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Potato *R1* resistance gene confers resistance against *Phytophthora infestans* in transgenic tomato plants

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Abstract Tomato is challenged by several pathogens which cause loss of production. One such pathogen is the oomycete *Phytophthora infestans* which is able to attack all the aerial parts of the plant. Although a wide range of resistance sources are available, genetic control of this disease is not yet successful. Pyramiding R-genes through genetic transformation could be a straightforward way to produce tomato and potato lines carrying durable resistance to *P. infestans*. In this work the R1 potato gene was transferred into tomato lines. The tomato transgenic lines were analyzed by using q-RT-PCR and progeny segregation to determine the gene copy number. To test the hypothesis that *R1* represents a specifically regulated R-gene, transgenic tomato plants were inoculated with *P. infestans* isolate 88133 and IPO. All the plants containing the *R1* gene were resistant to the late blight isolate IPO-0 and susceptible to isolate 88133. These results provide evidence for specific activation of the *R1* gene during pathogen challenge. Furthermore, evidence for enhancement of *PR-1* gene expression during *P. infestans* resistance response was obtained.

Keywords Disease resistance gene · Late blight · Heterologue system · Transformation · PR proteins

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Introduction

Late blight caused by *Phytophthora infestans* is a major disease in the Solanaceae family, attacking leaves, stems, and fruits of tomato (Rubin et al. 2001). Disease may be initiated by air-borne sporangia or by oospores harboured in the soil (Rubin and Cohen 2004). Within-season spread occurs via sporangia. Following the attack of this oomycete, the production of tomato fruits is seriously compromised; hence the pathogen is controlled with chemicals, the effect of which is reduced by selection for fungicide-resistant *P. infestans* isolates (Fry and Goodwin 1997).

For these reasons, much effort in recent years has been applied to discover resistance genes (R-genes) conferring complete and broad-spectrum resistance

against *P. infestans* in the *Solanum* genus. Four sources of resistance to late blight have been discovered in tomato wild relatives and transferred into tomato breeding lines: *Ph-1*, located on chromosome 7; *Ph-2* located on chromosome 10 (Moreau et al. 1998); *Ph-3* located on chromosome 9 (Chunwongse et al. 2002) and *Ph-5* (Foolad et al. 2008). Additional studies were performed to discover tomato QTLs giving resistance to *P. infestans* (Foolad et al. 2008). Despite the wide range of resistance sources available, genetic control has not yet been successful due to the partial resistance conferred by such genes and to the rapid evolution of new pathogen strains which are able to overcome the R-genes.

Extensive studies have been performed within potato germplasm, and cultivars resistant to different isolates as well as broad-resistance cultivars have been released. Several of the R-genes in question have been cloned and characterized in potato (Hein et al. 2009). The first was the *R1* gene originated from *S. demissum* (Ballvora et al. 2002). The *R1* resistance gene is a member of the leucine zipper/NBS/LRR class of plant resistance genes and is located on potato chromosome V in a cluster of several *R1* homologues (Ballvora et al. 2002). A QTL is mapped in the neighbourhood of the *R1* cluster and it confers broad resistance to *P. infestans* (Ballvora et al. 2007). The co-localization of R-QTLs and R-genes is frequently observed (Gebhardt and Valkonen 2001; Timmerman-Vaughan et al. 2001; Bai et al. 2003). Some evidence shows that, in potato, the *R1* gene might act synergistically with the QTL enhancing the durability of resistance (Ercolano et al. 2004; Ballvora et al. 2007). Pyramiding R-genes by conventional breeding is laborious, time-consuming and sometimes impossible. Making use of transgenic plants could be a straightforward approach to achieve durable resistance to *P. infestans*.

Pathogenesis-related (PR) proteins are reported to be important players in the *P. infestans* defence response (Hein et al. 2009). Several plant PR proteins are involved in potentially important activities in defence mechanisms through both direct degradation of the pathogen mycelia wall (Hoegen et al. 2002) and during effector-triggered immunity (Jones and Dangl 2006). Assessment of their expression during the infection process can be useful to understand the resistance mechanism activated by the *R1* gene and to elucidate reasons for the establishment of an incompatible plant pathogen interaction.

In this work, we transformed tomato plants with the potato *R1* gene and in attempting to identify its functionality we verified the correct genomic introgression and expression. We then assessed the primary transformants (T0) copy number by using a relative Real Time PCR. Finally, we tested whether *R1* gene confers resistance to *P. infestans* isolate IPO-0 in tomato and if it is able to activate a specific *PR-1* gene reported as expressed during effector-receptor interaction.

Material and methods

Plant materials

Solanum lycopersicum cv Moneymaker and cv San Marzano seeds were used for transformation experiments. Seeds were placed in 50 ml Falcon tubes and were then rinsed, twice in 70% ethanol, for 5 min and 1 min respectively. The solution was then decanted from the tubes. The seeds were washed in 10% of commercial NaOCl (4.95 active chlorides) with 0.1% of SDS twice for 10 min, and then rinsed five times in sterile dd water. The seeds were germinated in a Magenta box containing a germination medium (GM). Approximately 20–30 seeds were placed in each box. The cultures were placed at 27±1°C under a 16 h photoperiod for 10 days.

Plant expression vector, tomato transformation and plant regeneration

Agrobacterium tumefaciens strain LBA4404, containing in a recombinant plasmid pCLD04541 (Tao and Zhang 1998) the potato genomic clone 10-2, was used to generate transgenic tomato plants in this study. This clone is a 10 kb fragment of the potato genome carrying the *R1* gene (provided by Dr. Christiane Gebhardt MPZI, German). According to the procedure described by Park et al. (2003) with some modifications, *A. tumefaciens* was grown in YEP liquid medium (Table 1), supplemented with tetracycline, streptomycin and spectinomycin, at 28°C for approximately 3 days in the dark. One day before inoculation, 1 ml of pre-inoculums cells was inoculated in 50 ml of AB medium (Table 1) and the flask was shaken overnight at 28°C at the speed of approximately 200 rpm. The bacterial cells were collected by centrifugation at 1500 g for 5 min and then

Table 1 Media composition for *Agrobacterium tumefaciens* growth and induction

	AB	YEP
K_2HPO_4	3 g/l	
NaH_2PO_4	1 g/l	
NH_4Cl	1 g/l	
$CaCl_2$	0.01 g/l	
$FeSO_4 \times 7H_2O$	2.5 g/l	
$MgSO_4 \times 7H_2O$	0.3 g/l	
KCl	0.15 g/l	
Glucose	5 g/l	
Yeast extract		10 g
Bacto peptone		10 g
NaCl		5 g

resuspended in AB medium up to a final OD 600 of 0.8 for infection.

Cotyledons were sectioned into halves. The end of each cotyledon was cut off to allow it to adsorb the bacterial suspension. The explants were dipped in the bacterial suspension for 10 min and blotted on a sterilized paper towel placed on CM medium. The explants were placed with the upper leaf surface in contact with the medium. The plates were closed with parafilm, wrapped with aluminium foil and incubated for 2 days at 25°C. The explants were then grown on RM medium to induce callus formation. Every 2 weeks, the explants were propagated onto RM selection medium. When a stem developed a leaf, the stem was cut off at the maximum possible length and transferred to rooting medium (RTM). The plants were propagated every 4 weeks on MS30 supplied with kanamycin and carbacillin. Composition of the media used is reported in Table 2.

Molecular detection of transgenic plants by polymerase chain reaction

The presence of the construct was verified by PCR amplification. Genomic DNA was isolated according to the procedure of DNeasy-Kit (Qiagen, Hilden, Germany). PCR was carried out by using specific primer for the *NPTII* and *R1* genes (Table 3). The amplification was performed under the following conditions: 94°C for 5 min, 94°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min for 40 cycles and 72°C for 7 min. Finally, the PCR products were separated on a 1% agarose gel, and visualized by ethidium bromide staining.

RNA isolation and retrotranscription PCR analysis

Total RNA was extracted from tomato leaves, using RNeasy-Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed using 0.5 μg of oligo (dT) and 200 units of SuperScript III (Invitrogen, USA). The specific primers 76-2sf2 and 76-2SR (Table 3), were used for gene amplification. One μl of the retrotranscription reaction and 10 pmol of each oligonucleotide primer were used for RT-PCR, in a total volume of 25 μl. The PCR conditions used were as follows: 94°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min for 40 cycles, and 72°C for 7 min and 94°C for 5 min after 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min, followed by 7 min of final extension at 72°C. The amplified products were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining.

Transgene detection by q-RT-PCR

Genomic DNA was used in q-RT-PCR experiments. The q-RT-PCR reactions were performed by iCycler (Bio-Rad, Richmond, CA, U.S.A.) using the SYBR Green approach as a fluorescence reporter and hot-start *Taq* polymerase iQ SYBR Green (Bio-Rad, Richmond, CA, U.S.A.). Reactions were performed in triplicate using the following conditions: one cycle of 95°C for 10 min and 35 cycles of 95°C for 20 s, 60°C for 30 s. cDNA concentrations were normalized based on primers designed for *Elongation Factor 1* (*EF-1*) (Nicot et al. 2005) genomic DNA (Table 3). The software iQ5 (Bio-Rad, Richmond, CA, U.S.A.) was used to determine the fold between the endogenous and target sequences. Two DNA controls with respectively one and two copies *NPTII* gene number were used in the analysis. Transgenic DNAs containing one copy and two copy *NPTII* gene were kindly provided by Dr. M Tucci IGV-CNR Portici (Italy) and copy number was assessed by Southern blot analysis (Data not shown).

cDNA q-RT-PCR

cDNA of T1 L5 plants was used to perform q-RT-PCR amplifications with PR primers (Table 3). Reactions were

Table 2 Media used in different stages of tomato transformation experiments

	MS30	GM	CM	RM	RTM
MS[a]	4.3 g/l	2.2 g/l	4.3 g/l	4.3 g/l	4.3 g/l
Sucrose	30 g/l	15 g/l	30 g/l	30 g/l	30 g/l
Myo-inositol		50 mg/l	100 mg/l	100 mg/l	100 mg/l
Thiamine Hcl		0.2 mg/l	0.4 mg/l	0.4 mg/l	0.4 mg/l
Acetosyringone			100 mM		
Pyridoxine			0.5 mg/l	0.5 mg/l	
Zeatin Riboside			1.5 mg/l	1.5 mg/l	
Indole-3-acetic acid		0.2 mg/l	0.2 mg/l	0.2 mg/l	
Nicotinic acid			0.5 mg/l	0.5 mg/l	
Kanamycin				50 mg/l	30 mg/l
Carbenacillin				500 mg/l	250 mg/l
Agar		8 g/l	8 g/l	8 g/l	8 g/l
pH	5.8	5.9	5.9	5.9	5.9

[a] Murashige and Skoog (1962)

GM = germination medium

CM = co-culture medium

RM = regeneration medium

RTM = rooting medium

performed in triplicate using the following conditions: one cycle of 95°C for 10 min and 35 cycles of 95°C for 20 s and 60°C for 30 s. cDNA concentrations were normalized based on primers for the *Elongation Factor 1* (*EF-1*) (Nicot et al. 2005). The software iQ5 (Bio-Rad, Richmond, CA, U.S.A.) was used to determine the fold between the endogenous and target sequences.

Pathogen inoculation

P. infestans isolate IPO (race 0) and isolate 88133 (race 1,3,5,7,11), kindly provided by Dr Francine Govers, Wageningen University, The Netherlands, were grown in the dark at 26°C on V8 juice agar medium (200 ml V8 juice, 3 g $CaCO_3$, 800 ml DW, 15 g agar, pH 6.4). For the leaf infection assay, fully expanded leaves of 5- or 6-week-old plants were used for *P. infestans* inoculation. An oomycete plug (AGAR disc) of a five-day-old culture (0.4 mm in diameter) was placed at the center of the abaxial leaf surface. The inoculated plants were cultured in a growth chamber at 25°C and 100% humidity. Finally, three expanded leaves from three different plants of each T0 plant or T1 population were inoculated. The presence or absence of a hypersensitive response (HR) was scored after 8–10 days post-inoculation. Each experiment was replicated at least twice.

Statistical test

Statistical analysis, performed by GeneStat software q-RT-PCR, both at the genomic and cDNA level, was made with ANOVA and Tukey's test at 95% of significance. The chi-square test at $P \geq 0.5$ was used to analyze the segregation ratio of four independent T1 populations ($n=30$).

Table 3 List of primers used in PCR amplifications

Genes	Primers	Sequences (5′-3′)	Product gDNA	Product length cDNA
NPTII	NPTII f	tcggctatgactgggcacaacag	700	
	NPTII r	aagaaggcgatagaaggcgatgcg		
	NPTII RealTime f	tagccggatcaagcgtatgc	88	
	NPTII RealTime r	tcctgtcatctcaccttgctcc		
R1	76-2sf2	cactcgtgacatatcctcacta	1.400	1.200
	76-2SR	caaccctggcatgccacg		
EF-1	EF-1 f	ggctgactgtgctgttctca	83	
	EF-1 r	gttcacgggtctgaccatct		
PR-1	PR-1 f	gtgggtgtccgagagg		100
	PR-1 r	ctgagttgcgccagactactt		

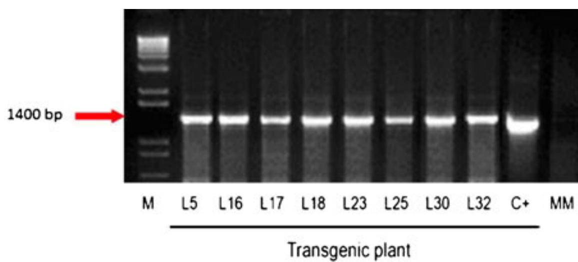

Fig. 1 PCR analysis of putative transgenic plants analyzed using *R1* gene-specific primers. DNA amplicons of eight kanamycin-resistant transgenic plants (L5–L32); Potato BAC clone containing *R1* gene (C+); untransformed control (MM)

Results

Tomato transformation

To generate transgenic tomatoes expressing *R1*, we used a *Agrobacterium tumefaciens* strain containing the clone 10-2, that has been demonstrated to be necessary and sufficient to transfer the HR response upon infection with *P. infestans* to the susceptible potato cultivar Desiree (Ballvora et al. 2002). Susceptible tomato Moneymaker and San Marzano cultivars were used in transformation experiments. In all, 190 explants were co-cultivated by *Agrobacterium tumefaciens* clone LBA4404/10-2. Thirty-two (17%) shoots were regenerated after 8 weeks of culture on RM selection medium containing 50 mg/l kanamycin. Generally, single shoots were produced from each explant. These 32 regenerated plants were finally transferred onto rooting medium (RTM) with carbanicillin. Twelve (6.3%) plants were rooted on the RTM. These shoots were propagated about five times on the rooting medium to suppress *A. tumefaciens* infection. Growing on rooting medium containing the antibiotic, the shoots were analyzed at molecular level to detect the stable integration of the *R1* gene by PCR and RT-PCR.

Molecular analysis of the transgenic plants

Twelve plants were analyzed by using *NPTII* and *R1* specific primers and all of them showed the presence of the heterologue gene (Fig. 1). Further RT-PCR analysis confirmed the transcription of the *R1* gene in the tomato plants. PCR analysis conducted with specific *R1* primers showed that the gene was introgressed and transcribed in the same way between potato and tomato (Fig. 2). The evidence is confirmed by the results of retrotranscription analysis: the *R1* primer pair was designed in a region spanning an intron which produced an amplicon 200 bp smaller than the cDNA amplicon with the gDNA. The results confirm the correct splicing of the gene in tomato (Fig. 2a, b). All the plants were propagated and transferred into soil pots. The plants were grown in a greenhouse and all produced progeny.

Assessment of gene copy number by using q-RT-PCR and progeny segregation analysis

The real time PCR approach on gDNA, in combination with segregation analysis, was used to determine the gene copy number in tomato transformed plants. The *EF-1* gene was used as a reference sequence for q-RT-PCR analysis. Although the introgressed gene was the *R1* gene, in order to detect the transgene copy number, specific primers for the reporter *NPTII* gene were used. By using the reporter gene as a target, we avoided aspecific amplification due to the presence of *R1* homologue genes in the tomato genome. Primary transformants were used in q-RT-PCR experiments. Two control plants, respectively carrying one copy and two copies for the *NPTII* target gene, were included in the analysis. The gDNA target gene was quantified as 0.5 times less than the endogenous control *EF-1* that is present as a double copy in the tomato genome (Fig. 3). Tukey's test

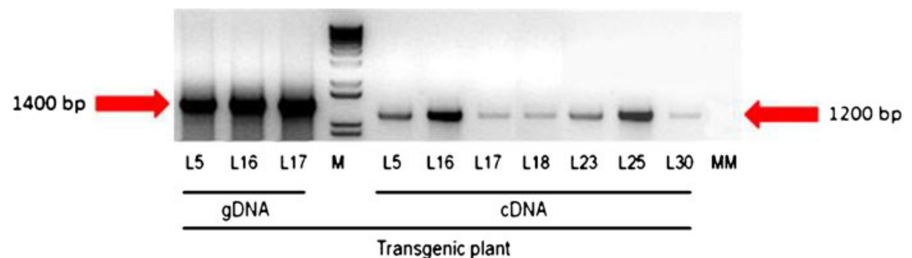

Fig. 2 RT-PCR analysis of transgenic plants using *R1* specific primers. M, marker; lines gDNA indicated gDNA amplicons; cDNA indicated cDNA amplicons; MM Moneymaker not transformed control

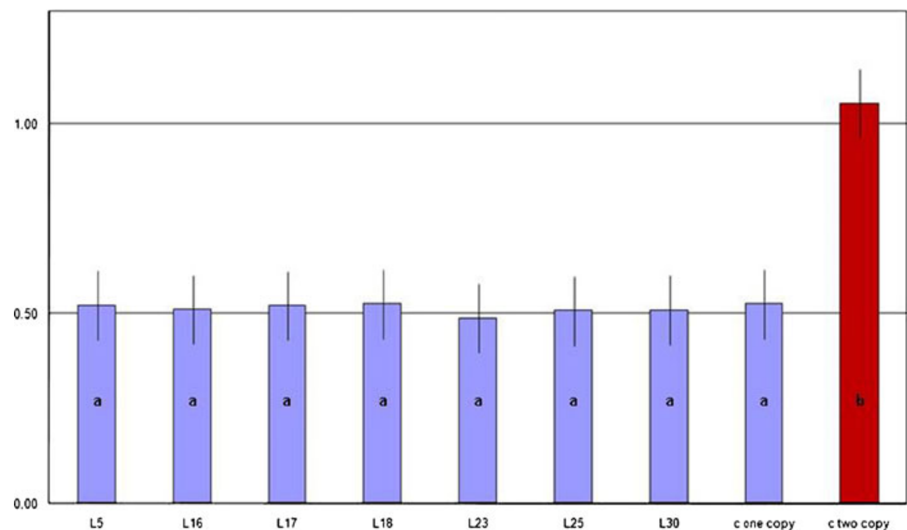

Fig. 3 q-RT-PCR on the primary transformants T0 compared with the "C" one-copy NPTII control (*violet bar*) and "C" two-copy NPTII control (*red bar*). The *letters bar* showed Tukey's test calculated from three different replicas. Amplification of T0 plants showed no significant differences with the one-copy control (*black bar*) but differences with the two-copy control (*white bar*) and the non-transformed Moneymaker (MM)

revealed significant differences between the two-copy control and the other assessed samples (Fig. 3). In order to confirm the gene copy number evaluated by q-RT-PCR, four different progenies from the single L5, L16, L18 and L23 primary transformants were investigated by PCR molecular analysis using specific *NTPII* and *R1* primers. The segregation ratio was analyzed by chi-square test; data were in accordance with a Mendelian segregation ratio of 3:1 reported for a single gene ($p>0.05$) (Table 4).

Disease evaluation in *R1* transgenic plants

To determine whether or not the *R1* transgene confers resistance to late blight isolate IPO-0, tests were carried out on seven T0 plants and fifty T1 plants, derived from different T0 individuals (Table 4). Completely expanded leaves of primary transgenic tomato plants were inoculated by using the agar plug method with IPO-0 and 88133 pathogen isolates. Sixty-three independent leaves of tomato, from seven different tomato transformed plants, were tested and the disease symptoms evaluated. Nine days after inoculation, prominent necrotic areas with late blight symptoms appeared on the leaves inoculated with isolate 88133 and in non-transformed plants, whereas a hypersensitive reaction developed in all transgenic lines inoculated with isolate IPO-0 of *P. infestans* (Fig. 4). An additional disease test was conducted using zoospores of virulent and avirulent *P. infestans* isolates, confirming the previous results. Disease tests were also performed on the full L5 and L16T1 segregating populations and selected individuals of L8 and L23 populations. All the plants carrying the R1 gene showed the resistant phenotype after 9 days, confirming molecular results (Table 4).

Leaves of three L5 inoculated individuals carrying *R1* were collected 0, 3 and 6 h after inoculation (hpi) to conduct expression analysis during infection. The *PR-1* gene was chosen as a marker gene to evaluate *P. infestans* infection in the resistant and susceptible pools of plants. The *PR-1* expression level, quantified by q-RT-PCR, showed a high transcription level already 3 h after inoculation in the resistant plant-pathogen interaction that increased after 6 hpi, while in the

Table 4 Molecular and disease test using P. *infestans* isolate IPO-0 and 88133 performed on the T1 segregating progenies

T0line	n.T1 molecular analyzed plants	n. T1 transgenic plants	n.T1 disease tested plants	n. isolate IPO-0 resistant plants	n. isolate 88133 resistant plants
L5	20	9*	19	0	8
L16	17	7*	17	0	7
L18	23	13*	11	0	11
L23	9	5*	4	0	4
TOT	117	62	47	0	46

* $p>0.05$

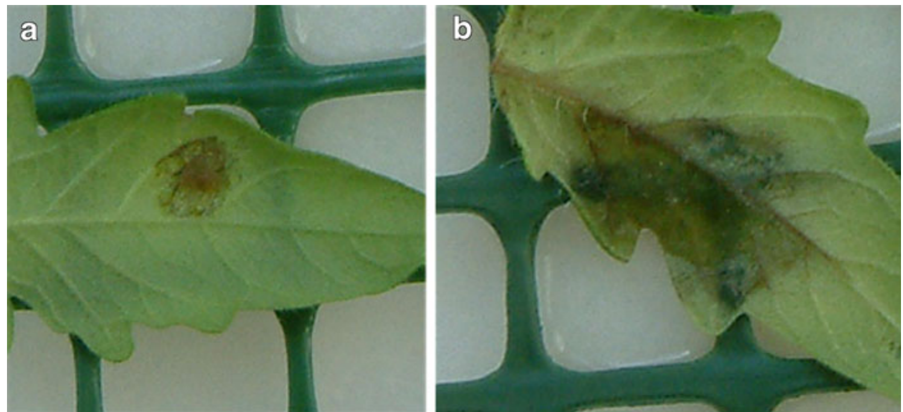

Fig. 4 *R1* transgenic (T0-L5) tomato plant disease evaluation. **a** Inhibition of disease development in transgenic plants infected by *P. infestans* isolate IPO-0. **b** Development of symptoms in transgenic infected leaves by isolate 88133 of *P. infestans*

susceptible plants the same amount of transcription was revealed at two time points (Fig. 5).

Discussion

Wild *Solanum* species represent an important source of resistance against diseases and pests in tomato. However, introduction of such resistance genes to commercial cultivars of *S. lycopersicum* by conventional breeding techniques often encounters serious difficulties due to high incompatibility barriers to hybridization (Bhatia et al. 2004). Cisgenic (Schouten et al. 2006) and/or transgenic approaches can be useful to overcome problems of incompatibility between species, extending the pool of genes of available for this crop.

In the present study, tomato plants were generated, carrying the heterologue potato *R1* gene. The putative transformants were selected in the presence of kanamycin, and the integration of the transgene into the host genomes was confirmed by molecular analyses. We then developed a sensitive and high-throughput q-RT-PCR technique for rapidly estimating the transgene copy number in our material. Indeed, the number of the transgene copy could greatly influence the expression level and genetic stability of the target gene (Yang et al. 2005).

Based on our approach, all transformed plants showed an amount of transgenic gDNA comparable with the one copy *NPTII* control providing evidence for a single gene copy insertion. Estimation of transgene copy number based on Southern analysis is laborious and time-consuming, may involve hazardous radioisotopes, and requires relatively large amounts of plant materials. The q-RT-PCR strategy has already proved useful for quantifying the transgene copy number

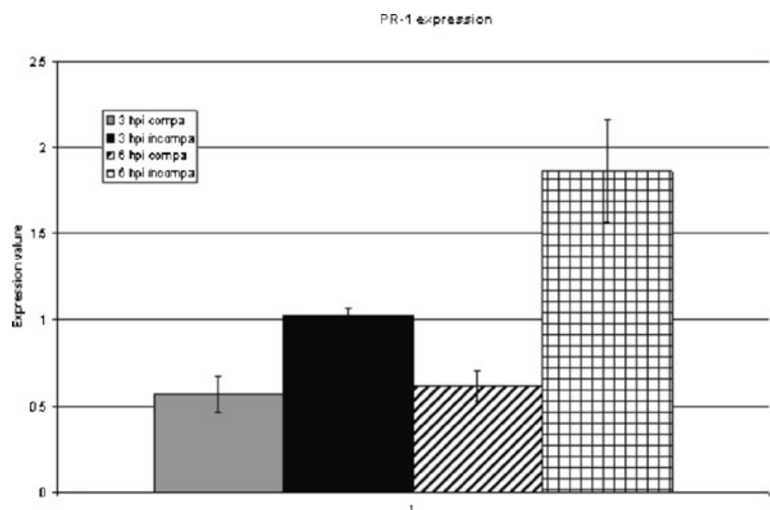

Fig. 5 Pr-1 expression during the early interaction in the compatible and incompatible *P. infestans* interaction. The *bars* showed standard error

(Ingham et al. 2001; Batista et al. 2008). However, in previous works a single copy gene was selected as reference gene in a relative q-RT-PCR (German et al. 2003; Prior et al. 2006). Bradeen et al. (2009) showed a high correlation between the transgene quantification obtained using both a single copy gene (*Urease*) and a double copy gene (*EF-1*). Here we have reported the combined use of the tomato *EF*-1 endogenous gene with *NTPII* quantified genes, as reference sources, for accurate measurement of the number of introgressed genes. To confirm our results, molecular and phenotypic segregation analysis was conducted on two progenies derived from different T0 as reported elsewhere (Bubner and Baldwin 2004).

To test the hypothesis that *R1* represents a specifically regulated R-gene, transgenic tomato plants were inoculated with *P. infestans* isolates 88133 and IPO-0. All the T0 and T1 plants containing the *R1* gene were resistant to the late blight isolate IPO-0 and susceptible to isolate 88133. These results provide evidence for specific activation of the *R1* gene during pathogen attack also in tomato. In Solanaceae, several R-genes isolated from a species have proved functional in sexually incompatible species. Interestingly, Van der Vossen et al. (2005) reported a functional complementation of resistance gene *Rpi-blb2* against *P. Infestans* isolated from wild potato species in susceptible tomato. The *Bs2* gene, which encodes *Xanthomonas* resistance in pepper, has been transferred into tomato and shown to be functional (Tai et al. 1999). The N gene isolated in tobacco is active also in tomato (Whitham et al. 1996); the tomato Sw-5 resistance gene was transferred into the tobacco susceptible plant proving its ability to confer resistance to tospoviruses (Spassova et al. 2001).

Analysis of the *PR* proteins expression and of molecules triggered by the HR response in the plant cell programmed death could be useful to better investigate resistance process. In our study the Pr-1 gene was analyzed during the first hours post inoculation in the compatible and incompatible interaction. Our results suggest an activation of the *PR-1* gene in *R1* incompatible reaction at a very early stage. Indeed, a *PR-1* expression increasing of one fold between 3 hpi and 6 hpi in incompatible interaction was displayed. Other data provided evidence of *PR-1* activation in the early defence stages. In particular, Ronning et al. (2003), reported *PR-1* activation during the first 12 h after inoculation. The Pr-proteins, particularly *PR-1* and *PR-5*, are reported as important components of the defence mechanism of potato against *P. infestans* (Wang et al. 2006). PR genes induced in this interaction have been described as “pathogen response” genes (Smart et al. 2003). Indeed, van Loon et al. (2006) demonstrated that, in tomato, *PR-1* was able to reduce sporangia germination and germ-tube length.

In our work we generated several tomato transgenic *R1* lines and estimated gene copy number by the real time PCR method using an endogenous tomato sequence which is not in a single gene copy. Furthermore, we provided evidence that the *PR-1* gene is expressed specifically during pathogen challenge also in tomato, as a defence response upon infection with *P. infestans* isolate 88133 is induced. Finally, we recognized enhancement of *PR-1* gene expression during the *P. infestans* resistance response. Future investigations will seek to elucidate the pattern of defence genes activated during *R1* compatible and incompatible reactions.

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