

Heterologous expression of the *Secale cereal* thaumatin-like protein in transgenic canola plants enhances resistance to stem rot disease

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Abstract

Canola (*Brassica napus* L.) is an important oilseed crop. A serious problem in cultivation of this crop and yield loss, are due to fungal disease stem rot caused by *Sclerotinia sclerotiorum*. The pathogenesis-related (PR) proteins have the potential for enhancing resistance against fungal pathogen. Thaumatin-like proteins (TLPs) have been shown to have antifungal activity on various fungal pathogens. In this study, the *tlp* gene isolated from cereal rye (*Secale cereal* L.) was introduced into canola plants. The amplified DNA fragment (about 500 bp) was analyzed and confirmed by restriction pattern and cloned into pUC19 and designated as pUCNG1. Comparison of the cloned fragment with the DNA sequence indicated that this gene contains no intron. The *tlp* gene was predicted to encode a protein of 173 amino acids with an estimated molecular mass of 17.7 kDa. The deduced amino acid sequence of TLP showed a significant sequence identity with TLP from *S.cereal* and other plants. We used a transgenic over-expression approach in order to investigate antifungal activity of expressed TLP on *Sclerotinia sclerotiorum*. TLP was overexpressed under the CaMV35S constitutive promoter in (*Brassica napus*, R line Hyola 308). Transformation of cotyledonary petioles was achieved via *Agrobacterium tumefaciens* LBA4404. The insertion of transgene was verified by the polymerase chain reaction (PCR) and genomic DNA dot blotting. Antifungal activity was detected in transgenic canola lines using detached leaf assay. The size of lesions induced by *S. sclerotiorum* in the leaves of

transgenic canola was significantly retarded when compared to that detected in non-transgenic plants.

Keywords: Canola; *Sclerotinia sclerotiorum*; *Secale cereal*; Thaumatin-like proteins; Transgenic plant

INTRODUCTION

Canola (*Brassica napus* L.) is one of the most important sources of the edible vegetable oil, industrial used oil, and protein-rich products in the world. Fungal diseases are rated either the most important or the second most important factor contributing to the yield losses in many important crops. Contribution of the fungal diseases towards the total yield losses in some important crops is about 30% (Adams, 2004). Like many other crops, the production of canola plant is challenged by phytopathogenic fungi. *Sclerotinia* stem rot, caused by *Sclerotinia sclerotiorum*, is a destructive disease of broadleaf plants including canola in warm and humid growing areas of the world, especially in Iran. In serious epidemic years in Iran, *sclerotina* stem rot incidence may be 40-50%, and yield is reduced by 20-25% (AfshariAzad, 2001).

Extensive use of the chemical fungicides, which have drawbacks such as environmental pollution, producing residual poisons to the human beings and animals, and expensive cost, is a custom way for controlling of fungal pathogens. Therefore, it is desirable to introduce the foreign fungal-resistant genes into the important crop plants. Theoretically, the foreign genes can be transferred into the genome of plants without

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altering any other agro-economically important traits (Chang *et al.*, 2002).

An important and alternative approach in the management of fungal diseases is over-expression of the genes that encode proteins with direct inhibitory effect on the growth of fungi through genetic engineering. The pathogen related (PR) proteins, which are able to inhibit the growth of pathogens, are divided into 17 subgroups (PR1-PR17) based on similarity in amino acid sequence data and molecular masses (Van Loon and Van Strien 1999; Okushima *et al.*, 2001; Park *et al.*, 2004). Various PR proteins, including chitinases (Swegle *et al.*, 1992), beta 1,3 glucanases (Velazhahan *et al.*, 2003), defensins (Terras *et al.*, 1992) and thaumatin like proteins (Roberts and Selitrennikoff, 1990) are present in some plant tissues. Among these PR proteins, thaumatin like proteins (TLPs) are induced in plants in response to pathogen and have been shown to enhance resistance to a variety of fungi (Vigers *et al.*, 1991; Yun *et al.*, 1997). The TLPs are thought to alter the membrane permeability and cellular signal transduction cascades (Yun *et al.*, 1998; Dudler *et al.*, 1994; Vigers *et al.*, 1991).

Since the TLPs have strong antifungal activity and could be used to improve the fungal resistance in plants, the purpose of this research was to amplify and clone the *tlp* gene from *Secale cereal* and transform canola (R line Hyola 308) by this gene via *Agrobacterium*-mediated transformation. The inhibitory effect of the expressed TLP in transgenic lines on the *S. sclerotiorum* growth was evaluated.

MATERIALS AND METHODS

Plant material: The rapeseed (*Brassica napus* L.) R line Hyola 308, was used as receptor, which was kindly provided by the Oilseed and Development Co. Tehran, Iran. The *Secale cereal* (TN-06-1430) seeds were prepared from National Plant Gene Bank of Iran, Seed and Plant Improvement Institute.

Microorganisms, plasmid vector, and culture conditions: *Sclerotinia sclerotiorum* was kindly provided by H. Afshari-Azad, Iranian Research Institute of Plant Protection, Tehran, Iran. The fungus strain was grown on the PDA (Potato Dextrose Agar) medium at 28°C. *Escherichia coli* DH5 α was used in all the molecular biological experiments and *Agrobacterium tumefaciens* LBA4404 was used for plant transformation.

Bacteria were grown in the LB medium at the appropriate temperatures (37°C for *E. coli* and 28°C for *Agrobacterium tumefaciens*) with shaking by shaker incubator (200 rpm). Plasmid pUC19 (MBI, Fermentas) was used for routine cloning and sequencing and plasmid pBI121 (Novagen) was used as a binary plant expression vector.

Cloning of the *tlp* gene: Leaf material from the *S. cereal* was harvested, lyophilized and grinded into the fine powder for extraction of the genomic DNA by the CTAB method (Doyle and Doyle, 1990). DNA fragment containing the *tlp* gene was amplified by PCR using the genomic DNA. The primers used for amplification of the *tlp* gene were designed based on the *tlp* sequences in the GenBank from NCBI web site (www.ncbi.nlm.nih.gov) under accession numbers of AF096927, AF099671 and AF100142.

The *tlp* gene was amplified by PCR using the specific primers, STLPr as forward primer and STLPr as reverse primer (Table 1). PCR reactions contained 2.5 units of Fermentas *Pfu* DNA polymerase, 1X buffer, 200 μ M of each deoxynucleotide triphosphate, 2 μ M MgSO₄ and 0.5 μ M primers. Reaction conditions for PCR amplification were initiated with 94°C for 5 min followed by 34 cycles of 94°C for 90 sec, 56°C for 45 sec, and 72°C for 150 sec, and final extension of 5 min. PCR products were separated by electrophoresis on a 1% agarose gel. The resulting PCR product (500 bp) was cloned into the pUC19 plasmid and sequenced from both directions with the M13 standard primers, using the dideoxy chain termination method (Sambrook and Russell, 2001).

General DNA procedures: Plasmid DNA preparation and electrophoresis of the DNA fragments were performed by routine procedures (Sambrook and Russell, 2001). The restriction enzyme analysis of the amplified DNA was carried out as recommended by the manufacturer (Fermentas, Germany). The bacteria were transformed by the heat shock method

Table 1. Oligonucleotides (primers) used in this study.

Name	Oligonucleotides sequence
STLPf	5'- GCTCTAGAATGGCGACCTCTGCGGTGC-3'
STLPr	5'- GGAGCTCTCATGGGCAGAAAGGTGATCTG-3'
35S	5'- GGCGAACAGTTCATACAGAGTCT-3'
Nos	5'- CGCGATAATTATCCTAGTTTGC-3'

(Sambrook and Russell 2001).

Construction of the binary vector: The *A. tumefaciens* strain LBA4404 harboring the recombinant binary vector pBIAZ1 with the *tlp* gene was used in the experiments. Within the T-DNA region, the plasmid pBIAZ1 contains a neomycin phosphotransferase II (*NPTII*) gene as the selectable marker that is a kanamycin-resistant gene for plant selection; a *tlp* gene, encoding the thaumatin like protein. The *NPTII* gene is regulated by the nopaline synthase promoter and terminator: the *tlp* gene is regulated by the Cauliflower mosaic virus 35S promoter (CaMV 35S) and terminated by the nos terminator (Fig. 1).

Preparation of the explants and bacterial strain for transformation: Seeds were sterilized by being submerged in 70% ethanol for 5 min and then in 0.1% HgCl₂ for 8 min. Then they were rinsed several times with the sterilized water and plated on the ½MS medium (Murashige and Skoog 1962) under light for 5 days. After germination, the cotyledonary petioles were cut and placed on the MS solid medium with 3.5 mg/l benzylaminopurine (BAP)(CM) for pre-culture. After 2 days, the explants were used for transformation.

Single colonies of the *A. tumefaciens* strain harboring pBIAZ1 containing the *tlp* gene were grown in the LB medium supplemented with 50 mg/l 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 a fresh medium with the amount of 0.1% and cultivated till OD₆₀₀ = 0.4 with liquid medium. The bacterial cells were collected by centrifugation (6000 rpm for 5 min) and re-suspended in ½MS medium for use.

Transformation and selection procedure: The explants were immersed in the bacterial suspension for 1.5 min with constant shaking, and then placed on the

sterile filter paper to remove the excessive moisture, and placed on the CM medium in the Petri dishes for co-cultivation at 25°C for 2 days.

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

Molecular analysis of the transgenic canola: The leaf materials from the transgenic and non transgenic canola plants were harvested, lyophilized and grinded into the fine powder for extraction of genomic DNA by the CTAB method (Doyle and Doyle, 1990). PCR amplification was used for initial evidence of the presence of the transgene in the putative transgenic plants. The DNA fragment containing the *tlp* gene was amplified by PCR using the transgenic genomic DNA of canola and specific primers.

Dot blot analysis: As a probe in hybridization experiments, a fragment (500 bp in size) was obtained from PCR amplification of the *tlp* gene, using the STLPr/STLPf primers and plasmid pBIAZ1 containing the *tlp* gene as template and subjected to the DIG DNA labeling (Roche Applied Science GmbH, Germany). The extracted genomic DNA (15 µg) from the fresh leaves of the putative transgenic and untrans-

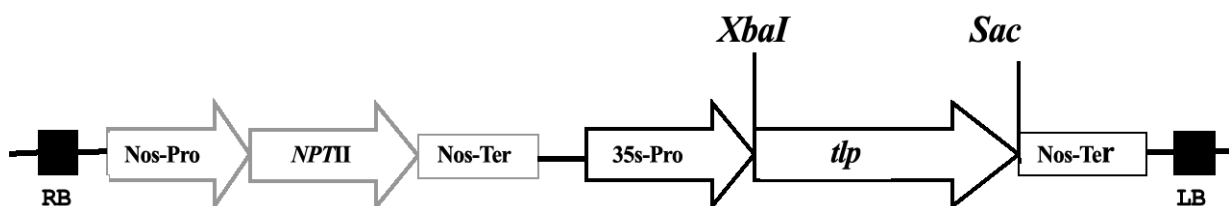


Figure 1. Schematic representation of the T-DNA region of transformation vector pBIAZ1. RB, right border; LB, left border; Nos-pro, nopaline synthase promoter; *NPTII*, gene for neomycin phosphotransferase; Nos-ter, terminator of nopaline synthase; 35s-pro, 35S promoter of cauliflower mosaic virus; *tlp*, Thaumatin like protein gene.

formed control plants, was denatured for 10 min in boiling water and chilled on the ice. The denatured genomic DNAs were spotted on a nylon membrane (Hybond N+, Amersham), and hybridized to Dig-dUTP labeled *tlp* probe.

Bioassay of the transgenic canola plants:

Susceptibility of the transgenic and non transgenic canola to *S. scelotiorum* was evaluated using a detached leaf assay described by Carstens *et al.* (2003). The leaves of the 5-6 week old greenhouse plants were detached and placed in the petri dishes, positioned over the wet filter papers. The upper surface of the leaves were inoculated with a piece of agar disk (5 mm in diameter) of the actively growing *S. scelotiorum* medium. Petri dishes were incubated at 28°C and the lesion growth was measured 48 hours after inoculation and then photographed.

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). The deduced amino acid sequence from the *tlp* gene was obtained by BLASTX Network Service (NCBI) and multiple alignments were generated using ClustalW (<http://www.ebi->

[ac.uk/ClustalW](http://www.ebi.ac.uk/ClustalW)).

RESULTS

In this study, we isolated the thaumatin like protein gene (*tlp*) from *Secale cereal*. To isolate the *tlp* gene, the oligonucleotide specific primers (STLPf and STLPPr) were designed based on the related DNA sequences available at the NCBI. PCR amplification was performed on the genomic DNA of *S. cereal* generating the specific band of approximately 500 bp (Fig. 2A).

Following the amplification of the *tlp* gene, the purified DNA fragment digested with *Xba*I/*Sac*I and cloned into the same sites in pUC19 and designated pUCNG1. The cloning of the *tlp* gene in the new construct (pUCNG1) was confirmed by the restriction enzyme pattern using the *Hind*III/*Eco*RI enzymes (Fig. 2B), and amplification by the specific primers. Comparison of the cloned DNA sequence with that of the *tlp* DNA sequences available in the GenBank database demonstrated that this gene is not interrupted by intron(s). The gene contains an open reading frame encoding a protein of 173 amino acids, with the calculated molecular mass of 17.7 kDa.

To clone the *tlp* gene in plant binary expression

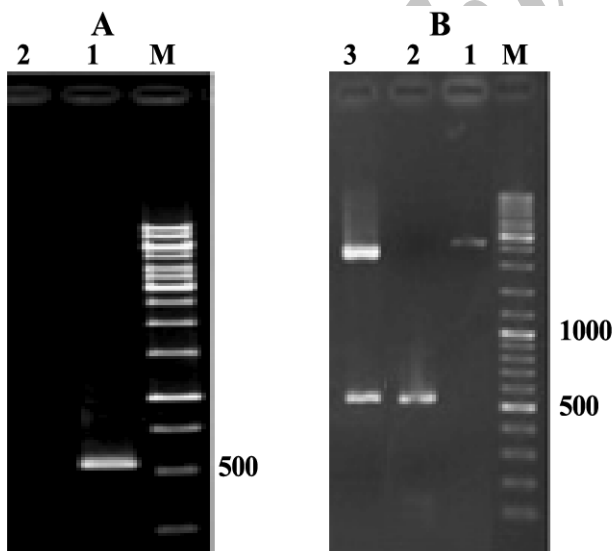


Figure 2. A: PCR amplification of the *tlp* gene using STLPf/r specific primers (line 1, approximately 500 bp), line 2, negative control; M, DNA ladder. B: Confirmation of pUCNG1 containing *tlp* gene by digestion pattern using *Hind*III and *Eco*RI enzymes, line 1, empty vector, line 2, PCR product using specific primers and PUCNG1 DNA as positive control, line 3, Digested PUCNG1 using *Hind*III and *Eco*RI enzymes, M, DNA ladder.

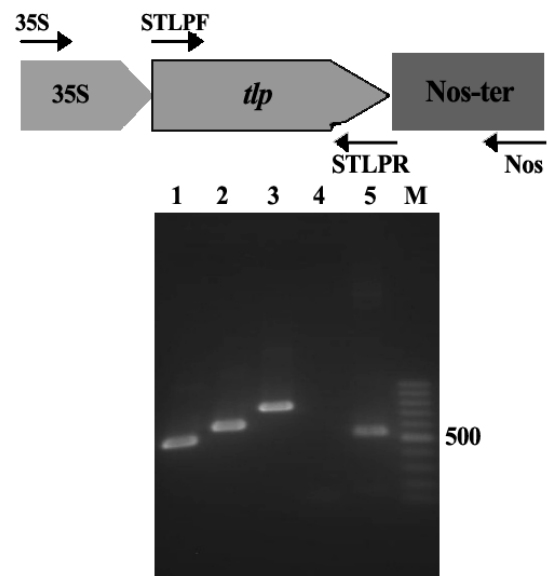


Figure 3. Confirmation of pBIAZ1 containing *tlp* gene by PCR line 1, PCR product using the STLPf/r as primers; line 2, PCR product using the 35S/STLPPr as primers; line 3, PCR product using the STLPf/Nos as primers, line 4, negative control, line 5, positive control (PUCNG1 DNA as template and STLPf/r as primers); M, DNA ladder.

vector pBI121 for transformation of *Brassica napus*, R line Hyola 308, the fragment containing the *tlp* gene was cloned into *Xba*I/ *Sac*I sites of pBI121. Cloning of the *tlp* gene into this expression vector was confirmed by amplified patterns using combination of the gene and vector specific primers (Fig. 3). The open reading frame of *tlp* gene in pBI121 (pBIAZ1 construct) which is between the CaMV 35S promoter and nopaline synthase terminator was confirmed by DNA sequencing (data not shown).

The pBIAZ1 mobilized into the *Agrobacterium tumefaciens* (LBA4404) and subsequently used for transformation of 5 days old cotyledonary petioles of the *B. napus*. The shoots were regenerated from the cotyledonary petioles 1-2 weeks after planting. Independent transgenic canola lines were successfully rooted on the kanamycin-containing selection media and then transferred to the greenhouse (Fig. 4). Amplification of products showed that the putative transgenic plants were contained a fragment corresponding to the size "500 bp" of the *tlp* transgene in the lines (Fig. 5). The *tlp*-specific primers did not

amplify the corresponding *tlp* fragment in the untransformed sample.

A set of *vir*-G primers (*vir*Gf and *vir*Gr) used for detection of any *Agrobacterium* contamination that might have escaped the selection. The PCR detection under various conditions showed no detectable band using the transgenic plants DNA as template. Using *Vir*G primers an expected 738 bp band was detected when the *Agrobacterium* DNA used as control (data not shown). Dot blot analysis on the PCR positive transgenic plants were performed and the results verified the integration of the exogenous gene into the genome of transgenic canola plants (Fig. 6). No hybridization signal occurred in the non-transgenic control plants.

In order to test the antifungal activity of the expressed TLP from transgenic canola lines on the phytopathogenic fungus *Sclerotinia sclerotiorum*, the experiment was performed using the greenhouse acclimated transgenic line in a detached leaf assay (Goth and Keane 1997). Lesion expansion occurred on the leaves and proceeded aggressively in untransformed

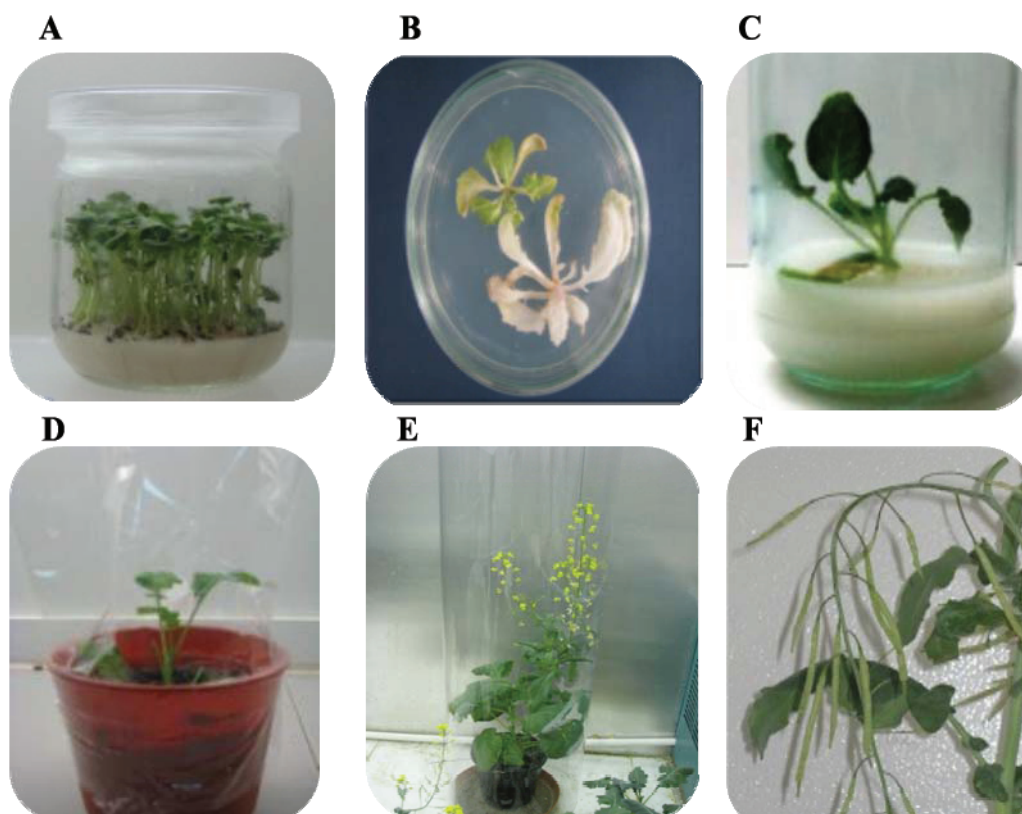


Figure 4. Transformation and regeneration of transgenic canola plants. A: Germination of the R line Hyola 308 seeds; B: Shoot regeneration from cotyledonary petioles after transformation (1-2 weeks); C: regeneration shoots in the growth medium; D: regeneration plantlet in the pot and acclimated to the non-aseptic environment; E and F: transgenic canola plant flowers and pods in the greenhouse.

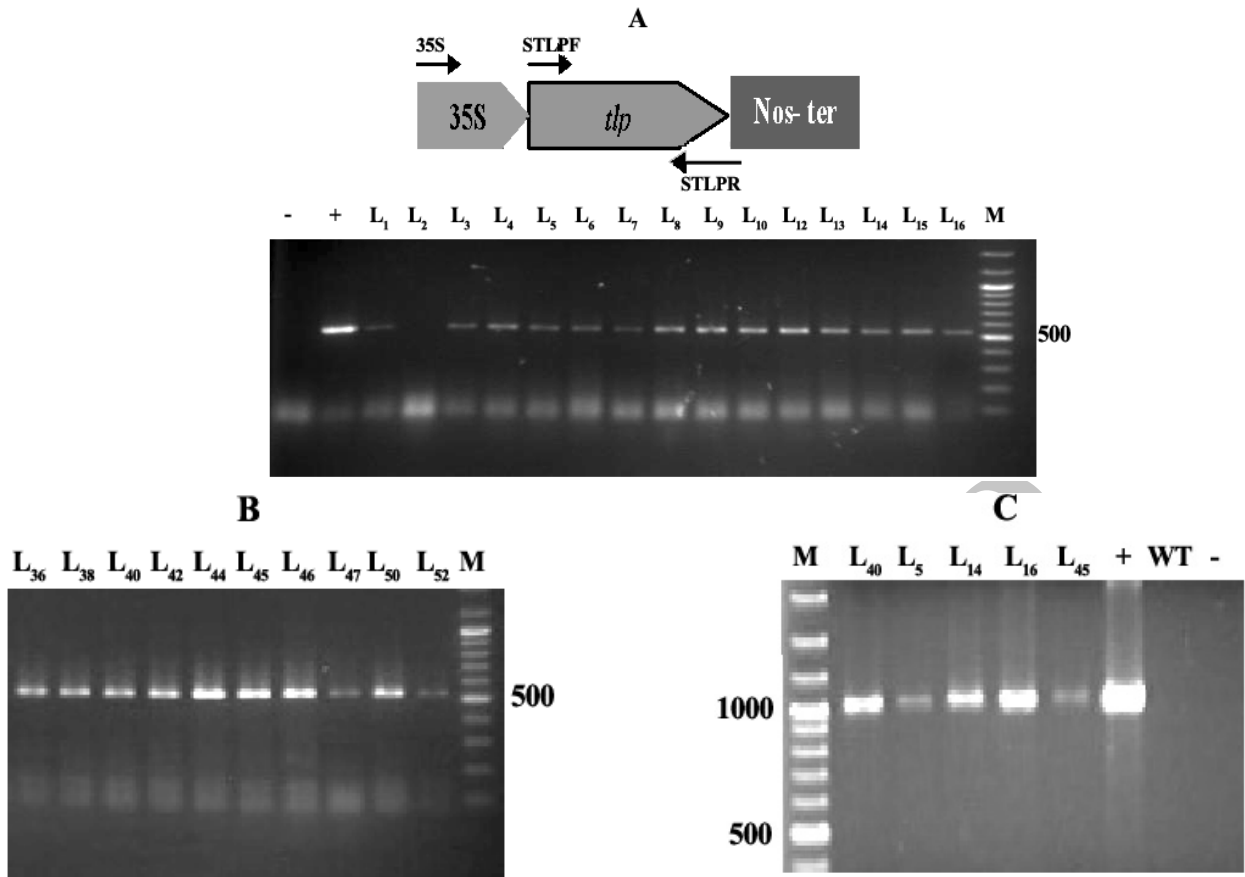


Figure 5. PCR analysis of putative transgenic canola plants. A and B: 500 bp band was amplified using the specific primers (STLPf/r) and DNA of different putative transgenic plants as template; C: amplified fragment (app. 1 Kb) using 35S/STLPR primers in selected lines. These PCR pattern confirm the transgenic lines; M, 1 Kb DNA ladder.

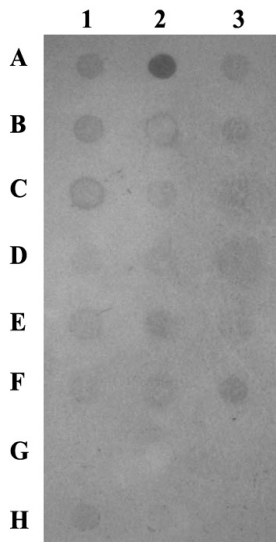


Figure 6. Genomic DNA dot blot analysis of putative transgenic canola with the total DNA loaded onto hybond N+ membrane hybridized with a digoxigenin- labeled *tlp* probe. A1(L₁₁), A3(L₁₄), B1(L₄₇), B2(L₄₂), B3(L₃₀), C1(L₁), C2(L₁₅), C3(L₄₀), D1(L₅₃) D2(L₄₄), D3(L₁₂), E1(L₅), E2(L₃₆), E3(L₂), F1(L₃₃), F2(L₅₂), F3(L₃), G1(L₅₀), H1(L₁₃) and H2(L₁₆) DNA from leaves of transgenic canola plants of T₀ generation; A2, DNA from pBIAZ1 plasmid as positive control; G2, DNA from non transgenic plant as negative control.

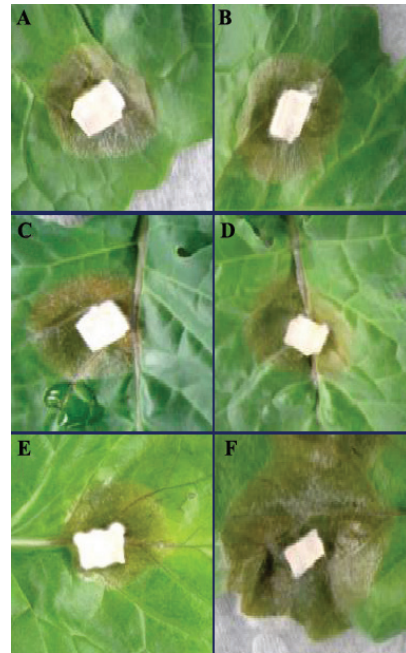


Figure 7. *Sclerotinia sclerotiorum* lesion development on detached leaves of selected transgenic (A, B, C, D and E) and non-transgenic (F) canola plants. Photographs were taken 48 hours after inoculation showing confined lesion growth in the transgenic plants as compared to the wild type ones.

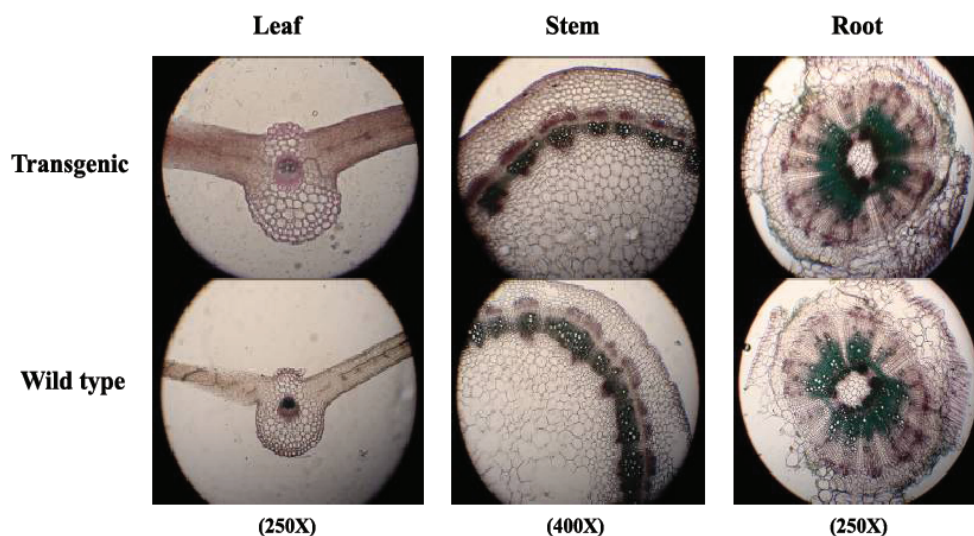


Figure 8. Light micrograph of cross sections from leaves, stems, and roots of transgenic and wild type canola plants: The screened transgenic canola by kanamycin, PCR pattern and bioassay were used to evaluate in term of anatomy in comparison with wild type plants. Leaves, stems and roots were cross-sectioned in order to observe the tissues structure under microscope. No abnormalities were observed in leaf, stem and root tissues.

control. The lesion sizes were significantly retarded in the transgenic canola lines (Fig. 7). The transgenic lines were phenotypically analyzed and compared to the untransformed controls and did not show any abnormalities with regard to the growth, size, reproduction or cross section of the leaf, stem and root tissues (Fig. 8).

DISCUSSION

With regard to the importance of yield losses due to the contribution of fungal diseases, some investigations have been conducted to develop transgenic crop plants with enhanced expression levels of pathogen related (PR) protein genes or producing fungal disease resistant varieties (Lawrence *et al.*, 2000; Vleeshouwers *et al.*, 2000; Gau *et al.*, 2004).

In the past two decades, extensive studies on PR proteins have been done by a large number of laboratories (selitrennikoff 2001; Velazhahan *et al.*, 2002; Edreva, 2005). This high level of interest in PR proteins is mostly due to the antifungal, antibacterial, insecticidal, nematicidal, and antiviral property of these proteins (Edreva, 2005). An important common feature of PR proteins is their antifungal effect (selitrennikoff 2001; Van Loon 2001). Thaumatin-like proteins (TLPs) are a group of PR proteins (PR-5) that are induced in plants in response to the infection by

fungi. TLPs have been shown to have antifungal activity on various fungal pathogens (Choi *et al.*, 1997; ye *et al.*, 1999; Velazhahan *et al.*, 2002).

Our previous study has shown that heterologous expressed TLP in prokaryotic system has antifungal activity against some phytopathogenic fungi (unpublished data). In this study the *tlp* gene from *S. cereale* was used for transformation of canola to confer resistance against *Sclerotinia sclerotiorum*. We showed that DNA sequence information of the cloned *tlp* gene contains no intron as the same result has been obtained in oat-stem rust study by from Lin *et al.*, 1996.

There are several reports indicating that transgenic plants expressing *tlp* gene enhanced fungal disease resistance in different species including potato (Liu *et al.*, 1994), rice (Datta *et al.*, 1999), orange (Fagoaga *et al.*, 2001), wheat (Chen *et al.*, 1999), and tobacco (Velazhahan and Muthukrishnan, 2003). In our study, under experimental conditions, a similar improvement in resistance to stem rot disease was demonstrated. The ability of the introduced *tlp* gene to enhance the antifungal potential of transgenic canola plants was studied by the detached leaf assay. The transgenic lines were able to delay formation of lesion by fungal pathogen *S. sclerotiorum* in the *in vitro* assay. No inhibition was detected in non transgenic lines.

Transformation of the R line Hyola 308 of *B. napus* was mediated by *Agrobacterium* and the cut surfaces of cotyledoneary 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 has been 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 *tlp* gene in the transgenic canola. Also, CaMV 35S promoter used to ensure high levels of gene expression in all tissues. *Agrobacterium* mediated transformation and use of CaMV 35S promoter have been reported by several research groups as effective parameter (Liu et al., 2010; Kahrizi et al., 2007; Cardoza and Stewart, 2003).

All the lines that gave positive results in the PCR analysis were further confirmed by the use of *virG* primers and showed that products have been obtained from stable T-DNA integrated into the canola genome and not from *Agrobacterium* contamination. The present study demonstrated a successful transformation of canola plants by the *tlp* gene which is able to retard the symptoms of the disease. Meanwhile, the expression of this gene had no deleterious phenotypic effect on the transgenic plants.

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