# EXPRESSION OF PATHOGENESIS-RELATED GENES IN TRANSGENIC BROCCOLI AND CANOLA PLANTS EXPRESSING THE *Trichoderma harzianum*-ENDOCHITINASE GENE

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## ABSTRACT

Expression of genes encoding two pathogenesis-related plant proteins (PR-1 and PR-2) was examined in transgenic broccoli plants expressing the *Trichoderma harzianum* endochitinase gene and in control plants. mRNA accumulation in two-month-old plants inoculated with *Alternaria brassicicola* and in non-inoculated plants was assessed by RNA hybridization, using PR-1 and PR-2 DNA sequences of *Arabidopsis thaliana* as probes. Non-transgenic controls and transgenic controls carrying a different transgene (*cry1Ac* from *Bacillus thuringiensis*) showed accumulation of PR-1 mRNA only after inoculation. In contrast, endochitinase-transgenic plants produced PR-1 mRNA with and without inoculation. These results indicate that the PR-1 gene, normally induced by fungal infection, is constitutively expressed in transgenic plants expressing the heterologous endochitinase gene. Accumulation of PR-2 mRNA was observed in all transgenic and non-transgenic plants, with or without inoculation. This suggests that the PR-2 gene may be permanently active or developmentally regulated and is involved in functions other than systemic acquired resistance. Similar results were seen when expression of PR-1 and PR-2 was examined in endochitinase-transgenic rapeseed (*Brassica napus*).

**ADDITIONAL KEY WORDS:** *Alternaria brassicicola, Brassica oleracea* var. *italica*, endochitinase, systemic acquired resistance.

## EXPRESIÓN DE GENES RELACIONADOS CON LA PATOGENICIDAD EN PLANTAS DE BRÓCOLI EXPRESANDO EL GEN ENDOQUITINASA DE *Trichoderma harzianum*

## RESUMEN

La expresión de genes que codifican dos proteínas relacionadas con la patogenicidad (PR1 y PR2) fue examinada en plantas transgénicas de brócoli que expresan el gen endoquitinasa de *Trichoderma harzianum*, y en plantas testigo. La acumulación de ARN mensajero (mARN) en plantas de dos meses de edad inoculadas con *Alternaria brassicicola* y en plantas no inoculadas, fue determinada por hibridación de ARN usando dos secuencias de *Arabidopsis thaliana* PR1 y PR2 como sondas. Los controles no transgénicos y transgénicos con diferente transgen (*cryIAc* de *Bacillus thuringiensis*) mostraron acumulación de mARN de PR-1 solamente después de la inoculación. En contraste, las plantas transgénicas con endoquitinasa produjeron la acumulación de mARN de PR-1 con y sin inoculación. Estos resultados indican que el gen PR-1 normalmente inducido por infección fúngica, es expresado constitutivamente en plantas transgénicas que expresan el gen heterólogo endoquitinasa. La acumulación de mARN de PR-2 fue observado en todas las plantas transgénicas y no transgénicas, con y sin inoculación. Esto sugiere que el gen PR-2 puede estar permanentemente activado o bien es regulado en función del desarrollo de la planta y está envuelto en otras funciones diferentes a la resistencia sistémica adquirida. Resultados similares fueron observados cuando la expresión de PR-1y PR-2 fue examinada en canola transformada con el gen endoquitinasa.

**PALABRAS CLAVE ADICIONALES:** *Alternaria brassicicola, Brassica oleracea* var. *italica,* endoquitinasa, resistencia sistémica adquirida.

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## INTRODUCTION

Pathogenesis-related (PR) proteins are induced concurrently with the appearance of the systemic acquired resistance (SAR) mechanism and are believed to contribute to its efficiency (Stintzi *et al.*, 1993). Synthesis of PR proteins has been reported to occur as a result of plant infection by viruses, viroids, bacteria or fungi (Carr and Klessing, 1989; Bol *et al.*, 1990; Bowles, 1990; Linthorst, 1991; White and Antoniw, 1991; Stintzi *et al.*, 1993; Van Loon, 1985; Yun *et al.*, 1997). At least some members of PR families are also induced after treatments with chemicals (Hanfrey *et al.*, 1996; Dixelius, 1994) or osmotic stress (Yu *et al.,* 1998) and/or are believed to be developmentally regulated (Hanfrey *et al.*, 1996; Lers *et al.*, 1998).

Basic or acidic PR protein forms are induced through stress signals or may be expressed constitutively in certain organs. For instance, an acidic PR-5 protein from *Arabidopsis* was induced by pathogen infection and salicylic acid and showed activity against several fungal pathogens (Hu and Reddy, 1997). Similarly, Chang *et al.* (1997) reported that the nonpathogenic mycelial extract of *Trichoderma longibrachiatum* and combination of salicylic acid and methyl jasmonate induced resistance to *Phytophthora parasitica* var. *nicotianae* (race 0) in tobacco seedlings and induced expression of acidic PR-1b and acidic osmotin (PR-5) genes. Another case of PR gene activation by a pathogen is the increase in mRNA, protein, and enzyme activity levels of 3 acidic forms of b-1,3-glucanase (PR-2a, -2b, -2c) caused by tobacco mosaic virus (TMV) infection (Cote *et al.*, 1991). Increases in steady state levels of PR-2 mRNA, protein, and enzyme activities indicated that levels of PR-2 protein were regulated by mRNA accumulation. Finally, expression of several PR genes was enhanced upon *Pto-avrPto* recognition in tobacco, induced after binding of Pti4/5/6 proteins in the PR gene promoter region (Zhou *et al.*, 1997).

Our objective was to determine whether the endochitinase transgene from *Trichoderma harzianum* induces the expression of other PR proteins in the SAR pathway.

## MATERIALS AND METHODS

### Plant Material

Two month-old T1 progeny of 'Green Comet' broccoli (*Brassica oleracea* var. *italica*) and of 'Westar' rapeseed (*B. napus*) plants expressing the *T. harzianum* endochitinase gene (Hayes *et al*., 1994; NCBI No. L14614) were used. Eight primary transgenic broccoli and four canola plants were obtained by transformation of cotyledonary-petioles with *Agrobacterium tumefaciens* strain LBA4404 carrying the endochitinase gene construct (Mora and Earle*,* 2001). Putative transgenic plants were selected via resistance to kanamycin, and the levels of endochitinase activity were determined by fluorometric analysis (Mora*,* 2000). Four transgenic Green Comet plants carrying the *Bacillus thuringiensis* (Bt) *cry1Ac* gene (Metz *et al.,* 1995) and non-transgenic Green Comet plants were used as controls. Both the endochitinase-transgenic and Bt-transgenic plants contained and expressed the *nptII* gene for resistance to kanamycin. The Bt-transgenic plants expressed the *cry1A*c gene, as shown by their toxicity to the Lepidopteran insect *Plutella xylostella* (Metz *et al*., 1995). Seeds were germinated in flats in Metro Mix™ at 18 °C during the day and 22 °C at night with a 16-h photoperiod supplied with incandescent lights (120 $E \cdot m^2 \cdot s^{-1}$). Seedlings were watered twice a day and fertilized once a week with 15-16-17 Peat-Lite Special water-soluble fertilizer, added to full soil saturation during watering (The Scotts Company).

### Fungal cultures

A culture of *A. brassicicola* (Schwein) Wiltshire (ATCC No. xxx), obtained from Hellen Dillard, Department of Plant Pathology, New York Agricultural Experiment Station, Geneva, N.Y.; American Type Culture Collection ATCC MYA-897 was grown on potato dextrose agar (PDA) medium (Difco) for approximately 7 days at 20 °C. *Sclerotinia sclerotiorum* (Lib.) provided by J. Tewary, University of Alberta, Edmonton, Alberta, Canada was grown on V8 medium for 5 days at 20 °C.

### Plant inoculation

Two month old broccoli plants grown in a greenhouse under the conditions described in "Plant Material" were sprayed with *A. brassicicola* at a concentration of $5 \times 10^4$ spores per ml (50 ml per flat) (King, 1994) and incubated in a mist chamber at 22 °C. High humidity was maintained with a mist interval of 6 seconds every 32 min for two days and 6 seconds every 64 min for another two days. Two month old rapeseed plants were inoculated at the base of the stem placing a 5 mm plug containing *S. sclerotiorum* mycelium. Humidity was maintained with transparent plastic covers, and flats were watered profusely up to field capacity. Seedlings were incubated in the greenhouse during 7 days at 22 °C.

### Isolation of total cellular RNA

Leaf material was collected from inoculated and non-inoculated plants eight days after inoculation in both species. Total RNA was extracted according to the protocol of Verwoerd *et al.* (1989). RNA (20 ng) denaturation and gel electrophoresis and blotting were performed according to Sambrook *et al*. (1988). Probe labeling with $^{32}P$ dCTP was done using the Random Primed DNA Labeling Kit (Boehringer Mannheim). Hybridization followed Church and Gilbert (1984) and Sambrook *et al*. (1989).

### PR Probes

Two PR gene sequences were found at the National Center for Biotechnology Information (NCBI) PR-1 (NCBI

No. M90508) and PR-2 (NCBI No. M90509). Both PR-1 and PR-2 are acidic types and are considered as pathogen-induced compounds in crucifers (Yun *et al.*, 1997). Primers were designed based on the regions that presented complete homology during the BLAST aligning [BLAST v.2 (Altschul *et al.* 1997)]. These primers were used to amplify *Arabidopsis* PR gene sequences. The expected band was cut out of the gel and purified using CleanGene II Kit (Bio 101). Primer sequences of PR genes assayed on *Arabidopsis* DNA were as follows: PR-1 5' primer: GCT GGA CAA ATC GGA and PR-1 3' primer: TCT CCG ACA CCA CGA; PR-2 5' primer: TCC CTC GAA AGC TCA AGA, and PR-2 3' primer: CGA GGA TCA TAG TTG CAA. PCR running conditions were 94 °C per 1 minute for denaturation, 50 °C per 1 minute for annealing, and 72 °C per 2 minutes for amplification for 35 cycles.

### Actin probe

An actin gene (1.5 kb) cloned from a *Brassica* anther cDNA library (Yu *et al.*, 1999) and kindly provided by Dr. J. Nasrallah, Dept. of Plant Biology, Cornell University was used as an internal control on RNA blots. The actin gene was isolated from plasmid DH5a by a PCR approach. Actin primers were designed from the cDNA sequence: Actin 5': AAT GGT ACC GGA ATG GTC AA, and Actin 3': GAA CCA CCG ATC CAG ACA CT. PCR conditions were 94 °C per 1 minute for denaturation, 55 °C per 1 minute for annealing, and 72 °C per 2 minutes for amplification during 35 cycles.

## RESULTS AND DISCUSSION

### PR-1 gene

A *Brassica napus* PR-1 gene of 683 bp (Zhang and Fristenskty, 1996) was compared to an *Arabidopsis thaliana* (strain Columbia) PR-1 gene of 757 bp (Uknes *et al.*, 1992), by using BLAST to determine the percentage of sequence identity between these two genes. It was found that the *A. thaliana* and *B. napus* PR-1 genes have 84 % identity. Identical regions were searched to determine the most suitable sequence region for designing of primers.

### PR-2 gene (glucanase)

A *B. campestris* PR-2 gene containing 1284 bp (Newman *et al.*, 1994) and an *A. thaliana* (strain Columbia) PR-2 gene of 1181 bp (Uknes *et al.*, 1992) were obtained from this search. These nucleotide sequences have 83 % identity between them.

### PCR assay

PCR assays were done to determine the suitability of the PR-1 and PR-2 primers on *A. thaliana* genomic DNA (kindly provided by Dr. S. Tanksley, Dept. of Plant Breeding, Cornell University). PR-1 primers amplified a single band of 400 bp in *Arabidopsis* DNA, whereas PR-2 primers amplified a band of approximately 700 bp (data not shown).

### Transcription of PR-1 in transgenic broccoli plants

Inoculated transgenic plants and controls both showed transcription of PR-1 gene (Figure1). Samples from non-inoculated transgenic plants where RNA was present as indicated by actin mRNA accumulation (Figure 1) also showed transcription; however, the non-inoculated control plant did not show the PR-1 band (Figure1). Transgenic broccoli with the *B. thuringiensis cry1Ac* gene transcribed the PR-1 gene after inoculation, but not without inoculation (Figure 2).

### Transcription of PR-1 in rapeseed plants

Rapeseed RNA blots gave similar results. Non-inoculated transgenic lines showed a transcription signal in every sample, whereas no signal was detected in the non-inoculated control.

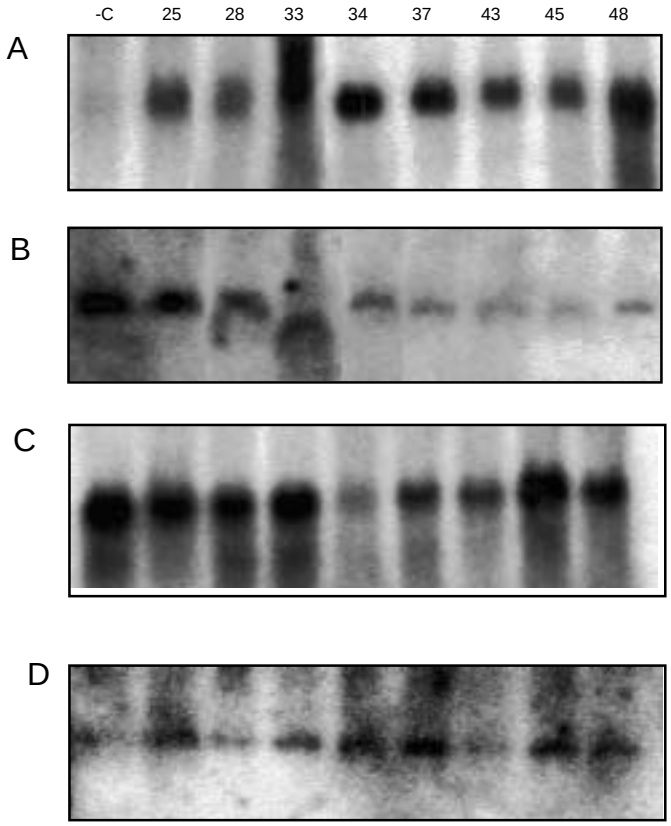


**FIGURE 1. Northern blots of T1 broccoli lines expressing the *Trichoderma harzianum* endochitinase gene and control plants, probed with PR-1. Lane -C: non-transgenic broccoli; Lanes 25 to 48: transgenic lines. A. PR-1 mRNA of non-inoculated plants; B. actin mRNA of non-inoculated plants; C. PR-1 mRNA of plants inoculated with *Alternaria brassicicola*; D. actin mRNA of inoculated plants.**

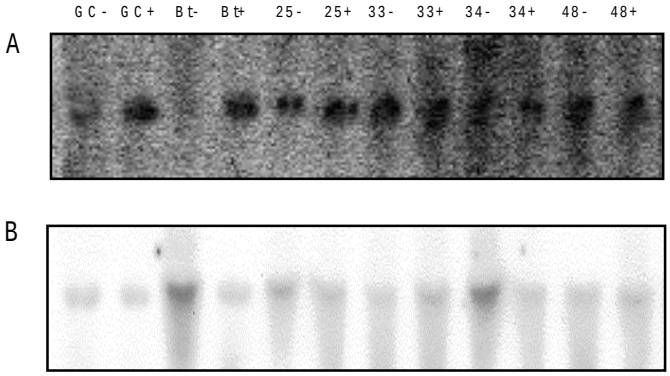


**FIGURE 2. Accumulation of PR-1 mRNA in transgenic and control broccoli lines not-inoculated (+) or inoculated (-) with *Alternaria brassicicola*. Lanes GC: non-transgenic broccoli; Lanes Bt: transgenic broccoli carrying the *Bacillus thuringiensis cryIAc* gene; Lanes 25-48: transgenic lines with the *Trichoderma harzianum* endochitinase gene. A. PR-1 probe; B. Actin probe.**

Transgenic and non-transgenic broccoli inoculated with *A. brassicicola* accumulated PR-1 mRNA, but only endochitinase-transgenic lines also accumulated PR-1 mRNA without inoculation. These results suggest that a) PR-1 mRNA accumulates in non-transgenic plants after *A. brassicicola* infection as a result of signal transduction triggered by plant-pathogen recognition; b) the heterologous endochitinase gene may be a transcriptional inducer of plant PR-proteins that compose the SAR mechanism; c) part of the SAR mechanism is constitutive in endochitinase-transgenic plants; d) expression of PR-1 is transgene-specific and is not related to the transformation process per se or to the *nptII* selectable marker gene present in both types of transgenic plants assayed.

T1 endochitinase-transgenic broccoli plants inoculated with *A. brassicicola* showed less severe symptoms of disease compared to control plants (Mora, 2000); however, it is not clear whether both the PR-1 and endochitinase mRNA are involved in the enhanced protection against *A. brassicicola*. On the other hand, the induction of PR-1 in controls after infection with *A. brassicicola* suggests that transcription of PR-1 in non-inoculated transgenic plants has a direct anti-fungal potential.

Induction of PR proteins by the biocontrol fungus *T. harzianum* was reported in cucumber seedlings (Yedidia *et al.*, 1999). An increased level of chitinase (PR-3) and peroxidase were observed in leaves and roots 48 hrs after application of *T. harzianum*.

### Transcription of PR-2 in transgenic plants

Inoculated and non-inoculated T1 broccoli plants and controls transcribed the PR-2 gene at the same level whenever RNA was present (Figure 3). Similarly, T1 rapeseed plants showed no differential transcription between transgenic lines and control samples (data not shown).

A direct role for PR-2 proteins (β-1,3-glucanase) in defense against pathogens has been suggested because glucan (the substrate of this protein) is a major component of cell walls of many fungi (Wessels and Sietsma, 1981; Farkas, 1990). In our hybridization of a PR-2 probe to RNA blots we found that inoculated and non-inoculated transgenic and non-transgenic plants all accumulated PR-2 mRNA. The appearance of PR proteins when pathogen invasion is not evident suggests that PR genes may have multiple functions or modes of induction and may have roles unrelated to SAR. Hanfrey *et al.* (1996) found that a protein with high sequence similarity to a PR-1-like protein is induced in healthy and uninfected leaves of *B. napus* during leaf senescence. They proposed that during leaf senescence tissues are vulnerable due to decompartmentalization of chloroplasts and translocation of proteins to other parts of the plant and that PR proteins are induced as part of the defense system during this process. Similarly, Lers *et al.* (1998) found that levels of proteins like PR-1, PR-2, and PR-3 increased in parsley during leaf senescence.

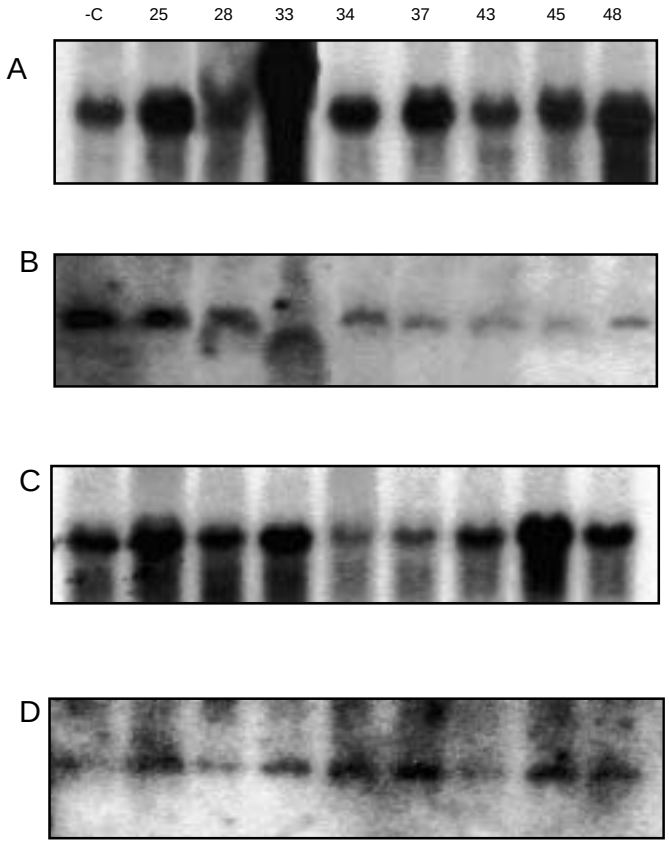


**FIGURE 3. Northern blots of T1 broccoli lines expressing the endochitinase gene inoculated with *Alternaria brassicicola* and probed with PR-2. Lane -C: non-transgenic plant; Lanes 25 to 48: transgenic lines. A. PR-2 mRNA of non-inoculated plants; B. actin mRNA of non-inoculated plants; C. PR-2 mRNA of inoculated plants; D. actin mRNA of inoculated plants.**

Expression of PR proteins has been studied in crucifers such *as A. thaliana, B. napus,* and *B. nigra*. Dixelius (1994) found that PR-1 and PR-2 mRNA accumulation was higher in *B. nigra* resistant to *Phoma lingam* than susceptible *B. napus,* in which no or extremely low expression was observed. Western blot analysis indicated that these proteins were more active and expressed much earlier in resistant *B. nigra*. *Arabidopsis* plants sprayed with the SAR-activator INA showed reduced pathogenesis after inoculation with *Peronospora parasitica*. This event correlated well with accumulation of PR-1, PR-2, and PR-5 mRNAs and proteins (Uknes *et al.,* 1992).

Levels of PR-1 and PR-2 mRNA accumulation varied among our transgenic broccoli lines. We did not measure accumulation of endochitinase mRNA, but have assessed the endochitinase activity of the transgenic lines by fluorometric analysis (Mora and Earle, 2001). Endochitinase activity levels in 2-month-old T1 plants varied from 75 to 208 times over control (Mora and Earle, 2001); however, these values did not correlate with mRNA accumulation of endogenous PR genes, since the levels of expression among non inoculated transgenic plants and inoculated non-transgenic plants did not varied regardless endochitinase levels.

The modest resistance to *Alternaria brassicicola* and lack of enhanced resistance to *Sclerotinia sclerotiorum* observed on inoculated plants after accumulation of PR mRNA may be due a post-transcriptional modification that limits the accumulation of protein to low levels (Mora and Earle, 2001).

We conclude that the endogenous PR-1 gene is constitutively transcribed in endochitinase-transgenic plants in absence of fungal infection. The fact that Bt-transgenic plants failed to accumulate PR-1 mRNA suggests that endochitinase and no other gene in the constructs (e.g. *nptII*) is responsible for the PR-1 induction. These results also suggest that foreign genes from different kingdoms cannot only be expressed in other organisms but can also modify substantially the expression of native genes. The PR-2 gene, on the other hand, was transcribed even in the absence of pathogen infection on control plants. This may confirm that some PR proteins are indeed activated by other metabolic signals.

Comparison of mRNA and/or protein accumulation from other PR genes in endochitinase-transgenic and control plants would increase understanding of the non pathogen-specific mechanism of resistance in plants. It would also be interesting to study various time periods after inoculation to assess the initiation of PR gene expression and so determine the speed of recognition of the pathogen by the transgenic plants versus controls. The plant materials available provide a good basis for further work on the effect of a heterologous PR gene on the induction of other PR genes, with and without pathogen attack.

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