Theor Appl Genet (1998) 97: 575–583

P. Bettini · E. Cosi · M. G. Pellegrini · L. Turbanti
G. G. Vendramin · M. Buiatti

Modification of competence for in vitro response to *Fusarium oxysporum* in tomato cells. III. PR-protein gene expression and ethylene evolution in tomato cell lines transgenic for phytohormone-related bacterial genes

Received: 25 February 1998 / Accepted: 7 April 1998

Abstract Previous work carried out in our laboratory has shown that, in tomato, the alteration of endogenous phytohormone equilibria through the integration of *Agrobacterium tumefaciens* genes for auxin and cytokinin synthesis can modify the active defense response to *Fusarium oxysporum* f. sp. *lycopersici*. The susceptible cv 'Red River' acquires a stable competence for active defense, particularly when the phytohormone equilibrium is altered in favour of cytokinins. Here, we analyse the expression of genes involved in the defense response against pathogens, i.e. pathogenesis-related (PR)-protein genes, in the susceptible 'Red River' and resistant 'Davis' cultivars transgenic for the aforementioned genes. Fungal cell-wall components, glutathione, salicylic acid and the ethylene-forming ethephon are used as "probes" for the induction of defense processes, including ethylene production. The data obtained show that the extracellular PR-proteins (acidic chitinase and PR-1 protein) that were inducible in the control tissue of the resistant 'Davis' cultivar and not expressed in the susceptible 'Red River' cultivar became constitutive in the transgenic tissues of both. On the other hand, expression of the intracellular PR-proteins (basic chitinase and β-1,3-glucanase) was found to be constitutive in all cases, both in the control and in the transgenic cell lines of the resistant and the susceptible tomato cultivars. Ethylene production was higher in 'Davis' than in 'Red River', and significantly increased in the transgenic cell lines, particularly when cytokinin synthesis was altered.

Key words *Lycopersicon esculentum* · *Fusarium oxysporum* f. sp. *lycopersici* · *Agrobacterium tumefaciens*-mediated transformation · Phytohormones · PR-proteins

Communicated by G. Wenzel

P. Bettini · E. Cosi · M. G. Pellegrini
L. Turbanti · M. Buiatti (✉)
Dipartimento di Biologia animale e Genetica "Leo Pardi",
via Romana 17, 50125 Florence, Italy
Fax: +39 55 222565
E-mail: mbuiatti@dbag.unifi.it

G. Vendramin
Istituto per il Miglioramento Genetico delle Piante Forestali,
Consiglio Nazionale delle Ricerche, via Atto Vannucci 13, 50134
Florence, Italy

Introduction

Plants respond to pathogen attack by two different mechanisms: one specific ("vertical" resistance); the other aspecific ("horizontal" resistance).

"Vertical" resistance is represented by the so-called gene-for-gene interaction (Flor 1946), where the product of a dominant resistance gene in the host interacts specifically with the product of a dominant avirulence gene in the pathogen, triggering a complex series of biochemical reactions (hypersensitive response) that ultimately leads to plant resistance (reviewed by de Wit 1992; Dixon et al. 1994). However, this kind of resistance is often easily overcome through pathogen coevolution.

"Horizontal" resistance, on the other hand, is represented by a series of rather aspecific defense responses that confers tolerance towards several different pathogens. The best studied example of horizontal resistance is Systemic Acquired Resistance (SAR), where a host challenged with an avirulent pathogen, or having survived the attack of a virulent one, develops a long-lasting, broad-spectrum systemic resistance to subsequent infections. SAR onset is associated with the expression of a specific set of genes, such as chitinases, β-1,3-glucanases, thaumatin-like proteins or "permatins", PR-1 protein, etc. (Ryals et al. 1996). The systemic signal responsible for the establishment of SAR has been proposed to be salicylic acid (SA). While

SA undoubtedly plays an important role in the induction of plant defense against pathogens and in SAR (Malamy et al. 1990; Gaffney et al. 1993), it is nevertheless certainly only one of a number of "critical" molecules involved in SAR, a complex network of reactions modulated by the overall physiological state of the plant.

Research in this field is therefore increasing with the aim of exploiting the potential of horizontal resistance for the development of new strategies to enhance plant resistance to pathogens, while assuring, at the same time, durable results and a reduced economical and environmental impact.

Within this framework, it is worth noting that phytohormones have been shown in several instances to be involved in the defense response of plants against pathogen attack. Treatment with growth regulators can act either by increasing (Sarhan et al. 1986; Gretenkort and Ingram 1993) or by inhibiting (Beckman and Ingram 1994) the hypersensitive response, and differences in endogenous auxin and/or cytokinin concentrations have been shown between resistant and susceptible plants (Dermastia and Ravnikar 1996; Lennox et al. 1993). Moreover, the expression of defense-related genes, such as those coding for pathogenesis-related proteins (PR-proteins), is regulated by hormone levels in *Nicotiana tabacum* L. PR-proteins represent a group of defense-related products that have been implicated in both the hypersensitive response against viral, bacterial or fungal pathogens and in the onset of SAR (reviewed by van de Rhee et al. 1994; Cutt and Klessig 1992). In tobacco cell cultures, β-1,3-glucanase (Felix and Meins 1986) and chitinase (Shinshi et al. 1987) production are consistently inhibited by the combination of auxin and cytokinin in the culture medium, while they increase on hormone-free medium or in the presence of only one of the two hormones. In tobacco protoplasts, auxins have been shown to have a negative effect on the accumulation of both the abovementioned PR-proteins and to interfere with the spontaneous induction of defense reactions by controlling in a concentration-dependent manner the elicitor-induced synthesis of β-glucanase (Jouanneau et al. 1991). On the other hand, in tobacco shoots subjected to cytokinin stress either through transformation with the *A. tumefaciens* cytokinin locus or through incubation on cytokinin-containing medium, the accumulation of several mRNAs was observed, including chitinase, PR-1 and PR-1-like proteins, the HRGP extensin (Memelink et al. 1987, 1990).

Previous work carried out in our laboratory (Storti et al. 1994) has shown through the analysis of physiological parameters in dual cultures that an increased competence for active defense can be obtained in a tomato cultivar ('Red River') susceptible to the phytopathogenic fungus *Fusarium oxysporum* f. sp. *lycopersici* by modifying the physiological background through the integration of *Agrobacterium tumefaciens* genes involved in phytohormone biosynthesis. The aim of the work presented here was to directly analyse the effect of transgenosis on the pattern of expression of defense-related genes in tomato cvs 'Red River' and 'Davis UC82', respectively susceptible and resistant to *F. oxysporum* f. sp *lycopersici*. For this purpose, the expression of PR-protein coding genes and ethylene production were monitored following treatment with a series of inducers known to be involved both in the active defense response (fungal cell-wall components and glutathione) (Wingate et al. 1988) and in SAR (salicylic acid and the ethylene-forming ethephon) (van Kan et al. 1995). These two parameters are in fact closely linked, as it is well known that ethylene produced during host-pathogen interactions induces the accumulation of PR-proteins (Felix and Meins 1987) and that ethylene-responsive elements have been identified in the promoters of several PR-protein genes (Shinshi et al. 1995; Sessa et al. 1995).

Materials and methods

Plant and fungal culture

The *Lycopersicon esculentum* Mill. cultivars used were 'Davis UC82' and 'Red River', respectively resistant and susceptible to *F. oxysporum* f. sp. *lycopersici*. Establishment and maintenance of callus cultures were as already described (Buiatti et al. 1987).

Transgenic cell lines were obtained by *Agrobacterium*-mediated transformation (Storti et al. 1994) using strains C58C1ClmR (pGV2250, gene 4^- pTiB6S3 mutant) and C58C1ClmR (pGV2282, gene 1^- pTiB6S3 mutant) (Inzé et al. 1984); these transgenic cell lines were called 4^- (iaaM$^+$, iaaH$^+$, ipt$^-$) and 1^- (iaaM$^-$, iaaH$^+$, ipt$^+$), respectively.

F. oxysporum f. sp. *lycopersici* race 1 was obtained from DI.VA.P.R.A. (Dipartimento di Valorizzazione e Protezione delle Risorse Agroforestali, University of Torino, Italy) and maintained on Czapek Dox Broth medium (Difco).

"Elicitor" treatments

Seven grams of callus from the control and the transgenic cell lines were inoculated in 40 ml of LS medium (Linsmaier and Skoog 1965) in 250-ml Erlenmeyer flasks and incubated in a growth chamber at $25° \pm 1°C$ with continuous shaking (100 rpm). Cells were routinely subcultured at 20-day intervals by transferring an aliquot to fresh LS medium. Seven days before elicitor treatment, 20 ml of cells were diluted with 20 ml of fresh medium and incubated as described above.

The elicitors used were: fungal cell-wall components (20 µg/ml glucose equivalents, 7 h) (Buiatti et al. 1985), glutathione (1 m*M*, 7 h, Sigma), salicylic acid (1 m*M*, 24 h, Sigma), ethephon (2.5 m*M*, 5 h, Sigma). Treated cells were harvested by filtration on 11-µm nylon mesh, immediately frozen in liquid nitrogen and stored at $-80°C$ for nucleic acid extraction.

Nucleic acid extraction

Total DNA extraction from callus tissue was carried out according to Doyle and Doyle (1989). Plasmid DNA was extracted either with

a standard miniprep procedure for restriction analysis (Sambrook et al. 1989) or with QIAGEN-tips (Plasmid midi kit, QIAGEN GmbH, Germany) for sequencing. DNA was quantitated by fluorometry (Hoefer Dyna Quant 200 Fluorometer) using Hoechst 33258 as dye.

Total RNA was extracted using the RNeasy plant total RNA kit (QIAGEN GmbH, Germany), and the concentration was evaluated spectrophotometrically by measuring absorbance at 230, 260, 280 and 320 nm. Prior to its use, RNA integrity was assessed by agarose gel electrophoresis (1% agarose, 1 × TBE buffer) (Sambrook et al. 1989).

Southern blotting and hybridization

Nucleic acids were transferred from agarose gels to positively charged nylon membranes (Boehringer Mannheim) by means of a VacuGene apparatus (Pharmacia Biotech) at 50 mbar for 2 h using 20 × SSC (Sambrook et al. 1989) as the blotting solution. After a short rinse in 2 × SSC, the membrane was air-dried, and DNA was then fixed to the membrane by UV-crosslinking (Spectrolinker XL-1000, Spectronics Corp).

The tomato cDNA probes used were a generous gift of Dr. J.A.L. Van Kan (Agricultural University Wageningen, NL). They were: an acidic extracellular 26-kDa chitinase, a basic intracellular 30-kDa chitinase, a basic intracellular 33-kDa β-1,3-glucanase and an extracellular PR-1 protein that corresponds to P4, a highly basic isomer of P14 serologically related to the PR-1 protein family of tobacco (van Kan et al. 1992).

The labelling of inserts eluted from agarose gels (QIAquick gel extraction kit, QIAGEN GmbH, Germany) and hybridization were carried out using the Nonradioactive DNA labelling and hybridization kit (Boehringer Mannheim) following the manufacturer's instructions.

Polymerase chain reaction (PCR)

PCR was performed on total 'Davis UC82' and 'Red River' DNA. The PCR reaction mixture contained 1 µg DNA, 0.1 µ*M* of each primer, 0.2 m*M* dNTPs, 2.5 U *Taq* polymerase (Boehringer Mannheim), 1 × polymerase buffer supplied with the enzyme, in a final volume of 50 µl. The primers used for the four PR-protein genes are listed in Table 1. Amplification was performed in a Perkin Elmer Cetus GeneAmp 9600 thermal cycler for 30 cycles of 1.5 min at 94°C; 1.5 min at 45°C for the acidic chitinase, the β-1,3-glucanase and the PR-1 protein, 50°C for the basic chitinase, 1 min at 72°C. The amplified products were analysed by agarose gel electrophoresis, blotted and hybridized to the appropriate probes as described.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was carried out as described by Van Der Straeten et al. (1992), with 1 µg total RNA in a GeneAmp 9600 thermal cycler (Perkin Elmer Cetus). Samples were incubated at 85°C for 10 min, followed by 5 min at 45°C. After reverse transcription (40°C, 45 min), the PCR amplification was performed as described in the previous section. The primers used were the same as those already reported for PCR. A 30-µl aliquot of the reaction was separated by agarose gel electrophoresis (1% agarose, 0.5 × TBE buffer), blotted and hybridized to the appropriate digoxigenin-labelled probe.

As a control for DNA contamination, samples were treated with 10 µg heat-treated RNase A (Boehringer Mannheim) at 37°C for 1 h, then with 1 vol of phenol : chlorophorm : isoamyl alcohol (50 : 49 : 1), ethanol-precipitated and used in a PCR amplification cycle. After electrophoresis, gels were blotted and hybridized with the appropriate probe. If a signal was visible, RNA extraction was repeated.

Cloning and sequencing

RT-PCR fragments were cloned either directly from the purified reaction mixture (QIAquick PCR purification kit, QIAGEN GmbH, Germany) or after re-amplification. Blunt-end cloning in the *Sma*I site of plasmid pUC18 was performed using the "SureClone™ Ligation kit" (Pharmacia Biotech). A standard calcium chloride procedure was used for the preparation of competent *Escherichia coli* CH5α bacteria (Sambrook et al. 1989). Clones containing inserts of the appropriate size were sequenced with an ALF DNA sequencer (Pharmacia Biotech), and sequence homology searches were performed with sequence databases at EMBL using FASTA version 3.0t71 November, 1996 (Pearson and Lipman 1988).

Measurement of ethylene evolution

Ethylene production was measured from 1 g callus (f.w.) of the control and transgenic cell lines, plated in 25-ml Erlenmeyer flasks containing 5 ml solid LS medium and sealed with silicon rubber.

Callus pieces were treated with 100 µg glucose equivalents of fungal cell-wall components, and ethylene production was measured every 6 h for 32 h, then at 48 and 72 h. Samples of gas (1 ml) from each flask were injected in a Packard gas chromatograph equipped with an aluminium 1/8-inch column packed with Porapak Q. Calibration of the column was performed with a gas mixture of ethylene and N_2 containing 13.12 ppm ethylene. Analysis was performed under isothermal conditions with the injector and oven at 50°C and the detector at 100°C. Ethylene accumulation was expressed as ppm/g callus (f.w.). Each sample was in triplicate, and for each time point three independent measures were taken.

Results

PR-protein gene expression

Prior to the analysis of gene expression, primers for basic chitinase, acidic chitinase, β-1,3 glucanase and

Table 1 Primers used in PCR and RT-PCR amplifications

Gene (GenBank accession no.)	Primer forward (5′ → 3′)	Primer reverse (5′ → 3′)	Amplified fragment size (bp)
Acidic chitinase (Z15141)	GCACTGTCTTGTCTCTTTTTC	ATGGTTTATTATCCTGTTCTG	496
Basic chitinase (Z15140)	TTCTGTGCTTTTGCTGTCTGC	TGGGCAAGGAAAGCAGCAATT	362
β-1,3 glucanase (M80604)	ATTGTTGGGTTTTTGAGGGAT	TTTAGGTTGTATTTTGGCTGC	454
PR-1 protein (M69247)	CACAAAACTATGCCAACTCAA	GTAAAGAACCTAAGCCACGAT	503

extracellular PR-1 protein were tested for their ability to correctly amplify genomic DNA of the two tomato cultivars used in this study. Results (Fig. 1) showed that fragments of the expected sizes (see Table 1) were amplified for basic chitinase, β-1,3-glucanase and the extracellular PR-1 protein. An unexpected result was obtained for the acidic chitinase, as the fragment amplified from the genomic DNA had a higher molecular weight than the corresponding fragment for cDNA. The possible presence of an intron, which could account for the observed size difference, will be further investigated. The sizes of the amplified fragments obtained for the four genes were the same for the 2CVS.

RT-PCR was used to qualitatively analyse the expression of PR-protein genes in the control and transgenic cell lines of 'Red River' and 'Davis' after treatment with a series of "elicitors" of the defense response (fungal cell-wall components, glutathione, salicylic acid, ethephon). Amplification products were separated by agarose gel electrophoresis, and their identity was confirmed by Southern hybridization (Figs. 2, 3). Results from a control experiment performed in order to check for DNA contamination (see Materials and methods) are shown in Fig. 4,

As multiple bands appeared upon hybridization with each probe used, even when a higher stringency of amplification was used through a 3° to 5°C increase of the annealing temperature (data not shown), sequencing of the amplification products was performed. A comparison of the sequences obtained with the sequence database at EMBL showed that all the observed bands indeed corresponded to the expected genes, the smaller ones being due to the internal annealing of one primer.

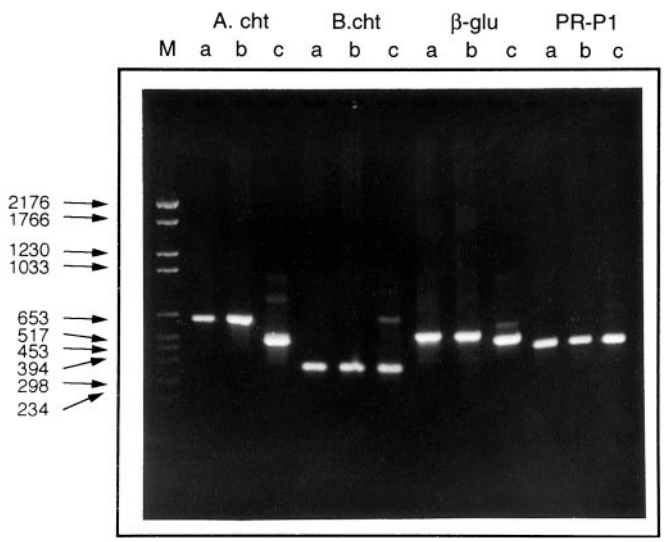

Fig. 1 Agarose gel electrophoresis of the PCR-amplification products obtained from genomic DNA of the tomato cvs 'Davis' (*a*) and 'Red River' (*b*) with the primers corresponding to PR-protein genes: acidic chitinase (*A. CHT*), basic chitinase (*B. CHT*), β-1,3-glucanase (*β-GLU*) and PR-1 protein (*PR-1*). The corresponding cDNAs (*c*) were used as controls. Molecular weights (M = MW marker VI, Boehringer Mannheim) are indicated in basepairs

Our results are summarized in Table 2. It is important to stress that these results have been interpreted only on a qualitative basis (presence/absence of signal after hybridization), and no meaning at all has been given to the apparent quantitative differences between samples.

In resistant cv 'Davis UC82' (Table 2, Fig. 2), basic chitinase and β-1,3-glucanase showed a constitutive expression in both the transgenic and the untransformed cell lines with all the inducing treatments and in the untreated control. For acidic chitinase, on the other hand, gene expression was present in the untransformed control only after treatment with the elicitors tested, but not in the absence of induction, being constitutive in both the transgenic cell lines. No PR-1 gene expression was detected in the control, both with and without induction, the transcription of the gene becoming constitutive in the transformants but inhibited by salicylic acid treatment.

In susceptible cv 'Red River' (Table 2, Fig. 3), the basic chitinase and β-1,3-glucanase genes showed constitutive expression in both the control and transgenic cell lines. The acidic chitinase was not induced in the untransformed control with any of the elicitors tested except SA but was constitutively expressed, as in 'Davis', in the two transgenic cell lines. The PR-1 protein gene was not expressed in the control, treated or not with the different inducers, but was constitutively expressed in the transgenic cell lines, with SA exerting an inhibitory action only in the 4^- tissue (iaaH^+, iaaM^+, ipt$^-$).

Ethylene evolution

Ethylene evolution was measured from 1 g callus tissue either treated or untreated with fungal cell-wall components at 6, 18, 24, 32, 48 and 72 h.

In resistant cv 'Davis' (Fig. 5), when transformation shifted the endogenous hormone equilibrium towards cytokinin production (1^-) ethylene evolution increased for the first 18 h in both the control and elicitor-treated tissues and reached a plateau from 18 to 72 h. In the 4^- cell line, however, a slight increase was observed from 6 h to 18 h without any further increase, and the amount of ethylene produced was much lower. On the contrary, in the untransformed 'Davis' tissue, elicitor treatment was effective in increasing ethylene evolution between 32 h and 48 h, while in the control ethylene evolution remained almost constant at a lower level from 32 h to the end of the experiment.

In susceptible cv 'Red River' (Fig. 6), the 1^- cell line showed an increase in ethylene production up to 32 h, followed by a plateau, while in the 4^- cell line the amount of ethylene produced remained lower even

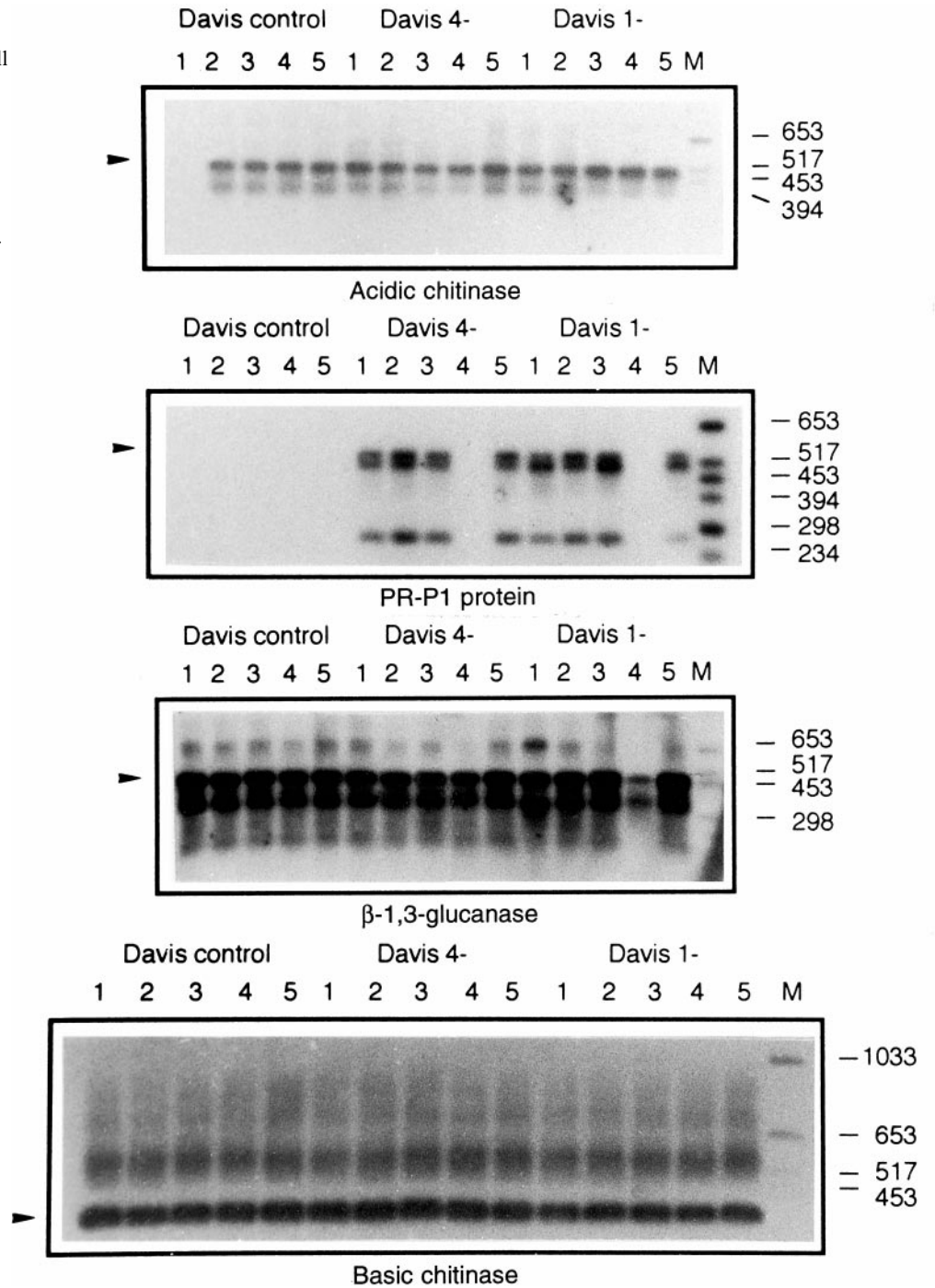

Fig. 2 RT-PCR analysis of PR-protein gene expression in the control and transgenic (*4-*, *1-)* cell lines of the resistant cv 'Davis UC82'. Amplification products were separated by agarose gel electrophoresis, blotted and hybridized to the digoxigenin-labelled appropriate probe. The band of the expected size is marked by an *arrow*. Molecular weights (*M* = MW marker VI, Boehringer Mannheim) are indicated in basepairs. *Lanes* *1* Untreated, *2* glutathione, *3* fungal cell-wall components, *4* salicylic acid, *5* ethephon

with respect to that of the untransformed control. In the case of the susceptible cultivar, however, elicitor treatment was effective in increasing the production of ethylene in both transgenic cell lines, while the amount and time course of ethylene were not affected in the control tissue. On the whole, the amount of ethylene produced by 'Red River' was lower than that produced by 'Davis'.

Discussion

In a previous paper we have shown that in tomato the modification of the endogenous phytohormone equilibrium through transformation with *A. tumefaciens* genes for auxin and cytokinin biosynthesis can bring a susceptible cultivar to a stable "competence" for

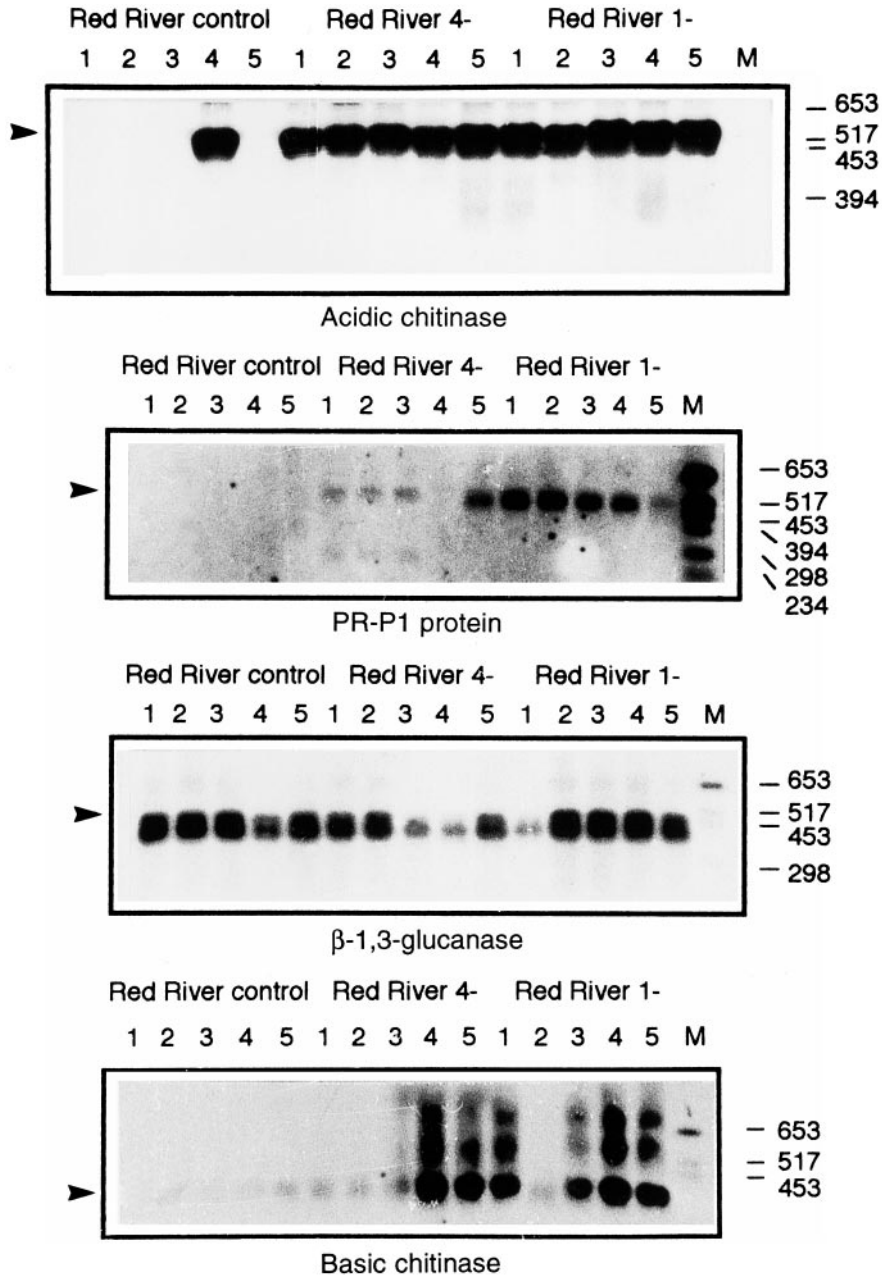

Fig. 3 RT-PCR analysis of PR-protein gene expression in the control and transgenic (*4-*, *1-*) cell lines of the susceptible cv 'Red River'. Amplification products were separated by agarose gel electrophoresis, blotted and hybridized to the digoxigenin-labelled appropriate probe. The band of the expected size is marked by an *arrow*. Molecular weights (M = MW marker VI, Boehringer Mannheim) are indicated in basepairs. *Lanes* *1* Untreated, *2* glutathione, *3* fungal cell-wall components, *4* salicylic acid, *5* ethephon

active defense against *F. oxysporum* f. sp. *lycopersici*. This was particularly true when the transformation resulted in a change in the phytohormone equilibrium towards a higher cytokinin production, as judged from a series of in vitro parameters known to be related to active defense (phytoalexin production, inhibition of fungal growth, peroxidase induction, ion leakage in the presence of fusaric acid, callose content) (Storti et al. 1992).

In this paper we report the results of an analysis of PR-protein gene expression and ethylene evolution in the same system, following treatment with a series of products with elicitor activity (fungal cell-wall components, glutathione, salicylic acid and ethephon).

Transgenosis was found to confer a "status of activation" of the defense response to susceptible cv 'Red River'. The extracellular PR-proteins (acidic chitinase and PR-1 protein) were in fact constitutively expressed only in the transformed 4^- and 1^- tissues, as in resistant cv 'Davis', while being absent in the control, except for the inducing effect of SA on acidic chitinase (Table 2, Fig. 3). With respect to ethylene evolution, again the response observed for the transgenic tissues was modified relative to that of the control, especially when cytokinin-biosynthetic genes were used for the transformation (Fig. 6), as the 1^- cell line produced more ethylene than the 4^- and control tissues. The most striking effect of transgenosis in this case was to confer

Table 2 PR-protein gene expression in the control and transgenic in vitro cultures of the resistant cv 'Davis UC82' and the susceptible cv 'Red River'

	Acidic chitinase	Basic chitinase	PR-1 protein	β-1,3 glucanase
Davis UC82				
Control	−	+	−	+
Glutathione	+	+	−	+
Fungal elicitor	+	+	−	+
Salicylic acid	+	+	−	+
Ethephon	+	+	−	+
Davis 4-				
Control	+	+	+	+
Glutathione	+	+	+	+
Fungal elicitor	+	+	+	+
Salicylic acid	+	+	−	+
Ethephon	+	+	+	+
Davis 1-				
Control	+	+	+	+
Glutathione	+	+	+	+
Fungal elicitor	+	+	+	+
Salicylic acid	+	+	−	+
Ethephon	+	+	+	+
Red River				
Control	−	+	−	+
Glutathione	−	+	−	+
Fungal elicitor	−	+	−	+
Salicylic acid	+	+	−	+
Ethephon	−	+	−	+
Red River 4-				
Control	+	+	+	+
Glutathione	+	+	+	+
Fungal elicitor	+	+	+	+
Salicylic acid	+	+	−	+
Ethephon	+	+	+	+
Red River 1-				
Control	+	+	+	+
Glutathione	+	+	+	+
Fungal elicitor	+	+	+	+
Salicylic acid	+	+	+	+
Ethephon	+	+	+	+

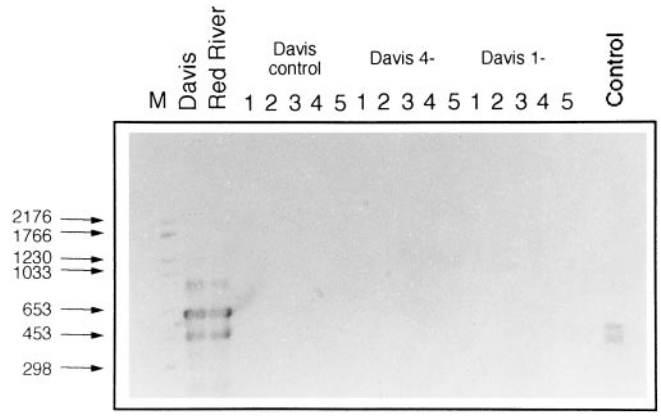

Fig. 4 Control experiment designed to check for DNA contamination of RNA samples. RNase-treated RNA samples of 'Davis' control and transgenic (*4-*, *1-*) cell lines were amplified with primers for the acidic chitinase, and hybridization was carried out with the corresponding cDNA probe. *Control* RT-PCR amplification of an untreated sample, 'Davis'-'Red River' PCR amplification of the genomic DNA of the two cultivars. Molecular weights (M = MW marker VI, Boehringer Mannheim) are indicated in basepairs. *Lanes 1* Untreated, *2* glutathione, *3* fungal cell-wall components, *4* salicylic acid, *5* ethephon

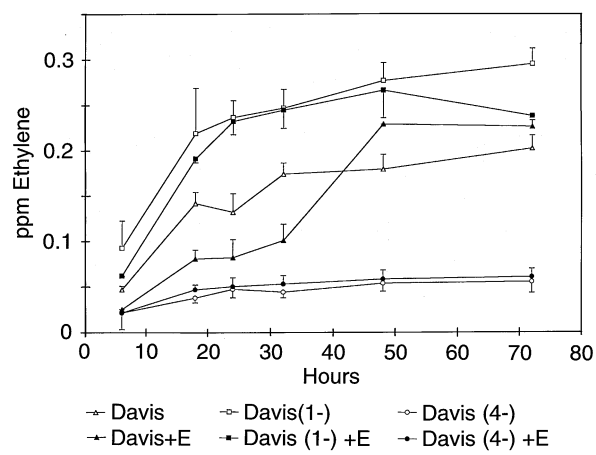

Fig. 5 Levels of ethylene produced from control and transgenic (*4-*, *1-*) callus of the resistant cv 'Davis', treated (+ *E*) or not with fungal cell-wall components. Each data point represents the mean (± SE) of nine independent measures (three independent measures per sample, each sample being in triplicate)

to both transformed tissues the capability to react to the treatment with fungal cell-wall components with a significant increase in ethylene production, a behaviour that was not observed in the control.

The expression of extracellular PR-protein genes in resistant cv 'Davis' was also affected by the integration of *Agrobacterium* genes as it became constitutive in the 4^- and the 1^- cell lines, with the exception again of the SA treatment which failed to induce the PR-1 gene in both transgenic tissues (Table 2, Fig. 2). Ethylene production was higher in the 1^- cell line than in the control and 4^- tissues (Fig. 5), thus confirming that this response is particularly sensitive to the shift of the endogenous phytohormone balance towards cytokinins.

Taken together, our data show that the four PR-proteins analysed have different behaviours with respect to gene expression and that they can be grouped according to the expression pattern. On one hand there are the extracellular proteins, acidic chitinase and PR-1, which were induced in the control tissue of the resistant, but not in the susceptible cultivar and which became constitutive in the transgenic tissues of both; on the other, there are the intracellular proteins, β-1,3-glucanase and basic chitinase, which showed no differences between the control and the transgenic cell lines of both cultivars. A similar behaviour of extracellular versus intracellular PR-proteins has been reported by

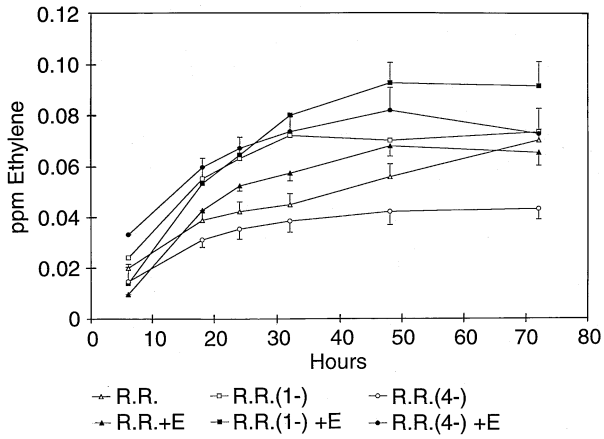

Fig. 6 Levels of ethylene produced from control and transgenic (*4-*, *1-*) callus of the susceptible cv 'Red River' (*R.R.*), treated (+*E*) or not with fungal cell-wall components. Each data point represents the mean (±SE) of nine independent measures (three independent measures/sample, each sample being in triplicate)

van Kan et al. (1992) and Danhash et al. (1993) in tomato during a compatible and an incompatible interaction with the fungus *Cladosporium fulvum*. In that case, the intracellular PR-proteins had the same pattern of induction in both the compatible and incompatible combinations, while the extracellular ones showed a rapid, transient induction in the incompatible interaction that was delayed in the compatible one. Also in tobacco, Memelink et al. (1990) have shown that genes encoding the basic PR-proteins are constitutively expressed and induced by stress signals, while the acidic proteins are expressed only after appropriate induction.

Moreover, in our system SA displayed either an inhibitory or an inducing effect on the expression of the PR-protein genes that can be attributed to the interactive action of at least two factors. On one hand, the possibly different endogenous wild-type hormonal background of the cultivars analysed has to be taken into account, as it is on this background that the integration of *A. tumefaciens* genes would act, leading to a genotype-dependent, and therefore not homogeneous, final auxin/cytokinin equilibria involved in the regulation of gene expression. On the other hand, the efficiency of the SA treatment itself must be considered. In some cases SA has in fact been reported to have varying degrees of effectiveness in inducing PR-proteins, such as basic β-1,3-glucanase (van de Rhee et al. 1993; Hudspeth et al. 1996), PR-5 proteins (Koiwa et al. 1994), and when administered in the absence of a pathogen it seems to be a weak inductive signal (Shirasu et al. 1997).

In conclusion, the modification of the hormonal background seemed to significantly alter the response of tomato tissue to *F. oxysporum* in vitro, especially by conferring to the susceptible cultivar a kind of "competence for active defense". This could open the way to valuable alternatives to the use of genes directly involved in the defense response (chitinase, β-1,3-glucanase, H_2O_2-generating glucose oxidase, stilbene synthase, etc.) in order to obtain plants with broad-spectrum resistance to pathogens. Within this frame, work is now in progress in our laboratory aimed at the production of tomato plants transgenic for *A. tumefaciens* and *A. rhizogenes* genes involved in hormone biosynthesis and utilization. These plants will provide a most important tool for obtaining pathogen resistance through a general or target-organ specific modification of hormone balance.

Acknowledgements Authors are grateful to Dr. M. Lambardi (Istituto per la Protezione delle Specie Legnose, C.N.R., Scandicci) and Dr. M. Michelozzi (Istituto per il Miglioramento Genetico delle Piante Forestali, C.N.R., Firenze) for helpful discussion and technical advice on ethylene determination, and to S. Bambi and R. Innocenti for photographic work. This research was supported by the Italian Ministry of Agricultural, Food and Forestal Resources (MIRAAF) in the frame of the National Plan: "Plant Biotechnology", Coordinated Research Project: "Physiological and molecular background and pathogen resistance". The experiments here reported comply with current Italian laws.

References

Beckman KB, Ingram DS (1994) The inhibition of the hypersensitive response of potato tuber tissues by cytokinins: similarities between senescence and plant defence responses. Physiol Mol Plant Pathol 45:229–246

Buiatti M, Scala A, Bettini P, Nascari G, Morpurgo R, Bogani P, Pellegrini MG, Gimelli F, Venturo R (1985) Correlation between in vivo resistance to *Fusarium* and in vitro response to fungal elicitors and toxic substances in carnation. Theor Appl Genet 70:42–47

Buiatti M, Simeti C, Marcheschi G, Scala A, Bettini P, Bogani P, Pellegrini MG (1987) Isolation of tomato cell lines with altered response to Fusarium cell wall components. Theor Appl Genet 75:37–40

Cutt JR, Klessig DF (1992) Pathogenesis-related proteins. In: Boller T, Meins F (eds) Genes involved in plant defense. Springer, Berlin Heidelberg, New York, pp 209–243

Danhash N, Wagemakers CAM, van Kan JAL, de Wit PJGM (1993) Molecular characterization of four chitinase cDNAs obtained from *Cladosporium fulvum*-infected tomato. Plant Mol Biol 22:1017–1029

Dermastia M, Ravnikar M (1996) Altered cytokinin pattern and enhanced tolerance to potato virus Y^{NTN} in the susceptible potato cultivar (*Solanum tuberosum* cv. Igor) grown in vitro. Physiol Mol Plant Pathol 48:65–71

De Wit PJGM (1992) Functional models to explain gene-for-gene relationship in plant-pathogen interactions. In: Boller T, Meins F (eds) Genes involved in plant defense. Springer, Berlin Heidelberg, New York, pp 25–47

Dixon RA, Harrison MJ, Lamb CJ (1994) Early events in the activation of plant defense responses. Annu Rev Phytopathol 32:479–501

Doyle J, Doyle J (1989) Isolation of plant DNA from fresh tissue. Focus 1:13–15

Felix G, Meins F Jr (1986) Developmental and hormonal regulation of β-1,3-glucanase in tobacco. Planta 167:206–211

Felix G, Meins F Jr (1987) Ethylene regulation of β-1,3-glucanase in tobacco. Planta 172:386–392

Flor HH (1946) Genetics of pathogenicity in *Melampsora lini*. J Agric Res 73 : 335–357

Gaffney T, Friedrich L, Vernoij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H, Ryals J (1993) Requirement of salicylic acid for the induction of systemic acquired resistance. Science 261 : 754–756

Gretenkort MA, Ingram DS (1993) The effect of environmental conditions on the expression of disease in cultured tissues of *Brassica napus* ssp. *oleifera* infected with *Leptosphaeria maculans*. J Phytopathol 138 : 293–307

Hudspeth RL, Hobbs SL, Anderson DM, Grula JW (1996) Characterization and expression of chitinase and 1,3-beta-glucanase genes in cotton. Plant Mol Biol 31 : 911–916

Inzé D, Follin A, Van Lijsebettens M, Simoens C, Genetello C, Van Montagu M, Schell J (1984) Genetic analysis of the individual T-DNA genes of *Agrobacterium tumefaciens*; further evidence that two genes are involved in indole-3-acetic acid synthesis. Mol Gen Genet 194 : 265–274

Jouanneau JP, Lapous D, Guern J (1991) In plant protoplasts, the spontaneous expression of defense reactions and the responsiveness to exogenous elicitors are under auxin control. Plant Physiol 96 : 459–466

Koiwa H, Sato F, Yamada Y (1994) Characterization of accumulation of tobacco PR-5 proteins by IEF-immunoblot analysis. Plant Cell Physiol 35 : 821–827

Lennox CL, Van Staden J, Rijkenberg FHJ (1993) Endogenous cytokinin levels and wheat stem rust resistance. S Afr J Bot 59 : 633–637

Linsmaier EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Plant Physiol 18 : 100–127

Malamy J, Carr JP, Klessig DF, Raskin I (1990) Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. Science 250 : 1002–1004

Memelink J, Hoge JHC, Schilperoort RA (1987) Cytokinin stress changes the developmental regulation of several defence-related genes in tobacco. EMBO J 12 : 3579–3583

Memelink J, Linthorst HJM, Schilperoort RA, Hoge JHC (1990) Tobacco genes encoding acidic and basic isoforms of pathogenesis-related proteins display different expression patterns. Plant Mol Biol 14 : 119–126

Mohnen D, Shinshi H, Felix G, Meins F Jr (1985) Hormonal regulation of β-1,3-glucanase messenger RNA levels in cultured tobacco tissues. EMBO J 4 : 1631–1635

Pearson WR, Lipman DJ (1988) Improved tools for biological sequence comparison. Proc Natl Acad Sci USA 85 : 2444–2448

Ryals J, Neuenschwander UH, Willits MG, Molina A, Steiner H-Y, Hunt MD (1996) Systemic acquired resistance. Plant Cell 8 : 1809–1819

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

Sarhan A, Kiraly Z, Sziraki I, Smedegaard-Petersen V (1986) Increased levels of cytokinins in barley leaves having the systemic acquired resistance to *Bipolaris sorokiniana* (Sacc.) Shoemaker. J Phytopathol 131 : 101–108

Sessa G, Meller Y, Fluhr R (1995) A GCC element and a G-box motif participate in ethylene-induced expression of the *PRB-1b* gene. Plant Mol Biol 28 : 145–153

Shinshi H, Mohnen D, Meins F Jr (1987) Regulation of a plant pathogenesis-related enzyme: inhibition of chitinase and chitinase mRNA accumulation in cultured tobacco tissue by auxin and cytokinin. Proc Natl Acad Sci USA 84 : 89–93

Shinshi H, Usami S, Ohme-Takagi M (1995) Identification of an ethylene-responsive region in the promoter of a tobacco class I chitinase gene. Plant Mol Biol 27 : 923–932

Shirasu K, Nakajima H, Rajasekhar VK, Dixon RA, Lamb C (1997) Salicylic acid potentiates an agonist-dependent gain control that amplifies pathogen signals in the activation of defense mechanisms. The Plant Cell 9 : 261–70

Storti E, Latil C, Salti S, Bettini P, Bogani P, Pellegrini MG, Simeti C, Molnar A, Buiatti M (1992) The in vitro physiological phenotype of tomato resistance to *Fusarium oxysporum* f. sp. *lycopersici*. Theor Appl Genet 84 : 123–128

Storti E, Bogani P, Bettini P, Bittini P, Guardiola ML, Pellegrini MG, Inzé D, Buiatti M (1994) Modification of competence for in vitro response to *Fusarium oxysporum* in tomato cells. II. Effect of the integration of *Agrobacterium tumefaciens* genes for auxin and cytokinin synthesis. Theor Appl Genet 88 : 89–96

van de Rhee MD, Lemmers R, Bol JF (1993) Analysis of regulatory elements involved in stress-induced and organ-specific expression of tobacco acidic and basic beta-1,3-glucanase genes. Plant Mol Biol 21 : 451–461

van de Rhee MD, Linthorst HJM, Bol JF (1994) Pathogen-induced gene expression. In : Basra AS (ed) Stress-induced gene expression in plants. Harwood Academic Publ, London, pp 249–284

Van Der Straeten D, Rodrigues-Pousada RA, Villarroel R, Hanley S, Goodman HM, Van Montagu M (1992) Cloning, genetic mapping and expression analysis of an *Arabidopsis thaliana* gene that encodes 1-aminocyclopropane-1-carboxylate synthase. Proc Natl Acad Sci. USA 89 : 9969–9973

van Kan JAL, Joosten MHAJ, Wagemakers CAM, van den Berg-Velthuis GCM, de Wit PJGM (1992) Differential accumulation of mRNAs encoding extracellular and intracellular PR proteins in tomato induced by virulent and avirulent races of *Cladosporium fulvum*. Plant Mol Biol 20 : 513–527

van Kan JAL, Cozijnsen T, Danhash N, de Wit PJGM (1995) Induction of tomato stress protein mRNAs by ethephon, 2,4-dichloroisonicotinic acid and salicylate. Plant Mol Biol 2 : 1205–1213

Wingate VPM, Lawton MA, Lamb CJ (1988) Glutathione causes a massive and selective induction of plant defense genes. Plant Physiol 87 : 206–210