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Full Length Research Paper

Expression of *TLP*-3 gene without signal peptide in tobacco plants using *Agrobacterium* mediated transformation

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Plants are exploited as a source of food by a wide range of parasites, including viruses, bacteria, fungi, nematodes, insects and even other plants. So paying attention to their protection is very important. One of the most important groups of defensive gene is pathogenesis-related (PR) proteins. PR proteins are a group of plant proteins which accumulate as a result of different types of pathogens attack. Thaumatin-like proteins (TLPs) belonging to the PR proteins, plays an important role in plant defense against pathogen invasions. *Agrobacterium*-mediated transformation is routinely used for the transformation of many plants. In the present study, thaumatin-like *TLP-3* gene without signal peptide was transferred into tobacco plants through *Agrobacterium*-mediated technology. The PCR and RT-PCR assays confirmed the presence of *TLP-3* gene and *TLP-3* mRNA in transgenic tobacco plants, respectively. The bioassay results showed the activity of TLP-3 against *Alternaria alternata* in microscopic experiment. This is the first report of the expression of *TLP*-3 gene without signal peptide in tobacco plants using *Agrobacterium* mediated transformation and it confirmed the ability of the gene action in another plant.

Key words: Thaumatin like protein, *Agrobacterium,* signal peptide, *Alternaria alternate*.

INTRODUCTION

Plants in nature are continuously exposed to a multitude of pathogens that attack them but they are generally resistant to most of the encountered pathogens (Santen, 2007). However, many pathogens can cause severe diseases, reduce product, destroy plant and cause significant yield loss (Agrios, 2005). The earliest defense responses are the opening of specific ion channels across the plasma membranes, the rapid production of active oxygen species (AOS) such as O_2 and H_2O_2, which are known as the oxidative burst, and phosphorylation and dephosphorylation of specific proteins (Doke et al., 1996; Conrath et al., 1997). The most common resistance exhibited by plants in response to various pathogens is the hypersensitive response (HR). Accompanying the HR is the *de novo* synthesis of a large number of novel proteins with roles in defense (Agrois, 1988). Following the activation of HR, uninfected distal parts of the plant may develop resistance to further infection, a phenomenon known as systemic acquired resistance (SAR) (Ryals et al., 1996). Pathogenesis-related (PR) protein are a group of plant proteins which accumulate as a result of different types of pathogens attack, certain abiotic stress, or through application of some plant growth regulators in the plant cells. Pathogenesis-related proteins were first classified in 1978; this classification was based on serology and sequencing details in tobacco (Van Loon and Strien, 1999, Van Loon et al., 1994). Up to now, 17 groups of PR proteins have been identified in various plants which consist of chitinases (Swegle et al., 1992; Velazhahan et al., 2000), glucanases (Velazhahan and Muthukrishnan, 2003), proteinase-inhibitor (Leah et al., 1991), thaumatin-like proteins (Roberts and Selitrennikoff, 1990) and defensins (Terras et al., 1992). Thaumatin-like proteins (TLP) shows high sequence similarity to thaumatin, a sweet-tasting protein from the West African shrub *Thaumatococcus danielli*. TLPs generally have 16 conserved cysteine residues, and their extensive disulfide linkages probably confer

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stability in varied thermal and pH conditions (Velazhahan et al., 1999). TLPs have physiological and developmental functions such as protection against osmotic stress (Zhu et al., 1995), roles in flower formation and fruit ripening (Salzman et al., 1998; Neale et al., 1990) and anti-freeze activities (Hon et al., 1995). Furthermore, TLPs have been suggested to bind either b-1,3-glucans or possess glucanase activity (Grenier et al., 1999, Trudel et al., 1998) and thus carbohydrates present on the fungal cell surface are a specific target of TLPs for their anti-fungal activity (Jami et al., 2007). The study showed the action of functional gene without signal peptide when transferred to another plant. The transgenic tobaccos that express the *TLP-3* gene without signal peptide showed enhanced resistance to *Alternaria alternata* in a microscopic experiment.

MATERIALS AND METHODS

Primer design-bioinformatics

First of all, the sequence of *TLP-3* gene (accession number AM94-3532) was studied to recognize the sequence of signal peptide. As a result, the nucleotide sequence of the gene was translated to protein sequence by translate software. After that, the succession of signal peptide was identified by signalp software (Figure 1).

After recognizing the sequence of signal peptide, this succession was cut and primer designation was done without this sequence. Forward primer of *TLP-3* gene was designed (5´-ATTCACCTTTG TAAACAACTGCC-3´) and identified with the name of TLP3Int-F. In designing *TLP-3* gene reverse primer, the restriction site of *Sac* I was created with the change of the sequence (5'-AATAGA-GCTCACACAATACAACCATC-3') and identified with the name of TLP3Ext-R. After designing specific primer, we used Oligo ver 5/0 software (National Bioscience, Inc) to investigate the primers (Figure 2).

The sequence that is obtained from TLP3Int-F/TLPExt-R primers had no start cod and restriction site for *Xba* I enzyme. To solve this problem, the adaptor was designed. The sequence of designed adaptor that was used for ligation with the PCR product is shown in Table 1.

DNA extraction and PCR product

One year-old alfalfa seeds (*Medicago truncatula* var *truncatula* cv jemalong A17) were prepared from the South Australian Research and Development Institute and were cultivated on a MS basal medium (Murashig and Skoog, 1962) for the purpose of rapid production of healthy plants. Modified CTAB method (Murray and Thompson, 1980) was used to extract the DNA. PCR was carried out in a 15 µl reaction mixture containing 2 µl (40 ng/µl) of genomic DNA, 0/5 µl $MgCl_2$ (25 Mm), 0/3 µl dNTP (10 Mm) mixed, 1 µl of each primer (10 pmol/µl) and 1/5 µl of *Taq* DNA polymerase (5 u/µl). Five microliters of DNA was mixed with one micro liter loading buffer and electrophoresed on 0.7% TAE agarose gel, in stable voltage of 60 V for two hours. After that, the gel was colored with ethidium bromide and through ultraviolet rays DNA band was observed. The extracted DNA had good quantity and quality and was used for PCR. The PCR programme was one cycle of 95°C for 5 min (denaturation), 35 cycles of 1 min at 94°C, 1 min at 61/3°C, 1 min and 30 s at 72°C. Finally, Five microliters of PCR product were mixed with one microliter loading buffer and then electrophoresed on 1/2% TBE agarose gel and in stable voltage of 70 V and viewed under UV light.

Adaptor connected with *TLP-3* gene without signal peptide

The PCR program was used to connect UTR-U and UTR-S together. The PCR programmed was one cycle of 65°C for 10 sec, one cycle of 50°C for 10 sec, one cycle of 37°C for 10 sec, one cycle of 25°C for 10 sec. After that the adaptor connected to the *TLP-3* gene without signal peptide. Ligation was carried out in a 35 µl reaction mixture containing 6µl (30ng/µl) of PCR product, 18µl adaptor (50pmol/µl), 1/5µl polyethylene glycol (50%), 2 µl T4 DNA ligase (5u/µl), 3/5 µl ligation buffer. This reaction was done for 12 hours at 22°C. Then the sequence was purified from ligation reaction and digested with *Sac* I and *Xba* I enzymes for three hours at 37°C and at the end of this time, the sequence was purified.

pSK plasmid construction

To prepare pSK plasmid, extracting plasmid was done by the use of plasmid extraction kit (Bioneer South Koree). For preparing pSK plasmid to ligated with gene, this plasmid was digested with *Xba* I and *Sac* I enzymes. After the purification, plasmid ligated with *TLP-3* gene for sixteen hours at 22°C. Then pSK plasmid transfer to *E. coli XL1*-Blue *and E. coli* MC 1061. To make sure for the existence of pSK with *TLP-3* gene in the MC 1061 and XL-1Blue the white colony which had grown in solid LB medium having penicillin were tested with Colony PCR and digesting. In this step pSK plasmids sent to Macrogen Company (South Korea) for sequencing.

pBI121 plasmid construction

For making the construction of pBI121 plasmid that have *TLP-3* gene without signal peptide, plasmid was digested with *Xba* I and *Sac* I enzymes and therefore, the gene were replaced with glocuro-nidase**.** After the purification, plasmid was ligated with *TLP-3* gene without signal peptide for sixteen hours at 22°C. Then pBI121 plasmid was transferred to *E. coli* XL1-Blue. For recognizing the *E. coli* XL1-Blue that has the construction, colony PCR and digesting were used.

Tobacco transformation by *Agrobacterium tumefaciens*

In order to transfer the pBI121 to *A. tumefaciens* GV3850 and *A. tumefaciens* AGLO, the method of freeze and thaw was used. To confirm the existence of construction in *A. tumefaciens*, the colony PCR was done for the colony that grew on LB medium containing kanamycin. Tobacco plants (*Nicotinia tabacum* cv. Xanthi) were prepared from Biotechnology Department at Isfahan University of Technology and propagated in MS medium through bud cultivating. Plant grew in the temperature of 22(±2)°C, under a 16/8 h (light/darkness) photoperiod and re-cultivated in MS medium every three weeks. After the tobacco plants leaves infected with *A. tumefaciens* were suspended in liquid LB medium, pieces of leaves were dried on a sterile paper and finally transferred to the vessels containing solid MS which had BAP (1 mg/l) and NAA (0/1 mg/l). Vessels containing the samples were kept at 28°C for three days in darkness. After exceedinging the mentioned time, samples were washed in water containing cefotaxime antibiotics (500 mg/l) and were placed on MS medium with kanamycin (100 mg/l), cefotaxime (500 mg/l), BAP (1 mg/l) and NAA (0/1 mg/l). Samples were kept at 22 to 25°C and dark conditions and were transferred to a new medium every ten days. After samples regeneration, produced buds separated and were propagated in MS basal medium which contains the kanamycin (100mg/l) and salicylic acid (500 mg/l).

MASSSSSSTNSLLISTFIFLTIFSPSOALIFTFVNNCPYTVWPAIIPNGGFPVLSSSGFELRHFTHLSIPVPDTHWAGRAWARTGCSTANN
KFSCLTGDCGNSLQCHGAGGSPPATLVQFDVHHGNKDFSSYSVSLVDGFNTPLTVTPHEGKGECPVVGCKADLVASCPPVLQHRVP
MGHGPVVACKSGCEAFHSDEHCCRNHFNNPQTCKPTVYSKFFKDACPATFTFAHDSPSLIHQCSSPGELKVIFCHStop

Figure 1. The sequence of *TLP-3* gene without signal peptide and the identified signal peptide.

CTAATATTCACCTTTGTAAACAACTGCCCGTACACCGTCTGGCCAGCTATCATCCCCAACGGCG
GCTTCCCCGTCCTATCCAGCAGCGGCTTCGAACTCCGTCACTTCACTCACCTCTCCATCCCCGT
CCCAGACACCCACTGGGCCGGCCGTGCATGGGCCAGAACCGGCTGCTCCACCGCCAACAACAAA
TTCTCCTGTCTCACCGGCGACTGTGGCAACAGTCTCCAATGCCACGGAGCAGGTGGCTCACCAC
CTGCAACCCTAGTACAGTTCGACGTACACCACGGTAACAAAGACTTTTCATCTTACAGTGTTAG
TCTTGTCGATGGTTTCAACACTCCTTTGACAGTTACACCGCATGAAGGTAAAGGTGAGTGTCCA
GTGGTAGGTTGCAAAGCTGATCTTGTTGCTTCATGTCCTCCTGTTTTGCAGCACCGTGTTCCAA
TGGGTCATGGTCCTGTTGTGGCTTGTAAGAGTGGTTGTGAAGCTTTTCACAGTGATGAACATTG
TTGTAGGAACCATTTTAACAACCCACAAACTTGTAAACCTACTGTGTACTCCAAGTTCTTTAAG
GATGCTTGTCCTGCAACGTTTACGTTTGCTCATGATTCTCCTTCTTTGATTCATCAGTGTTCTT
CACCGGGTGAACTTAAGGTTATCTTTTGCCATTGAAACTTGAAAGTGATGAGGAAAATAAAACA
TGTTTGAGTTGGGTTATTGTTAAGTGACCCTGTTAATGGTGTACTTCTTGAAATAAGAATGTGG
GTTGAGGGTAGAATGGTAATTAGGAAAAGGCAATCTATGTTTTTAGTTTTTGTTTTATGTTGTG
TTTGGAGATGATGGTTGTATTGTGTGATTTTTATT

Figure 2. The sequence and designed primer of *TLP-3* gene without signal peptide.

Table 1. The name and the sequence of the adaptor that was designed.

Name	Sequence
UTR-S	5´-CTAGAGAAGTTTCATTCAAGCAACAATGGCAAT-3´
UTR-U	5´-TCTTCAAAGTAAGTTCGTTGTTACCGTT-3´

Tobacco plants after sufficient growth were transferred to small vases containing equal amount of pitmas and perlit.

PCR assay

DNA was extracted from three transgenic and one untransformed tobacco plants leaves and then PCR assay was done by the help of PCR specific primers. Gradient PCR was used to achieve the optimum annealing temperature for primers. The PCR programmed was one cycle of 95°C for 5 min (denaturation), 35 cycles of 1 min at 94°C, 1 min at 61°C, 1 min and 30 sec at 72°C Finally, PCR product was electrophoresed on a 1/2% TBE agarose gel and viewed under UV light.

RT-PCR assay

Extracting mRNA from three transgenic and one untransformed tobacco plants leaves was done by the use of mRNA capture kit (Roche, Germany). For cDNA synthesis, reverse transcription reaction were performed in a 20 µl reaction. After performing reverse transcription reaction, PCR was done through specific primers. Finally, RT-PCR product was electrophoresed on 1/2% TBE agarose gel and in stable voltage of 70 V.

Bioassay

To affirm the expression of TLP-3 protein in transgenic tobacco plants, *Alternaria alternata*, which is the agent of leaf spot disease, was used for microscopic examination. For microscopic investigation, the extraction from the three transgenic and control plants was used for mixtures with potato dextrose agar (PDA) medium and then distributed into separate Petri dishes. *A. alternata* was grown on PDA medium, for seven days at 25°C and under a 16/8 h (light/darkness) photoperiod. Spores were harvested after 2 weeks by flooding the agar with sterile water. The concentration was adjusted at 6 x 10^6 spores/ml. In continuation, suspension was vortexed for one minute and was spread on the Petri dishes. Then the Petri dishes were incubated for eight hours in the 25°C. The numbers of germinated and not germinated *A. alternata* spores were counted in four eyesight from each Petri dish. For investigating the plant extracts anti-fungal activity, the ratio of not germinated/ all spore's number, was analysis through proc GLM.SAS computer software.

RESULT

The *TLP-3* gene that expresses the thaumatin-like protein was firstly recognized in one-year old alfalfa (*M. truncatula).* After cultivating alfalfa seeds in MS medium, they produced some small plants. When these small plants reached the appropriate size, they were separated and used for DNA extraction. Extracting DNA from various tissues including leaf, stem and root was done with the help of Murray and Thompson modified CTAB method. The results from electrophoreses, demonstrated that the earlier mentioned method is a useful method for

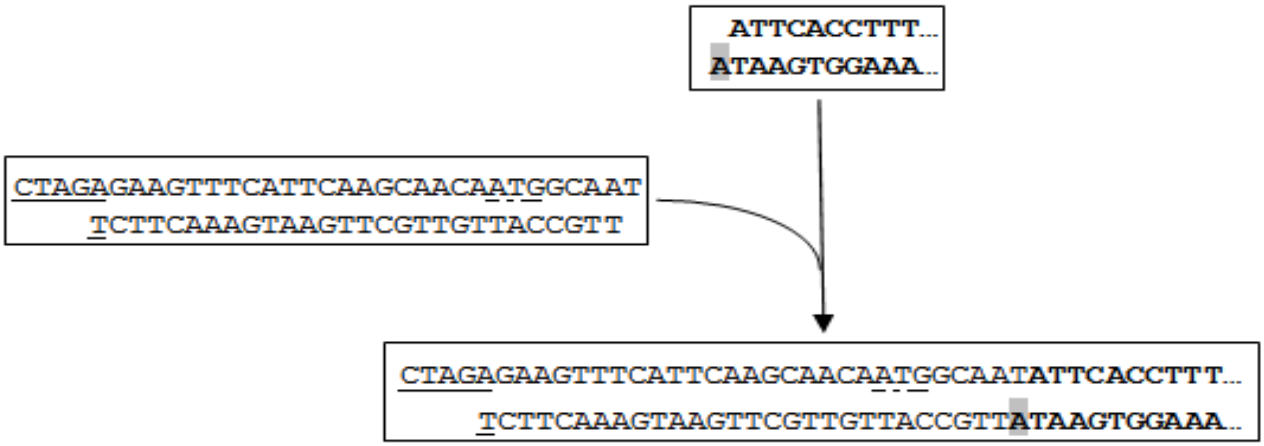

Figure 3. The *Taq* polymerase added A nucleotide in *TLP-3* gene without signal peptide and then ligated with adaptor.

extracting DNA. For separating *TLP-3* gene without signal peptide, PCR was used in the presence of *Taq* polymerase enzyme and special primers. The annealing temperature for the specific primers was defined at 61/3°C. On the basis of predictions, specific primers (TLP3Int-F/TLP3Ext-R) produced the band with the size of 862 bp. After that, the PCR product ligated with the adaptor and the size of the band was changed to 895 bp (Figure 3).

pSK and pBI121 plasmids containing *TLP-3* gene without signal peptide

In this step, the *TLP-3* gene without signal peptide ligated into pSK plasmid and then transferred to *E. coli* XL1-Blue and *E. coli* MC 1061. The colony PCR with specific primer and digestion with *Xba* I and *Sac* I enzymes confirmed the existence of *TLP-3* gene in the pSK plasmid. After sequencing, recognizing and omitting plasmids sequences, the tblastx search tools (fined in NCBI website) was used to compare the sequence with the other sequence that exist in GeneBank. The result showed that the *TLP-3* gene without signal peptide belonged to the thaumatin-like gene. After that, *TLP-3* gene without signal peptide ligated into pBI121 plasmid that was necessary for using the *Agrobacterium* method gene transfer and then the plasmid was transferred to *E. coli* XL1-Blue. For the purpose of increasing the number of pBI121 plasmids containing *TLP-3* gene without signal peptide and also for increasing these plasmids in essential times, cloning of the pBI121 was done. The existing resistance gene against kanamycin antibiotic in pBI121 plasmid provided the growth possibility on the medium containing the kanamycin antibiotic only for the *E. coli* XL1-Blue that has pBI121 with *TLP-3* gene without signal peptide. But because of the pBI121 plasmid that can be ligated without gene, it is necessary to analyses if they have *TLP-3* gene without signal peptide. Digestion with *Xba* I and *Sac* I enzymes and colony PCR with specific primer confirmed the existence of *TLP-3* gene without signal peptide in the pBI121 plasmid.

Tobacco transformation by *A. tumefaciens*

After becoming confident of TLP-3 existence in pBI121 plasmid, the plasmid was then transferred to *A. tumefaciens*. Then, colony PCR with specific primers was used for the *A. tumefaciens* which grew on LB medium that contained kanamycin antibiotic. The colony PCR confirmed the existence of constructed plasmid and then it was transferred to *A. tumefaciens.* Before transferring *TLP-3* to the important agricultural plant, it was necessary to ensure its ability of creating resistance in the model plants. In this research, tobacco *(Nicotina tabacum* cv. Xanthi) was used for transformation. For appropriate control of the experiment steps, a number of tobacco leaves pieces that were not infected by *A. tumefaciens* were cultivated in MS medium having NAA and BAP hormones (positive control) and a number of tobacco leaf pieces that were not infected by *A. tumefaciens* were cultivated in MS medium having kanamycin and cefotaxime antibiotic and the earlier mentioned hormones (negative control). Convenient growth of positive control demonstrated the ability of plants to regenerate and death of negative control demonstrated that they had no ability to grow on MS medium that contained kanamycin and cefotaxime.

PCR and RT-PCR assay

After DNA extraction from transgenic and untransformed plants, PCR was done by the *TLP-3* gene without signal peptide specific primers. The result confirmed the gene transfer and gel showed the band in desired size for

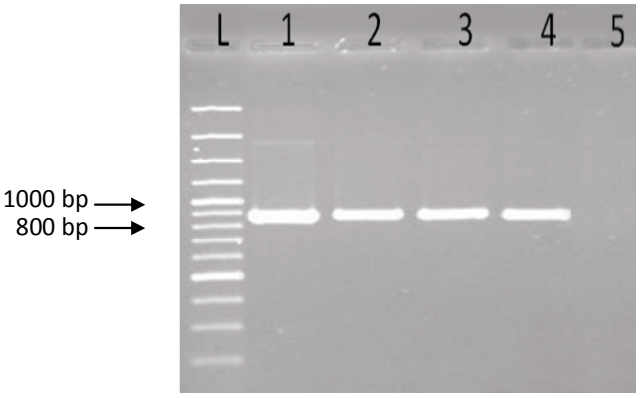

Figure 4. PCR assay of transgenic tobacco plants. Lane 1: positive control, the pBI121 containing *TLP-3.* Lanes 2 to 4: transgenic tobacco plants*;* Lane 5: negative control, untransformed tobacco plant.

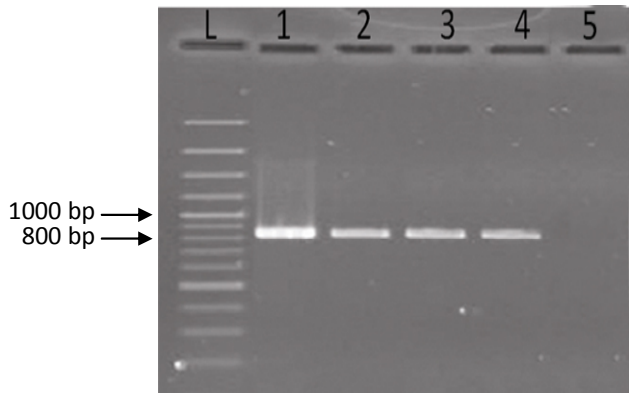

Figure 5. RT-PCR assay of transgenic tobacco plants. Lane 1: positive control, the pBI121 containing *TLP-3.* Lanes 2 to 4: transgenic tobacco plants; Lane 5: negative control, untransformed tobacco plant.

transgenic tobacco plants (Figure 4). Existing poly A tail at the end of 3´ mRNA led to choosing mRNA capture kit with oligo dT primer (Roche, Germany), for extracting mRNA and affirming *TLP-3* gene expression in transgenic plants. Producing desired band in PCR with transgenic tobacco's cDNA demonstrates the act of transcription and transferring the information form DNA to RNA (Figure 5)

Bioassay

In order to affirm *TLP-3* gene without signal peptide expression in transgenic tobacco plants, *A. alternata* was used in microscopic examination. First of all, for becoming confident on creating the disease on tobacco plant by *A. alternata*, the untransformed tobacco leaves were inoculated. After 10 days, the disease symptom appeared. After that, for investigating TLP-3 protein expression in transgenic tobacco plants, the microscopic tests were designed.

Microscopic experiment

A. alternata is able to produce complete individual and countable spores on PDA medium. So the TLP-3 antifungal activity that exists in plant extract was investigated base on comparing the germinated and non germinated spores. Then, germinated and non germinated spore numbers were counted and data analysis was done through Proc GLM SAS computer software (Tables 2 and 3).

Results from data variation analysis showed that, there was a significant difference in the level of 1% between the treatments containing TLP-3 proteins and control treatment.

DISCUSSION

When plants are invaded by a fungal pathogen, major physiological and chemical changes are activated (Pellegrineschi et al., 2001). Up to now, there have been several reports of improved resistance of plants to diseases as a result of gene transformation and over-expression of PR-proteins such as TLPs (Mackintosh et al., 1989; Vigers et al., 1992; Liu et al., 1994; Choi et al., 1997; Ye et al., 1999; Chen et al., 1999; Datta et al., 1999; Fagoaga et al., 2001; Velazhahan et al., 2002; Punja, 2005; Radhajeyalakshmi et al., 2005). Tobacco plants constitutively overexpressing a rice PR-5 showed enhanced resistance to *A. alternata* (Velazhahan and Muthukrishnan, 2003). Overexpression of TLP in tomato exhibited increase resistance to *Alternaria solani* (Radhajeyalakshmi et al., 2005). Overexpression of tobacco PR-5 in potato delayed the development of disease symptoms of *Phytophtora infestans* (liu et al., 1994). Constitutively expression of tomato PR-5 in transgenic orange showed increased tolerance to *Phytophtora citrophthora* (Fagoaga et al., 2001). Overexpressing the TLP in transgenic rice plant showed enhanced resistance to *Rhizoctonia solani* (Datta et al., 1999). This study separated *TLP-3* gene from *M. truncatula* and regenerated transgenic tobacco plants that expressed *TLP-3* gene without signal peptide under the control of the CaMV 35S prompter. For separating *TLP-3* gene without signal peptide, PCR was used in the presence of *taq* polymerase enzyme and special primers. Because of using the *Agrobacterium* method for gene transfer to tobacco plants, the *TLP-3* must ligate without signal peptide gene into pBI121 plasmid and for the purpose of increasing the number of pBI121 plasmids containing *TLP-3* gene without signal peptide and also increasing these plasmids in essential times, cloning of the pBI121

Table 2. The analysis of variance for prevention of *A. alternata* spore germination.

SV	df	MS
Treatment (extract of transgenic plants)	1	0/57193292**
Error	14	0/00651162

** Significant at 1% probability level.

Table 3. Mean comparison for prevention of *A. alternata* spore germination.

Treatment	Replication number	Mean
Extract of transgenic plants	12	0/5244[a]
Extract of control plant	4	0/10350[b†]

† Means following the same letters have no significant differences.

was done. The existing resistant gene against kanamycin antibiotic in pBI121 plasmid provided the growth possibility only for the *E. coli* XL1-Blue that has pBI121 with *TLP-3* gene without signal peptide on the medium containing the kanamycin antibiotic. Since the pBI121 plasmid can be ligated without gene, it is necessary to analyze if they have *TLP-3* gene. Before transferring *TLP-3* to the important agricultural plant, it was necessary to ensure the ability of creating resistance in the model plants. In this research, tobacco *(Nicotina tabacum* cv. Xanthi) was used for transformation. The transformed and positive control plants has a convenient growth but death of negative control demonstrated that they had no ability to growth on MS medium that contained kanamycin and cefotaxime. PCR analysis confirms the gene transformation and RT-PCR analysis confirm the gene expression in transgenic tobacco plants. For extracting TLP-3 mRNA in RT-PCR assay, mRNA capture kit with oligo dT primer (Roche, Germany) was used since the existing poly A tail at the 3´end of mentioned mRNA. After that for bioassay performing and becoming confident about creating the disease on tobacco plant by *A. alternata*, the untransformed tobacco leaves were inoculated. After 10 days the disease symptom was appeared. After that for investigating TLP-3 protein expression in transgenic tobacco plants, the microscopic test was designed. *A. alternata* is able to produce completely individual and countable spores on PDA medium. So the TLP-3 antifungal activity that existing in plant extract was investigated base on comparing the germinated and non germinated spores. The results of microscopic test show the ability of plant extract to prevent of *A. alternata* spore germination. The results suggested that the *TLP-3* without signal peptide can act as a useful gene for transferring to important plants and controlling the disease.

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