Bean Polygalacturonase-inhibiting Protein Expressed in Transgenic *Brassica napus* Inhibits Polygalacturonase from its Fungal Pathogen *Rhizoctonia solani*

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Abstract

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Polygalacturonase-inhibiting proteins (PGIPs) selectively inhibit polygalacturonases (PGs) secreted by invading plant pathogenic fungi. The objective of present research was to clone and introduce the *pgip*2 gene from bean (*Phaseolus vulgaris*) cv. Goli, with antifungal potential, into the commercially important canola (*Brassica napus*, R line Hyola 308) via *Agrobacterium tumefaciens* mediated transformation. Here we used a transgenic overexpression approach in order to investigate the inhibitory activity of the PGIP on the PG from *Rhizoctonia solani,* the causal agent of damping off and root rot of canola. PGIP expression was determined in the functional inhibition assays against fungal PGs. Crude protein extracts prepared from transgenic canola leaves were found to inhibit the *R. solani* PG from 29% to 37% as compared to untransformed plants. The putative transgenic canola lines harbouring the *pgip*2 gene encoding polygalacturonase-inhibiting proteins were identified by polymerase chain reaction and Southern blot analysis.

Keywords: canola; bean; transformation; PGIP

Rapeseed (*Brassica napus* L.) is one of the most important sources of edible vegetable and industrially used oil in the world. Like many other crops, the production of this crop is challenged by phytopathogenic fungi. The conventional fungal pathogen control method is mainly dependent on the intensive and extensive use of chemical fungicides, which have drawbacks such as doing harm to the ecological system, producing residual poisons to human beings and animals, and its expensive cost. Therefore, it is desirable to develop fungus-resistant plants through the introduction of foreign fungus-resistant genes into them. Among fungus-resistant genes, the *pgip* gene encoding polygalacturonase-inhibiting protein has been proved effective in controlling the development of fungal pathogens (Powell *et al.* 2000; Oelofse *et al.* 2006; Richter *et al.* 2006).

During plant infection, most phytopathogenic microorganisms produce enzymes like polygalacturonases that are capable of degrading the plant cell wall and of penetrating the tissue (Johnston & Williamson 1992). The PGs are the first enzymes to be secreted by pathogens when they encounter plant cell walls, and their contribution to the pathogenicity of some fungi and bacteria has been assessed (De Lorenzo *et al.* 1997; Idnurm & Howlett 2001). Inhibitors of these cell wall-degrading fungal enzymes have been proposed to be part of the plant defence to

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limit fungal development and colonisation. The polygalacturonase-inhibiting proteins (PGIP) are the cell wall-located glycoproteins that specifically inhibit the fungal PGs. They belong to a large family of leucine-rich repeat (LRR) proteins (Stotz *et al.* 1994; De Lorenzo *et al.* 2001). The LRR is a versatile structural motif found in many proteins and is implicated in protein-protein interactions. By acting as both inhibitors and regulators of the PG, PGIPs favour the release of oligogalacturonides, which are elicitors of a variety of defence responses (De Lorenzo *et al.* 2001). Plants have evolved the PGIPs with specific modes of expression and specific recognition capabilities for many PGs secreted by phytopathogenic fungi (reviewed by Yao *et al.* 1995; Desiderio *et al.* 1997; Leckie *et al.* 1999; Di Matteo *et al.* 2006), they are ineffective against the other pectic enzymes of different microbial or plant origin (Cervone *et al.* 1990).

Antifungal properties of PGIPs were confirmed by transgenic approach (Powell *et al.* 2000; Aguero *et al.* 2005; Joubert *et al.* 2006). Heterologous expression of pear PGIP in transgenic tomatoes and grapevine PGIP in transgenic tobacco demonstrated that the PGIP inhibition of fungal PGs slows down the expansion of disease lesions and the associated tissue maceration (Powell *et al.* 2000; Joubert *et al.* 2006).

In canola, *Rhizoctonia solani* is one of the most important causal agents of hypocotyl rot in young seedlings (Khangura *et al.* 1999), pre-emergence and post-emergence damping-off (Yitbarek *et al.* 1987; Keinath 1995; Baird 1996), seedling rot in plants up to 6 weeks of age, brown girdling root rot (Yitbarek *et al.* 1987), and seedling death (Baird 1996). The infection of canola by *R. sola*ni occurs in most canola growing regions of the world and reduces yield by 8–30% (Huber & Christmas 1992; Verma 1996; Khangura *et al.* 1999). PGs are among the enzymes produced by *R. solani* which participate in fungal penetration in order to initiate and expand necrotic infections or to establish the colonisation sites for biotrophic infections within plants.

In this paper we report the cloning of the *pgip*2 gene from *Phaseolus vulgaris* cv. Goli that was introduced into the R line of Hyola 308 of *Brassica napus* by *Agrobacterium*-mediated transformation. The introduced gene was expressed in transgenic plants and showed the inhibition of PG activity of *R. solani* to some extent.

MATERIAL AND METHODS

All general molecular biology techniques were carried out according to Sambrook and Russell (2001), unless otherwise stated.

Plant material. The rapeseed (*Brassica napus* L.) R line Hyola 308, used as a receptor, was kindly provided by the Oilseed and Development Co., Tehran, Iran.

Enzymes and chemicals. All chemicals, culture media, plant growth regulators and antibiotics were purchased from Merck (Darmstadt, Germany) at the highest purity available, unless stated otherwise. The restriction enzymes and other DNA-modifying enzymes were obtained from Roche (Applied Science GmbH, Mannheim, Germany) and Fermentas (Bourlington, Canada).

Bacterial strains and plasmids. *E. coli* DH5α was used in all molecular biological experiments and *Agrobacterium tumefaciens* LBA4404 was used for plant transformation. The bacteria were grown in LB (Luria-Bertani) medium at appropriate temperatures (37°C for *E. coli* and 28°C for *Agrobacterium tumefaciens*) with shaking (200 rpm). Plasmid pUC19 (Fermentas, Bourlington, Canada) was used for routine cloning and sequencing and plasmid pBI121 (Novagen; Merck, Darmstadt, Germany) was used as a binary plant expression vector.

Cloning of the pgip2 gene. Leaf material from the *Phaseolus vulgaris* cv. Goli was harvested, lyophilised and ground into fine powder for extraction of genomic DNA by the method of Doyle and Doyle (1990). DNA fragment containing the *pgip*2 gene was amplified by PCR using genomic DNA. The primers used for amplification of the *pgip*2 gene were designed based on the *pgip*2 sequence in the GenBank from NCBI web site (www.ncbi.nlm.nih.gov).

The *pgip*2 gene was amplified by PCR using the specific primers: 2RB1 as forward primer: 5'-GCT CTA GAA TGT CCT CAA GCT TAA GCA TAA TTT TG-3' and 2RB2 as reverse primer: 5'-GCA CGA GCT CTT AAG TGC AGG CAG GAA GAG G-3' with the *Xba*I and the *Sac*I sites at the 5' end of primers (underlined), respectively. PCR reactions contained 2.5 units of Fermentas *Pfu* DNA polymerase, 1× buffer, 200μM of each deoxynucleotide triphosphate, 2μM $MgSO_4$, and 0.5μM primers. Reaction conditions for PCR amplification were 94°C for 90 s, 56°C for 45 s, and 72°C for 150 s, for 34 cycles followed by a final

extension of 5 minutes. PCR products were separated by electrophoresis on a 1% agarose gel. The resulting PCR product (1 kb) was cloned into the pUC19 plasmid and sequenced from both directions with the M13 standard primers, using the dideoxy chain termination method.

The resulting plasmid pBIAE2 contains, within the T-DNA region, neomycin phosphotransferase II (*NPT*II) gene as a selectable marker that is kanamycin-resistant gene for plant selection; *pgip*2 gene, encoding the polygalacturonase inhibiting protein (PGIP) from the *Phaseolus vulgaris* L. cv. Goli. The *NPT*II gene is regulated by the nopaline synthase promoter and the terminator; the *pgip*2 gene is regulated by the *Cauliflower mosaic virus* 35S promoter (CaMV 35S) and terminated by the nos terminator (Figure 1). The *A. tumefaciens* strain LBA4404 harbouring the binary vector pBIAE2 harbouring the *pgip*2 gene was used in the experiments.

Preparation of explants and the bacterial strain for transformation. Seeds were sterilised by submerging in 70% ethanol for 5 min and then in 0.1% $HgCl_2$ for 8 minutes. They were then rinsed with sterilized water several times and plated on ½MS medium (Murashige & Skoog 1962) and incubated in the presence of light for 5 days. After germination, the cotyledonary petioles were cut and pre-cultured on CM solid medium (MS with 3.5 mg/l of benzylaminopurine – BAP). After 2 days, the explants were used for transformation.

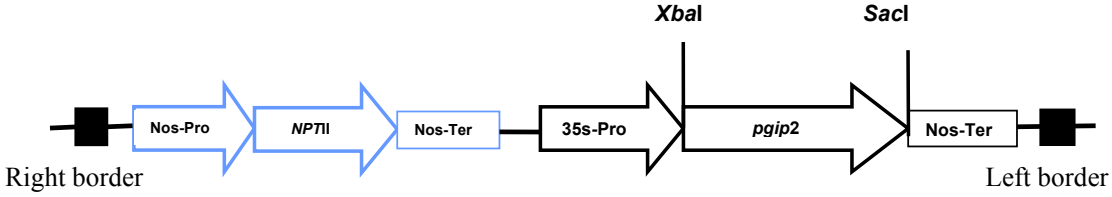
Single colonies of *A. tumefaciens* strain harbouring pBIAE2 containing the *pgip*2 gene were used to incubate LB medium supplemented with 50 mg/l of kanamycin, and allowed to grow overnight at 27–28°C with constant shaking (200 rpm) to mid-log phase. The bacterial culture was then transferred to fresh medium and cultivated until the optical density (OD_{600}) of 0.4 was obtained. The bacterial cells were then collected by centrifugation and re-suspended in fresh ½MS medium before use.

Transformation and selection procedure. Explants were immersed in a bacterial suspension for 1.5 min with constant shaking, then placed onto the sterile filter paper to remove excessive moisture, and placed on CM medium in Petri dishes for co-cultivation at 25°C for 2 days.

After co-cultivation, the explants were washed with sterile water containing 200 mg/l cephatoxim to inhibit the growth of *A. tumefaciens* attached to the explants and then transferred to MS solid medium containing 3.5 mg/l of BAP, 15 mg/l of kanamycin, and 200 mg/l of cephatoxim. After shoot initiation, the explants were transferred to MS solid medium with 25 mg/l of kanamycin and 200 mg/l of cephatoxim. Regenerating shoots (about 3 cm in length) were excised from the explants and transferred to MS solid medium with 2 mg/l of 3-indolebutyric acid (IBA), 25 mg/l of kanamycin, and 200 mg/l of cephatoxim for rooting and recovering complete plants. All the above media contained 3% (w/v) of sucrose and 1.5% of agar, pH 5.8. The explants were cultured at 23–25°C and under 16/8 h photoperiod, with light intensity of 2000 Lux.

Expression analysis. Specific mRNAs of the transgene were checked using reverse transcriptase (RT)-PCR. Total RNA was isolated from leaves of transgenic and control canola plants using an RNA isolation kit (Roche Applied Science, Mannheim, Germany). First strand cDNA was generated using the bean *pgip*2 specific primer (2RB2). PCR amplification of the 1002 bp fragment of the above gene was achieved using the first strand synthesis as template with specific primers (2RB1/2RB2) as described for PCR amplification of the *pgip*2 gene.

Southern blot analysis. Genomic DNA was extracted from fresh leaves of putative transgenic plants and untransformed control plants by the Cetyl Trimethyl Ammonium Bromide (CTAB) method (Doyle & Doyle 1990). PCR positive plants and untransformed control plants were



Nos-pro – nopaline synthase promoter; *NPT*II – gene for neomycin phosphotransferase; Nos-ter – terminator of nopaline synthase; 35s-Pro – 35S promoter of cauliflower mosaic virus; *pgip*2 – bean polygalacturonase inhibiting protein gene

Figure 1. Schematic representation of the T-DNA region of the transformation vector pBIAE2

analysed by Southern blot analysis to confirm the integration of the introduced genes.

Genomic DNA (15 µg) was digested with *Xba*I. The digested genomic DNAs were fractioned on 0.7% (w/v) agarose gels, transferred onto a nylon membrane (Amersham Hybond N^{TM}+; Amersham International Plc, Amersham, UK) and hybridised to the Dig-dUTP labelled *pgip*2 probe. A partial internal fragment (493 bp in size) was obtained from PCR amplification of the *pgip*2 gene using M2f/M2R1 primers and plasmid pUC19 containing the *pgip*2 as template and subjected to DIG DNA labelling (Roche Applied Science GmbH, Mannheim, Germany) and used as a probe in hybridization experiments.

PGIP activity assay of transgenic canola expressing the pgip2 gene. The extraction of PGIP from transgenic canola was adopted by Desiderio *et al*. (1997). Canola leaf material (3 g) was ground to a fine powder in liquid nitrogen using a mortar and pestle. Two volumes of 1M NaCl in 20mM NaOAc, pH 4.7 were added to the leaf material. The extracts were then shaken at 4°C for 1 hour. Extracts were subsequently centrifuged at 13 000 g at 4°C for 20 minutes. The pellets were discarded and the supernatants were used in the dialysis step. Samples were dialysed twice at 4°C for 2 h against 20mM NaOAc (pH 4.7). A 12 000 MW cut-off dialysis membrane was used. The extracts were subsequently centrifuged at 13 000 g at 4°C for 20 min and the supernatants were stored at –20°C.

For PG extraction, *Rhizoctonia solani* was grown on 10 ml of pectic zymogram (PZ) medium (Sweetingham *et al.* 1986) in 25 ml Erlenmeyer flasks for 6 days at 21°C. The mycelium was removed by vacuum filtration and the filtrate was clarified by centrifugation at 12 000 g for 5 min at 4°C. The supernatant was collected and used for enzyme assays. The assays were repeated three times. All the controls were performed using the heat-denatured enzyme.

The inhibition of the PG activity was determined by measuring the release of reducing groups using the Somogyi assay with Nelson's arsenomolybdate reagent (Berger *et al*. 2000) in the absence and presence of the PGIP. The PG activity was determined in 0.1 ml of the reaction mixture containing 0.5% (w/v) polygalacturonic acid as substrate, 50mM sodium acetate (pH 5.2) and suitable amounts of the culture filtrates. The samples were maintained at 37°C for 60 minutes. One unit of the PG activity was defined as the amount of PG enzyme producing one microequivalent of reducing group at 37°C/min with 0.5% polygalacturonic acid as substrate (Salvi *et al.* 1990). The same mixture containing the PGIP was used to assay the PGIP activity. One unit of the PGIP activity was defined as the amount of protein required to reduce the activity of 1 U of the PG by 50% (Salvi *et al.* 1990). Protein concentrations were determined using the micro-assay protocol of Bradford (1976).

Sequencing and computer analysis. The cloned DNA fragments in pUC19 and pBI121 (70–220 ng/µl) were sequenced by a Commercial Service (Seqlab, Gottingen, Germany). Computer analysis of the sequences was carried out and the deduced amino acid sequence from the *pgip*2 gene was obtained by BLASTX Network Service (NCBI) and multiple alignment was generated using ClustalW (http://www.ebi-ac.uk/ClustalW).

RESULTS

We isolated the polygalacturonase inhibiting protein encoding a gene (*pgip*2) from *Phaseolus vulgaris* cv. Goli. To isolate the *pgip*2 gene, the

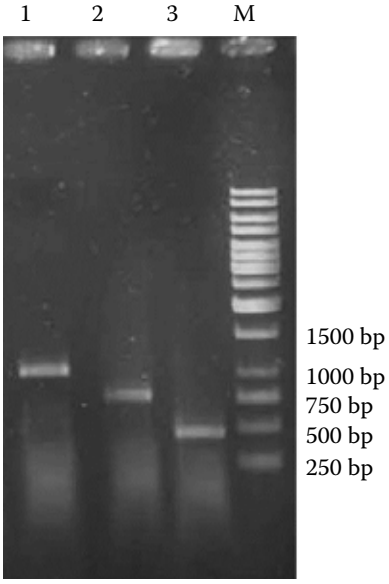


Figure 2. PCR analysis to confirm the cloning of *pgip*2: to differentiate the *pgip*2 sequence from that of the *pgip*1 which is highly similar, a set of mismatch primers was used: line 1 – the PCR amplified *pgip*2 by RB1/RB2 (1002 bp) primers, line 2 and 3 – amplification of 750 bp and 493 bp DNA fragments using the M2F/M2R2 and M2F/M2R1 mismatch primers, respectively. These two sets of primers amplify the related fragment only from the *pgip*2 gene; M = molecular size marker 1 kb ladder

oligonucleotide primers were designed based on the reported bean *pgip*s (Leckie *et al.* 1999; D'Ovidio *et al*. 2004) and compared to series of the related DNA sequences available in the GenBank database. PCR amplification was performed on the genomic DNA generating specific band of approximately 1 Kb which was cloned in pUC19 and confirmed by PCR using the specific and mismatch primers (Figure 2) and sequencing. This sequenced *Phaseolus vulgaris* cv. Goli *pgip*2 was highly similar and the coding polypeptide was identical to those isolated independently by D'Ovidio *et al*. (2004) from *Phaseolus vulgaris* cv. Pinto (with accession No. AJ864507) and Hosseinzadeh *et al*. (2005a,b) from *Phaseolus vulgaris* cv. Derakhshan and cv. Naz (with accession No. DQ105561 and DQ105560, respectively). The analysis of the *pgip*2 sequence revealed an open reading frame, 1002 bp in length, encoding a protein of 333 amino acids. The calculated molecular mass of the predicted product is 36034.5 dalton.

For a high level of expression, the complete coding region of the *pgip*2 gene was inserted between the CaMV 35S promoter and the nopaline synthase terminator in the plant expression vector pBI121 (Figure 1). DNA sequencing confirmed that the recombinant plasmid had been correctly constructed. The new construct (containing the *pgip*2 gene) designated as pBIAE2 was mobilised into *Agrobacterium tumefaciens* and subsequently used for *B. napus,* R line Hyola 308 transformation. The independent transgenic canola lines were successfully rooted on kanamycin-containing selection media. The transgenic plants were hardened off in the glasshouse and shown to contain the *pgip*2 transgene (a fragment corresponding to the size "1002 bp" of the *pgip*2 gene in all of the lines tested) using PCR (Figure 3). The transgenic lines were phenotypically analysed and compared with the untransformed controls and they did not show any abnormalities with regard to the growth, size or reproduction. The same primers did not amplify *pgip* in the untransformed sample.

Southern blot analyses were performed to verify the integration of the transgenes and to determine the respective copy number. When DNA from the *pgip*2 transgenic plants was digested with *Xba*I, which recognises one restriction site between the right border and the coding region of the *pgip*2 gene (Figure 1), the Southern blot analysis indicated variation in the copy number in the different transgenic lines. Between one to three insertion events of the *pgip*2 gene were estimated from the Southern results in the transgenic clones. Four *pgip* independent lines were identified: plant No. 1 with one band, plant No. 2 and No. 3 with two bands, and plant No. 4 with 3 bands (Figure 4). No hybridization signal occurred in the non-transgenic control plants.

Transcription of the specific mRNA transgene in the transformed canola lines was proved by means of RT-PCR. RNA was isolated from the leaf tissue for cDNA generation. The expected size of the amplified cDNA fragment was detected in the transformed lines (Figure 5). Non-transformed plants were used as negative controls and no transcripts could be detected.

Crude extracts from the independent transgenic canola plants overexpressing the *pgip*2 demonstrated the PGIP activity against the polygalacturonases produced by *Rhizoctonia solani* 4-day old culture.

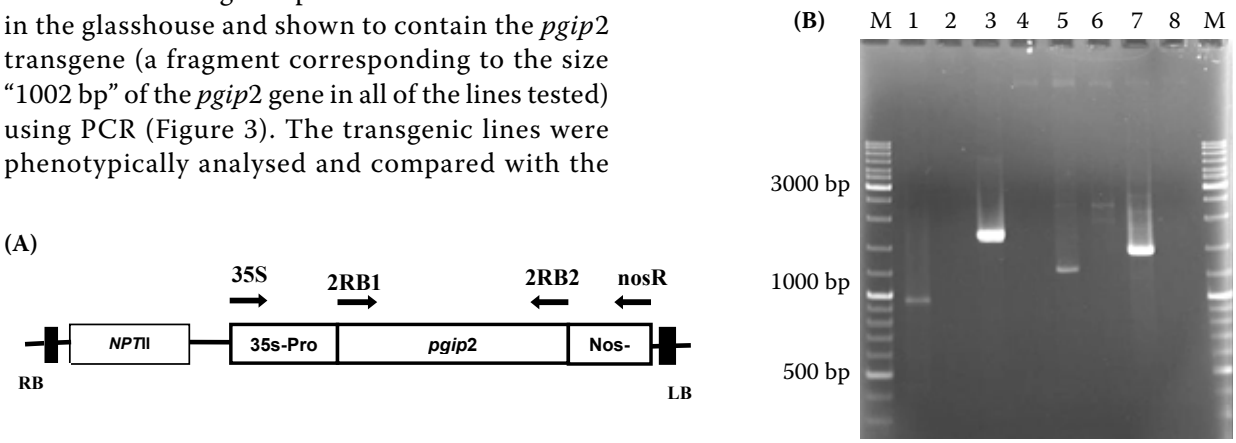


Figure 3. (A) Schematic representation of the position of the primers on the T-DNA used to identify the transgenic plants by PCR. (B) lines 1–4 PCR amplification using the transgenic plant as template DNA with the 2RB1/2RB2, 35S/nosR, 2RB1/nosR, and 35S/2RB2 primers, respectively. All the amplified fragments confirm the integration of the transgene. Lines 2, 4, 6 and 8 PCR reactions using the non transgenic plant as template DNA with the same set of primers used in lines 1, 3, 5, and 7, respectively (negative controls); M= molecular size marker 1 kb ladder

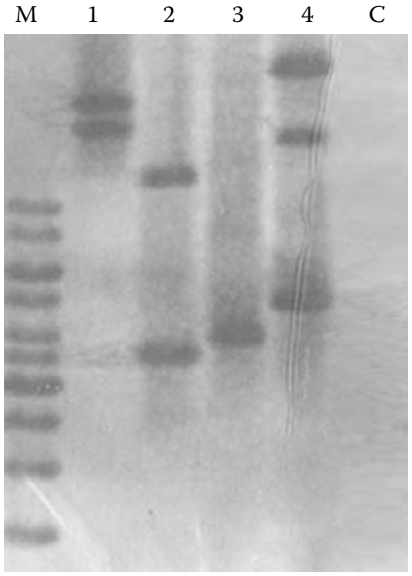
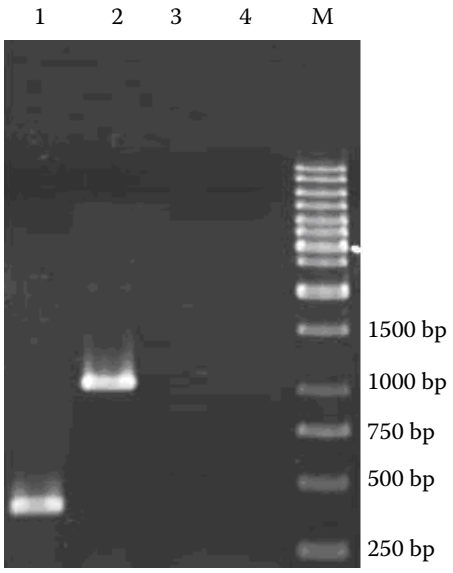


Figure 4. Southern blot analysis of the transgenic canola plants transformed with the bean *pgip2* encoding gene. Genomic DNA from the canola plant was digested with XbaI and hybridised with the digoxigenin-labelled 493 bp partial internal fragment of the *pgip*2 gene as a probe, to show integration of the DNA into the plant genome and the number of integrations. The numbers identify each independent transgenic plant tested. Untransformed canola genomic DNA digested with XbaI is shown in lane C; M = molecular marker



Line 1 – RT-PCR based on 18s rRNA as control; line 2 – RT-PCR of transgenic plant No. 1 using the specific primers; line 3 – PCR amplification using RNA extraction (without reverse transcriptase) as negative control (it ensures the amplified fragment in line 1 and 2 are not due to the DNA contamination); line 4 – RT-PCR of untransformed plant using the specific primers (as negative control); M = 1 kb DNA ladder

Figure 5. RT-PCR analysis of the transgenic canola expressing the *pgip2* gene. 4 transgenic lines were analysed by RT-PCR. We have shown the results of only one transgenic line (plant No. 1). All transgenic lines showed the same RT-PCR pattern

To assess the activity of PGIP in transgenic canola plants overexpressing the *pgip*2 gene, the crude protein extracts prepared from the greenhouse acclimatised transgenic canola leaves were used to assess the inhibition of the crude PG preparations from *Rhizoctonia solani* by a method described by Salvi *et al.* (1990). The results confirmed the PGIP activity in the crude extracts from all the transgenic lines, showing clear reductions in the PG activity when the plant extracts were incubated with the PGs on the pectic substrate (PGIP activities of > 1492 units/mg protein; Table 1). All the experiments were tested in three independent replicates. The levels of the PG inhibition ranged from 29% to 37% as compared to the untransformed plants. The *pgip*2 transgenic canola plant No. 4 had the highest PGIP activity (2277 units/mg; Table 1), which represents 37% inhibition of the *R. solani* PGs (Table 1). The pgip2 transgenic canola plant No. 2 and No. 3 showed 1954 and 1492 units/mg of PGIP activity (Table 1), which represents 32% and 29% inhibition, respectively.

DISCUSSION

Canola is a major oilseed crop and *Rhizoctonia solani* is associated with hypocotyl rot, damping off, seedling rot and root rot of this economically important oilseed crop (Kaminski & Verma 1985; Yitbarek *et al.* 1987; Keinath 1995; Baird 1996; Khangura *et al.* 1999), which causes an annual yield loss of 8–30% (Verma 1996; Khangura *et al.* 1999).

Polygalacturonases (PGs) are produced by many plant fungal pathogens (including *R. solani*) and are the first pathogenicity factors to be secreted. They can degrade plant polygalacturonic acid, cause cell wall collapse and provide nourishment for fungi (Desiderio *et al.* 1997; De Lorenzo & Ferrari 2002). Fungi produce many different PGs, each with its own expression pattern *in vivo* and *in vitro* (Wubben *et al.* 1999), and in order to interact with all these different PGs, plants have evolved different PGIPs with specific PG recognition capabilities (De Lorenzo *et al.* 2001). Since plants express more than one PGIP,

it is difficult to investigate the inhibitory activity of a single PGIP without going through a laborious purification protocol. The expression of the cloned *pgip* genes in a heterologous system is a convenient way of investigating the inhibitory activities of a single *pgip* gene product. Two options are currently available for heterologous expression of the PGIP in plant systems: by transient expression (Desiderio *et al.* 1997; Leckie *et al.* 1999) and through the production of stably transformed transgenic plants (Desiderio *et al.* 1997; Berger *et al.* 2000; Powell *et al.* 2000). In this study, *B. napus* R line Hyola 308 was stably transformed by the bean *pgip*2 gene to evaluate the inhibitory effect of expressed PGIP on the *R. solani* PG activity. Extracts of four individual T0 transgenic canola plants showed some variation in the polygalacturonase inhibition assay against polygalacturonases from *R. solani* ranging from 29% to 37% (Table 1). The variable expression of the PGIP is in agreement with the results of De Bolle *et al.* (2003) and Richter *et al.* (2006). The inhibition of the pathogen PGs by PGIP *in vitro* suggests that the plant PGIP is a deterrent to pathogen degradation of plant cell walls. There are several reports indicating the use of the *pgip* genes with the target of increasing disease resistance to fungal pathogens. Powell *et al.* (2000) and Joubert *et al.* (2006) introduced the *pgip* gene from pear and grapevine to tomato and tobacco, respectively. They demonstrated that the inhibition of the fungal PGs slows down the expansion of the disease lesions and the associated tissue maceration. Oelofse *et al.* (2006) demonstrated that the apple *pgip* gene expressed in transgenic tobacco inhibits the PG of *Botryosphaeria obtusa* and *Diaporthe ambigua*, which are two important pathogens of apple trees. The same results were reported when the raspberry PGIP expressed in transgenic pea interacted with the PGs from *Stenocarpella maydis* and *Colletotrichum lupini* (Richter *et al*. 2006).

Transformation of the R line Hyola 308 of *B. napus* was mediated by *Agrobacterium* and the cut surfaces of cotyledonary petioles containing the target cells. Results showed that this target is a vigorous source of new shoot material leading to very rapid shoot development. The origin of these shoots was shown by Sharma (1987) to be cells located around the cut end of the petioles.

The success of *Agrobacterium*-mediated plant transformation can be a function of the genotype of the species to be transformed, the strain (virulence) of *Agrobacterium*, the selectable marker, the regeneration capacity of the target cells and the accessibility of the bacterium to the regenerable cells. We examined the expression of the *Phaseolus vulgaris* cv. Goli *pgip*2 gene in transgenic canola. Also, CaMV 35S promoter was used to ensure high levels of gene expression in all tissues.

PCR data on the transgene confirmed the integration of the *pgip*2 gene into the rapeseed genome. Southern blot analysis of four transgenic lines provided additional evidence for the T-DNA integration. The transgenic lines were found to carry one to three copies of the *pgip*2 gene. This finding (more than one copy of the transgene) is an agreement with the results of Moloney *et al.* (1989), who reported multiple copy insertion into the canola genome. Many factors such as the transgene localisation and the copy number (reviewed by Finnegan & McElroy 1994; Iyer *et al.* 2000; Matzke *et al.* 2000) can contribute to the variation in the transgene expression.

Transgenic techniques provide us with the probability of introducing the foreign genes into the plants to improve their resistance to fungal pathogens. In the present study it was demonstrated that the specific product of the *pgip*2 gene inhibited the PGs of the economically important pathogen *R. solani*, which is the first important step in disease control strategies.

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