 mg/l indole-3-acetic acid, 1 mg/l 2-isopentenyladenine, 2 mg/l 2,4-dichlorophenoxyacetic acid, and 0.8% (w/v) agar, pH 5.7.

After co-cultivation, the explants were blotted dry with sterile filter paper and transferred to SRM medium, which consisted of MS salts, 0.2 mg/l thiamine hydrochloride, 1 mg/l pyridoxine hydrochloride, 1 mg/l nicotinic acid, 3% (w/v) sucrose, 1% (w/v) agar, pH 5.7, plus 100 mg/l kanamycin for the selection of transgenic shoots and 250 mg/l vancomycin and 500 mg/l cefotaxime to control bacterial growth, and supplemented with 3 mg/l benzylaminopurine. Cultures were maintained in the dark for 4 weeks at 26 °C and then were transferred to 16 h photoperiod, 45 μEm^{-2} s^{-1} illumination, 60% RH, and 26 °C.

Small pieces of the shoots emerging from the explants, were assayed histochemically for GUS activity [11], and then apical portions were grafted *in vitro* onto Troyer citrange (*C. sinensis* L. Osbeck × *Poncirus trifoliata* L. Raf.) seedlings [20]. A new grafting of the *in vitro*-growing plants on vigorous rootstocks allowed the rapid acclimatisation and development of plants under greenhouse conditions [21].

Molecular analyses of the transgenic plants

Standard PCR techniques were used to detect the presence of the tomato *PR-5 (P23)* transgene in leaf samples from the regenerated putative transgenic plantlets. Primers used were: 5′-CGGATCCTACAACTTCTTATAC-3′ and 5′-GGAGC TCCAAATGCACTCTTG-3′, for the amplification of a 967 bp *PR-5* gene fragment. Reactions were performed in a thermal cycler under the following conditions: 40 cycles of 1 min at 94 °C, 2 min at 60 °C and 1 min at 72 °C plus a final segment of 72 °C for 2 min.

Southern analyses were performed to confirm the stable integration of the *PR-5 (P23)* transgene in the transgenic plants. DNA was isolated from fully expanded leaves of growing flushes according to Dellaporta et al. [4]. *Hin*dIII and *Dra*I-digested DNA samples (20 μg) were electrophoresed on 1% (w/v) agarose gels and transferred onto Hybond-N$^+$ membranes (Amersham). Filters were probed with a digoxigenin (DIG; Boehringer-Mannheim) labelled fragment of the coding region of the *PR-5* gene prepared by PCR, according to suppliers' instructions. Chemiluminescent detection of target DNA was undertaken using CSPD-Star substrate (Tropix, Bedford, USA).

Leaf tissue was also used to obtain crude protein extracts for Western analysis. Extraction buffer was 100 mM Tris-HCl pH 6.8, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.3% (v/v) β-mercaptoethanol. Protein content of these crude extracts was quantified according to Bradford [2], using the Protein Assay Dye Reagent (Bio-Rad, USA) and bovine serum albumin as standard. Protein extracts (10 μg) were fractionated by electrophoresis on SDS-PAGE (14% polyacrylamide) and electroblotted onto Immobilon-PVDF membranes (Millipore) using a semidry transfer system (Biorad), following suppliers' instructions. Immunodetection was performed using a 1:500 dilution of a P23 antiserum as primary antibody [25], and alkaline–phosphatase-conjugated anti-rabbit IgGs (Boehringer-Mannheim) as secondary antibody. Crude acidic extracts from CEVd-infected tomato leaves were used as positive control [7].

Pathogenicity assays

Phytophthora citrophthora was obtained from the IVIA culture collection of plant pathogenic fungi and oomycetes. It was grown in the dark at 22–24 °C for 10 days in 8.5 cm diameter Petri dishes containing potato dextrose agar (PDA). Transgenic sweet orange plant lines containing the T-DNA from pBI121.*P23* and from pBI121 (controls) were propagated on vigorous rootstocks and grown in a temperature-controlled greenhouse with 24–26/15–16 °C day/night temperature, and 60–80% RH. Plants were grown in individual 2.5 l pots containing a mixture of 55% sphagnum peat and 45% siliceous sand, and were fertilized weekly. Two types of pathogenicity bioassays were performed:

Detached bark assay

Nine to 12-month-old transgenic plants with a stem diameter of about 1 cm were used for these experiments, which were carried out as described by Tuset et al. [32]. Stem portions of about 12 cm long were cut from the basal part of the tree, externally disinfected with sodium hypochlorite at 5% (v/v) for 10 min, and rinsed 3–5 times with sterile distilled water. Subsequently, the bark was detached from the wood and a 0.5 cm diameter disk from the oomycete culture grown in PDA was placed at the inner side of the bark piece. These bark pieces were then kept in a moisture chamber (100% RH) at 22–24 °C in the dark. Three pieces of bark with two inoculation sites were prepared per *PR-5*-transgenic and control line. After 3–4 days, the rot area was measured. The experiment was repeated three times in different seasons (November, March, and June). The data for each *PR-5*-transgenic and control line were averaged over each experiment, and the standard error of the means was calculated.

Whole plant assay

Ten vegetatively propagated plants of each PR-5-transgenic and control line grafted on vigorous rootstocks were used for this assay. Plants (9 to 12-month-old) were transferred to a greenhouse (maximum temperature of 27 °C). A wound was performed in the basal portion of the stem of each transgenic plant, and *Phytophthora citrophthora* was inoculated by placing a quarter of a 0.5 cm diameter disk from the oomycete culture grown in PDA on each wound. Inoculation

points were covered with wet cotton and aluminium foil to maintain high relative humidity. After five days, the disk piece was removed. Tolerance to oomycete disease was assessed by estimating the plant survival rate one year after inoculation.

Statistical analyses were performed using the SAS statistical software package (SAS Institute, Cary, NC).

Results

Recovery of transgenic orange plants constitutively expressing the tomato PR-5 protein

Nine GUS-expressing shoots (lines 1 to 9) were regenerated from orange explants co-cultivated with *A. tumefaciens* EHA 105/pBI121.*P23*, and also two GUS-positive shoots (lines A and B) were recovered from explants transformed with *A. tumefaciens* EHA 105 harbouring the binary plasmid pBI121, which were used as controls.

Insertion of the tomato *PR-5 (p23)* transgene into the orange genome was tested by PCR analysis, showing amplification of the expected 967 bp fragment for the nine putative transgenic plantlets (results not shown). This was confirmed by performing a Southern analysis. DNA digested with *Hin*dIII liberated the *PR-5* gene cassette (2.1 kb approx.) (Figure 2c) and hybridised with a probe of the *PR-5* coding region for all the nine transgenic lines (Figure 2b, lanes 1–9). No hybridising bands were detected in DNA from the two lines transformed with the pBI121 control vector (Figure 2b, lanes A and B). DNA digested with *Dra*I, that cut once the T-DNA near the RB (Figure 2c), and hybridised with a probe of the *PR-5* coding region, revealed the number of inserted loci of the *PR-5* transgene in the plant's genome. It ranged from one locus in lines 1, 2, 5, 7, 8 and 9, to four loci in line 4 (Figure 2a).

Western analysis from the transgenic plants revealed variable levels of expression of a 23 kDa protein that immunoreacted with the P23 antiserum and co-migrated with the tomato pathogenesis-related P23 protein induced by CEVd infection (Figure 3). In most transgenic lines, the expression level was high, which is consistent with the regulatory properties of the CaMV 35S promoter. Only transgenic line 5 showed almost no detectable immunoblot reaction. Total soluble proteins extracted from transgenic control lines (Figure 3, lanes A and B) did not show any reaction in the Western analysis.

Plants from all the transgenic lines growing at the greenhouse were phenotypically indistinguishable from Pineapple sweet orange non-transformed plants developed under the same environmental conditions.

Evaluation of oomycete tolerance in transgenic orange plants

Transgenic plants expressing the tomato PR-5 (P23) protein were assayed for protection against *Phytophthora citrophthora* infection. In order to determine whether the effect on tolerance to the oomycete disease was due only to the *PR-5 (P23)* transgene product, and not a result of some epigenetic effect of the transformation/regeneration process, orange lines A and B transformed with the vector pBI121 were included as controls in every bioassay.

Penetration of *Phytophthora* in the suberized tissues at the basal portion of the stem in citrus is usually produced through lesions made on the periderm. This behaves as a barrier preventing penetration of the fungi. Wounds or natural cracks in the bark are the natural means of infection. Colonization of cortical tissues starts once the zoospores have passed over the barrier of cork cambium. We have used a detached bark inoculation technique as standard bioassay to detect oomycete colonization on the bark of transgenic tissues, which only requires a small number of plants and short time for each test. Expanding dark-brown rot area around the mycelial disks was measured and the oomycete activity was rated as the reduction in rot area in *PR-5*-expressing bark portions compared to controls. Transgenic control lines A and B showed similar sensitivity to *Phytophthora*, since rot area was not significantly different in A and B samples. Thus, transgenic control lines were averaged and considered as a single control line in Table1. Transgenic *PR-5*-orange lines displayed different levels of tolerance to the pathogen. At 3 days post-inoculation, only line 8 showed higher tolerance to *Phytophthora* than the control line in the three bioassays performed. Lines 3, 7, and 9 were more tolerant than the control in two bioassays, and lines 2 and 4 were more tolerant only in one bioassay. At 4 days post-inoculation, line 8 remained more tolerant to the oomycete infection in two bioassays, and lines 3, 7, and 9 only in one bioassay (Table 1). Rot area for the same transgenic line was very variable in different bioassays, probably due to the fact that the experiments were performed in different seasons with plants of different sizes, ages and physiological state.

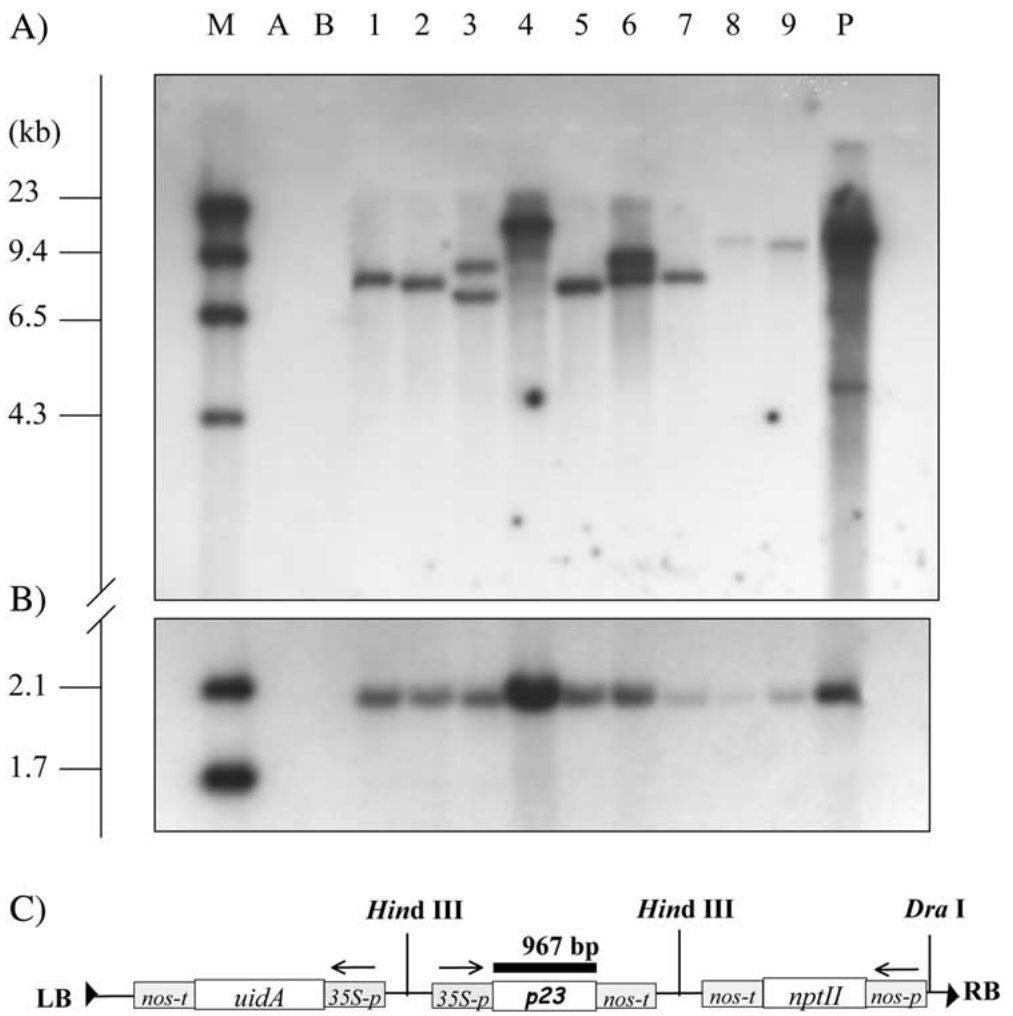

Figure 2. Detection of the *P23 (PR-5)* transgene integrated in orange plants by Southern analysis of genomic DNA. The probe used was a PCR-amplified and digoxigenin-labelled fragment of the tomato *P23 (PR-5)* gene. Lane P: pBI121.*P23* plasmid digested with *Hin*dIII, used as positive control. Lanes A and B: DNA extracted from control orange plants transformed with the vector pBI121. Lanes 1–9: DNA extracted from *P23 (PR-5)*-transgenic orange lines. Lane M: molecular weight marker. Molecular weight is indicated in kilobases. A) Southern blot from DNA digested with *Dra*I, that cut once the T-DNA near the RB; B) Southern blot from DNA digested with *Hin*dIII, that excise the *P23 (PR-5)* expression cassette; C) Schematic representation of pBI121.*P23* T-DNA, indicating restriction sites and the 967 bp probe used.

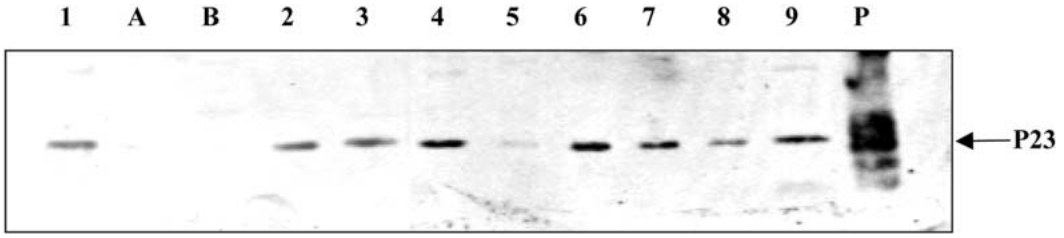

Figure 3. Western blot analysis of proteins extracted from transgenic orange plants. Lane P: protein extract of a tomato plant infected with CEVd, used as a positive control. Lanes A and B: orange lines transformed with the pBI121 control vector. Lanes 1–9: individual *PR-5*-trangenic citrus lines. The arrow shows the position of the P23 protein.

Table 1. Rot area induced by *Phytophthora citrophthora* inoculation on internal bark portions from transgenic orange plants expressing the tomato *PR-5* protein gene (lines 1–9) and control lines A and B, represented as a single control line. Three bioassays A), B) and C) were performed in different seasons (A, winter; B, spring; C, summer). Values are means of six replicates per transgenic line and 12 replicates from the controls.

Line	Rot area ($cm^2 \pm$ se)	
	3 D.P.I.[1]	4 D.P.I.
A		
1	2.41 ± 0.16	NT[2]
2	1.90 ± 0.10	NT
3	1.62 ± 0.17*	NT
4	2.92 ± 0.18	NT
5	2.37 ± 0.12	NT
6	2.32 ± 0.20	NT
7	1.50 ± 0.07*	NT
8	1.16 ± 0.36*	NT
9	1.13 ± 0.08*	NT
Control	2.32 ± 0.16	NT
B		
1	5.20 ± 0.50	13.78 ± 0.37
2	5.80 ± 0.86	13.68 ± 1.27
3	5.62 ± 0.48	9.58 ± 0.91*
4	4.70 ± 0.40	12.14 ± 0.56
5	6.90 ± 0.83	15.77 ± 1.27
6	5.98 ± 0.80	15.08 ± 1.13
7	7.47 ± 0.51	13.39 ± 0.52
8	3.45 ± 0.60*	7.09 ± 0.85*
9	3.53 ± 0.36*	6.57 ± 0.59*
Control	6.32 ± 0.35	13.74 ± 0.70
C		
1	10.93 ± 0.65	20.55 ± 1.01
2	10.48 ± 0.82*	14.88 ± 0.53
3	7.70 ± 0.92*	13.88 ± 0.69
4	10.02 ± 1.05*	17.10 ± 1.19
5	13.65 ± 0.78	20.60 ± 1.79
6	13.48 ± 0.18	17.40 ± 0.68
7	8.48 ± 0.91*	13.77 ± 0.58*
8	5.83 ± 0.69*	11.33 ± 0.49*
9	13.23 ± 0.86	17.17 ± 1.04
Control	13.96 ± 0.55	16.99 ± 0.71

[1]D.P.I.: days post-inoculation.
[2]N.T.: not tested.
*indicate significant differences at the $P \leq 0.05$ level between transgenic and control lines according to Dunnett's one way T-test.

To confirm these results in whole trees, one-year-old transgenic orange trees were inoculated with *Phytophthora citrophthora*. All the plants developed a dark-brown rot in the bark along the stem about ten days after inoculation and some of them produced gum exudates. However, in the following weeks stem area affected by necrosis was smaller in lines 8 and 3 than in control plants (results not shown). Two months after inoculation, the most susceptible transgenic lines showed pale green leaves, defoliation and withered state (Figure 4A). These *Phytophthora* disease symptoms finally caused the death of the susceptible orange plants (Figure 4B). However, other plants remained symptomless even one year after inoculation (Table 2).

Between 70 and 80% of the inoculated plants from transgenic lines 3, 4, 7, and 9 were alive one year post-inoculation, whereas only 50% of the inoculated plants from control lines A and B, averaged as a single control line, survived after *Phytophthora* infection (Table 2). The highest survival rate was achieved by transgenic line 8, since only one of ten inoculated plants died at the end of the assay. In addition, using a generalised linear model approach with a logit link function (Genmod test; SAS [27], Institute Cary, NC) line 8 showed a survival rate significantly different ($P < 0.10$) from the control line (Table 2). These results were in good correlation with those obtained with the detached bark bioassays, strongly indicating that at least transgenic line 8 showed increased protection against *Phytophthora citrophthora* infections.

Table 2. Survival rate of transgenic orange plants expressing the tomato *PR-5* protein gene (lines 1–9) and control lines A and B, represented as a single control line, after one year of inoculation with *Phytophthora citrophthora*. Ten vegetative propagated plants per transgenic line and 20 control plants (10 plants per line) were inoculated with the oomycete pathogen.

Line	Plant survival (%)
1	40
2	40
3	70
4	80
5	50
6	40
7	70
8	90*
9	80
Control	50

*indicate a significant difference from the control at $P < 0.10$ based on Genmod test using a generalised lineal model (GLM) procedure in SAS (Institute Cary, NC).

Discussion

In this study we describe the expression of a tomato PR-5 (P23) pathogenesis-related protein in transgenic orange plants and provide evidence of an *in vivo* biological role for this protein in plant defence against the oomycete pathogen *Phytophthora citrophthora* that causes gummosis and foot rot of citrus trees. Among citrus, we have chosen orange as a host to perform this research because of its well-known susceptibility to *Phytophthora* [31] and because of its economic importance, since it accounts for approximately 70% of the total citrus production in the world.

A common approach for the enhancement of antifungal protection has been the over-expression in transgenic plants of single genes encoding PR proteins, which have *in vitro* activity against one or more plant pathogens. Possibly, the most extensively used of these single genes have been chitinase genes, whose products catalyse the hydrolysis of chitin, the major component of the cell wall of most filamentous fungi [8, 15, 22, 29, 30]. However, this strategy seems not to be useful against oomycetes, such as *Phytophthora* spp. and others. In contrast to filamentous fungi, oomycetes have been described as insensitive to a mixture of hydrolytic enzymes such a β-1,3 glucanases and chitinases [17, 28]. They contain little or no chitin in their cell walls and therefore unlikely are affected by chitinases expressed in transgenic plants [3]. Furthermore, biochemical data in conjunction with phylogenetic analyses suggest that they share little taxonomic affinity to filamentous fungi. Oomycetes might have evolved strategies for interacting with plants that differ from those used by fungi [13, 14]. Considering that resistance against oomycetes is inducible in several plants species [9, 18], Woloshuk et al. [35] identified a pathogen-induced osmotin (AP24) with inhibitory activity *in vitro* toward *Phytophthora infestans*, causal agent of late blight in potato and tomato. Since then, a few reports on the production of transgenic plants that express osmotin proteins with improved resistance to oomycete diseases have been published. Liu et al. [16] demonstrated that over-expression of a tobacco osmotin of 24 kDa, that accumulates in NaCl and desiccation-adapted tobacco

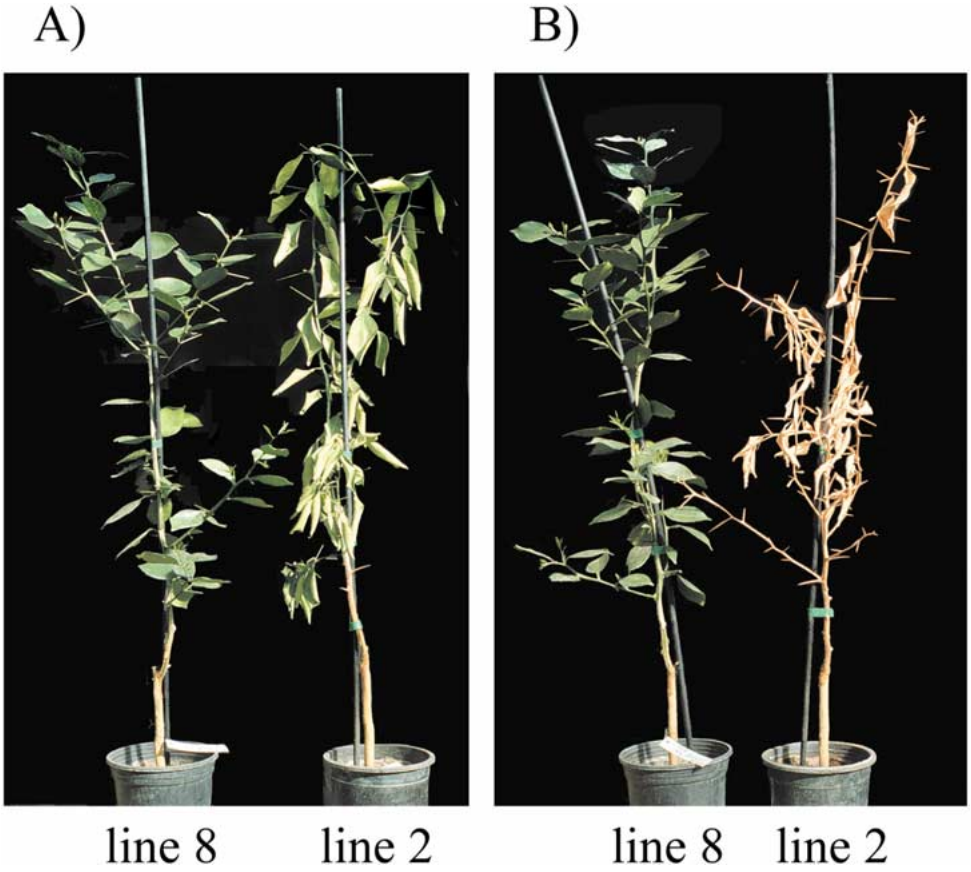

Figure 4. Protection in *PR-5*-transgenic orange plants against *Phytophthora citrophthora.* Comparison between a plant from the highly protected transgenic line 8 (left) and a plant from the susceptible transgenic line 2 (right) at (A) two months, and (B) six months after inoculation.

cells, delayed the development of disease symptoms in transgenic potato plants when challenged with *Phytophthora infestans*, but had no effect in transgenic tobacco plants inoculated with *P. parasitica* var. *nicotianae*. Although some inhibition of *P. parasitica* was observed in *in vitro* experiments, no *in vivo* effect could be established. More recently, Zhu et al. [36] reported that transgenic potato plants expressing high levels of the pA13 osmotin-like protein showed an increased tolerance to the late-blight (*P. infestans*) pathogen. Here, our results provide clear evidence of a biological role for a tomato PR-5 protein in plant defence against the economically important oomycete pathogen *Phytophthora citrophthora*. It is shown for the first time antipathogenic activity *in vivo* of a PR-5 against a oomycete in a transgenic woody tree species.

In previous reports, it has been considered that selection of transgenic lines with the highest protein expression levels might increase the likelihood of obtaining a disease tolerant phenotype [12, 22]. Broglie et al. [3] reported that the level of resistance to *Rhizoctonia solani* in transgenic tobacco plants expressing a basic chitinase gene was correlated with the level of chitinase expression. Liu et al. [16] selected transgenic plants showing the highest levels of osmotin accumulation for oomycete resistance assays. In contrast, we did not see correlation between disease development and level of PR-5 protein accumulation. However, disease rating of individual lines coincided in experiments performed under very different conditions, using detached bark pieces in the laboratory or using whole plants in the greenhouse. In all cases, line 8 exhibited the highest level of tolerance to *Phytophthora* infection, despite the small amount of PR-5 protein that accumulated. Lack of correlation between PR protein expression and protection has also been reported by others [1, 36]. When we tested histochemical GUS activity in different leaf and bark pieces from the transgenic orange lines, high and low GUS activity correlated with high and low levels, respec-

tively, of PR-5 accumulation. In addition, we found the same, high or low GUS activity, in different bark and leaf tissues from the same transgenic line in all cases (results not shown). These results indirectly indicated that *uidA* and *PR-5* expression were correlated and that their expression was constitutive in different orange tissues. This is not strange since *uidA* and *PR-5* transgenes were located at the same T-DNA and thus were inserted at the same plant genome sites, and their expression was under the control of the CaMV 35S promoter also for both transgenes. All this suggests that position effect of the integrated transgenes and consequently timing of protein expression, together with interaction with other defence factors, is more important than the absolute level of PR protein expressed.

The exact nature of tolerance to *Phytophthora citrophthora* provided by constitutive tomato P23 (PR-5) expression in transgenic orange plants is unknown. Woloshuk et al. [35] indicated that osmotins could cause sporangia lysis of *P. infestans*. Some lines of evidence suggest that proteins of the PR-5 group inhibit hyphal growth and promote hyphal and spore lysis probably by a oomycete membrane permeabilizing mechanism [23]. Thus, tomato P23 could exert a direct non-hydrolytic fungicidal effect on hyphal growth.

Further enhancement of the level of protection provided by P23 could possibly be obtained by manipulating the level, timing and location of protein expression, and by using this strategy combined with integration of other PR genes which could show synergistic effect against this and other oomycetes and fungi affecting citrus. However, we have got consistent protection against *Phytophthora citrophthora* over long time periods in greenhouse experiments by constitutive expression of only one antifungal protein in transgenic plants. The results presented here are promising, but our technical challenge is now to obtain the same results under field conditions and determine whether these transgenic plants could be useful in management of *Phytophthora* diseases in citrus. These experiments are part of a long-term project that is already under way in our Institute.

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