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Agrobacterium mediated production of transgenic tomato plants harbouring *AP24* gene from tobacco

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Abstract

The tobacco (*Nicotiana tabacum*) antifungal AP24 protein identifies as osmotin encodes by its corresponding gene and contributes to pathogen resistance in plants .In the present study the AP24 gene was obtained from tobacco genome by PCR amplification. Following of this successful amplification, transgenic tomato (*Solanum lycopersicum*) which constitutively expressing the AP24 gene was generated by *Agrobacterium tumefaciens* mediated transformation. In order to express AP24 protein in tomato, the gene was inserted into a plant expression vector PBI121 and the recombinant pBI-AP24 plasmid was proliferated in *E.Coli TG1*. The new construct was used to transform the Strain *Agrobacterium LBA4404* before plant transformation. Then transgenic tomato plants were developed by introducing AP24 gene in the plant genome under the control of CaMV35s promoter. In order to show the stability of AP24, transgenic T1 tomatoes analyzed by PCR and high transformation proficiencies obtained. Releasing this one new transgenic variety may be a considerable progress toward release varieties enables to show resistant antifungal characteristics.

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Introduction

Antimicrobial proteins are the most important part of the natural defense system which produces in plants to control diseases causing by microorganisms (Garcia-Olmedo *et al.,* 1998; Somssich and Halbrock, 1998). There are so many literatures about inducing plant resistance to pathogens through introduction of plant antimicrobial genes into the plant (Carmona *et al*., 1993; Mourgues *et al*., 1998; Iwai *et al*., 2002; Khan *et al* 2012; Sadumpati *et al*.2013).Introduction of antimicrobial genes and the production of new disease resistant plants maybe suitable for commercial agriculture. Fungicides provide a considerable degree of protection, but their application causes higher production costs, induction of fungal resistance, and higher environmental risks. Strategies explored to generate resistance by means of genetic engineering in order to transfer pathogen resistance gene into host plant genome which finally resulted to inhibition of bacterial or fungal virulence factors contamination. In tobacco one group of induced protein known as pathogenesis related (PR) protein has been classified into five families (PR1-PR5) based on their structures and functions (Melchers *et al*., 1994). AP24 is a 24 k Da thaumatin like pathogenesis related protein belonging to the PR-5 family that was early characterized as an antifungal protein (Abad *et al*., 1996). AP24 induces cell lysis by a mechanism involving pore formation and dissipation of membrane potential (Selitrennikoff, 2001). Potato plants expressing AP24 gene showed increased resistance to *Phytophthora infestans* which is an oomycete fungus that causes the serious potato disease known as late blight or potato blight. (Liu *et al*., 1994) and tomato plants transformed with tobacco AP24 and chitinase transgene beans showed improved resistance to *Fusarium oxysporum* (Ouyang *et al*., 2005).

In addition other protein similar to AP24 isolated from seeds of maize, oats, sorghum and wheat were reported to have antifungal activity for variety of fungi. The introduction of exogenous genes into the adequately modified T-DNA of *Agrobacterium* cells and following infection of a explants led to gene's stable integration in the plant genome. By using optimized *Agrobacterium* mediated transformation, we demonstrate constitutive expression of the AP24 gene in tomato plants which can increase their resistance to the fungi infections.

Materials and methods

Bacterial Strains and culture media

Escherichia Coli Strain TG1 and *Agrobacterium* LBA4404 were obtained from Cinnagen. *E.Coli* strains were grown in Luria-Bertani (LB) broth (1% (w/v) tryptone (Bacto; Cat. No. 211701), 0.5% (w/v) yeast extract (Bacto; Cat. No. 212730), 1% (w/v) NaCl (Fisher; Cat. No. 642-500)). *E. coli* TG1 cells containing pTZ57R plasmid were grown in LB broth containing 100 µg/mL ampicillin (Sigma; Cat. No. A9393). *E-Coli* and *Agrobacterium* cultures were incubated at 37ºC and 28ºC respectively in a shaking incubator.

Plasmid Construction

Nicotiana tabacum ap24 gene (1549 bp) that contains the intact osmotin gene open reading frame was amplified through PCR from tobacco genome (NCBI GenBank GI: 19782). The AP24 encoding region was amplified using the designed primers Back: 5´-GAAAAGCCGCCATTCCCCTA -3´ and Forward: 5´ -ACTTTCAACGTCTTGGGTCC-3´. Each PCR mixture was prepared in a final volume of 50 µl containing 50 ng template DNA, 50 pM forward primer, 50 pM of the corresponding back ward primer, 40pM dNTPs, 25 mM MgCl2, 1.25 U Taq DNA polymerase (Cinnagen) and 5 µl 10×PCR buffer II (Cinnagen). Hot start PCR was performed at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 45 s and elongation at 72 °C for 1 min. The reaction was completed by a final extension time at 72 °C for 1 min. The amplified variable regions were purified by electrophoresis on a 1.5% agarose gel and subsequently extracted with an AccuePrep Gel Extraction Kit (Bioneer) according to the manufacturer's instructions. Amplified DNA was cloned into T/A vector (pTZ57R)using the InsT/A clone PCR product Cloning kit from Fermentas (Cat. No.k1214) which is is a convenient system for direct one-step cloning of PCR-amplified DNA fragments.

pTZ57R plasmid was cut with BamH I and Sac I (Roche Applied Science). After another electrophoresis on a 1.5% agarose gel, the variable regions were excised and extracted with the AccuPrep Gel Extraction Kit (Bioneer). The fragment of interest was ligated with the correspondingly cut vector pBI121 in a 10 µl volume containing 50 ng vector DNA, threefold molar excess of the PCR product and 5 U of T4 DNA ligase (Fermentas) for 16 h at 14 °C. *E. coli* TG1 cells (50 µl) were transformed with 2 µl of the ligation product via Cacl2 method. One milliliter of prewarmed SOC medium (Sambrook and Russell , 2001) was immediately added, and the cells were grown at 37 °C for 1 h shaking with 250 rpm. Cells were placed on LB agar medium containing Kanamycin (25 µg/ml) and incubated overnight at 30 °C. Colonies were picked for plasmid extraction using the Accuprep Plasmid Extraction Kit (Bioneer). The engineered pBI 121 plasmids were used to transform the *Agrobacterium* strain LBA 4404 using a Freeze and Thawing standard protocol (Sambrook *et al.*, 2001).

Transformation

Standard protocol (Wang & Campbell, 2008) was followed for tomato transformation. Tomato seeds were sterilized and sown on Murashige and Skoog (MS) medium supplemented with 3% sucrose and solidified with 0.8% agar with vitamins. Seven day old cotyledons from the seedlings were cut at the petioles and at the tips. The explants were incubated upside down on MS plates with appropriate vitamins and hormones at room temperature for overnight. *Agrobacterium* LBA 4404 strain containing a gene construct was cultured on the same day for transformation of these explants the next day. The explants were added to 20 mL of *Agrobacterium* cell and incubated for 15 minutes with periodic shaking. The explants were then returned to their plates upside down, sealed with micropore tape and incubated at room temperature for two days in 16 h photoperiod. Then explants were transferred to the regeneration medium (MS medium, supplemented with 2.0 mg/l BAP, 0.01 mg/l NAA, 200 mg/l cefatoxim, and 100 mg/l kanamycin). The explants were transferred to the fresh medium at 2 weeks intervals. As a control, non-inoculated explants were cultured in the same medium without hormones and antibiotics. The induced shoots were then dissected from the explants and transferred to MS medium containing cefatoxim (200 mg/l) for *Agrobacterium* elimination and kanamycin (100mg) as a selective marker. As soon as shoots appeared (about 4–8 weeks), they were transferred to free hormones rooting medium, they were transplanted into greenhouse.

PCR analysis

After the shoots developed adequate roots on free antibiotic medium, the regenerated tomato plants were transferred into the greenhouse and maintained to maturity. Genomic DNA from 200 mg each of non-transgenic plants as negative control and all putative kanamycin resistant plants was extracted from transgenic plants according to Albani *et al.* (1992). Transformed and control plant genomic DNA was used as a template to detect the AP24 gene by Polymerase chain reaction (PCR) under the conditions that were described before and with specific primers. The 1500 bp amplified DNA fragments were electrophoresed on a 1.5% agarose gel and visualized by staining with EtBr. The primers and the composition of PCR system was the same to that in 2.2. PCR was performed also in the same way as described in 2.2.

Results

Construction and transformation of vectors

The isolation and PCR amplification of the gene encoding the AP24 was performed. AP24 gene of the tobacco was subcloned into the expression vector pTZ57R. Targeting the restriction sites BamH1 and Sac1 in pTZ57R plasmid facilitated subcloning process. New construct (plasmid) was transformed into TG1 *E-coli* and the colonies were appeared on the kanamycin containing plate. After the extraction, pTZ57R and pBI121 plasmids were cut by BamH1 and Sac1 restriction enzymes. Electrophoresis showed 1500 bp bands from pTZ57R and 12000 bp and 1900 bp GUS removed gene from pBI121 (Fig.1).The amplified 1500 bp and 12000bp bands were gel-

purified from agarose. During the ligation, AP24 gene inserted into pBI121 plasmid (pBI-AP24) and new recombinant plasmid was transferred to *Agrobacterim* strain LBA 4404 (Fig. 2). When the cotyledons explants were inoculated with *Agrobacterium* immediately after excision, shoots on the regenerated shoots were observed in the presence of 100mg/l kanamycin and 200mg/l cefatoxim after two weeks. The genes that had been stably integrated into the plant genome were translated under the control of CaMV35S, resulting in the expression of the AP24 gene and the growth of transformants on medium supplemented with kanamycin. The putatively transformed shoots were excised when they were about 1 cm tall and transfered to a shoot elongation medium containing cefatoxim and kanamycin.Consequently shots were transferred to rooting mediato generate a complete seedling. Finally in order to put in the hardenization step, transgenic plants transfered to the greenhouse.

Table 1. Stability of AP24 gene in different T1 transgenic plant lines according to obtained results from PCR analysis of 7 lines of T0 transgenic plants.

Line	Total	AP24+	Transformation proficiency percentage
T(1)1	25	18	72%
T(1)2	25	12	48%
T(1)3	25	20	80%
T(1)4	25	19	76%
T(1)5	25	11	44%
T(1)6	25	22	88%
T(1)7	25	15	60%

Expression of AP24 fragment in plants

We obtained kanamycin-resistant putative transformants on selective media and carried out further analysis of AP24 gene expressions on selected transgenic plants. Untransformed tomato plants that had been regenerated from explants without kanamycin selection, were used as negative controls. PCR analysis was carried out as the first method to confirm the transgenic nature of the regenerated plants. The presence of AP24 DNA in the genomic DNA isolated from regenerated tomato was confirmed. Transformants were detected by PCR amplification of inserted AP24 with specific primers. The expected 1500 bp AP24 bands were found in the transformants (Fig.3). The same DNA band was not observable in untransformed control plant DNA.

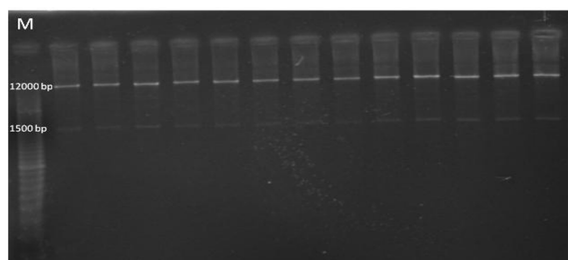

Fig 1. Agarose gel electrophoresis of Digested PBI Plasmid showed 12000 bp and 1900 bp bands related to linear PBI and GUS genes respectively.

Stability of AP24 gene in T1 Progeny

In order to determine the stability of foreign AP24 gene in obtained T1 progeny of survived T0 tomato transgenic plants were analyzed by PCR analysis of isolated DNA from leaves of transgenic tomatoes using the same primers for T0 plants. The obtained results (Table 1) showed the different transformation proficiencies from lowest 48% to highest 88% for T1 (2) and T1 (6) progenies respectively.

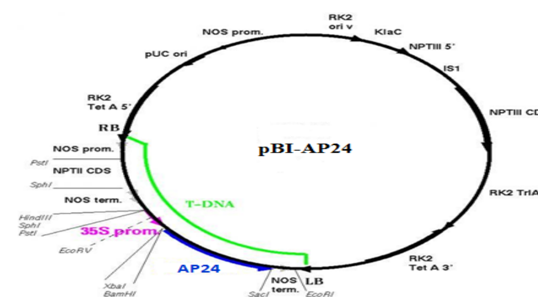

Fig. 2. Schematic representation of pBI-AP24 construction .AP24 gene inserted between CaMV 35S promoter and NOS terminator.

Discussions

We have described here, the extraction and amplification of AP24 gene from tobacco plant genome and following its overexpression in tomato plants through Agrobacterium mediated

transfomation. To facilitate gene transfer ability high level of AP24 is required therefore we used the CaMV 35S promoter which is very strong constitutive promoter, causing high levels of gene expression in dicot plants. In order to take advantages of MCS (Multi Cloning Site) which is beneficial for cloning the T/A cloning vector (PTZ57R) have been used. The presence of M13 primers around the MCS facilitated the gene sequencing through this plasmid and PCR product was able to insert into a linear vector. Among different candidate parts of plant includes leaves, stem fragments, and root fragments, the optimum explants used in transformation of tomato are cotyledons. The reason is that the frequency of shoot induction of leaves is the highest and moreover *Agrobacterium* has the strongest ability to infect leaves. Although Agrobacteroum strain C58GV3101 showed more priorities rather than LBA4404 for infection of tobacco leaf disks in the previous study (Korouhdehi *et al* 2011) but in this study *Agrobacterium* strain LBA4404 was selected for infection of tomato explants because cotyledon was selected as an explant. The activating potential of genes in *Agrobacterium* virulence region are important factors could improve the infecting ability of LBA4404. The free hormone solid MS medium has been used as rooting medium in our study and I was so effective for explants rooting.

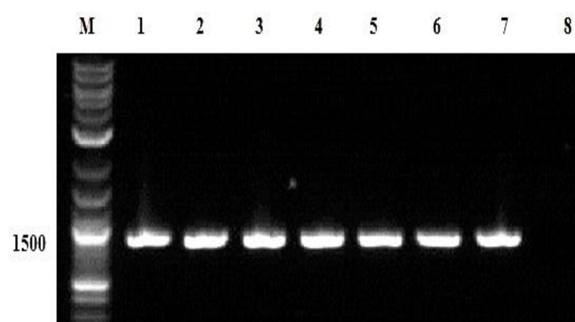

Fig. 3. Agarose gel electrophoresis of AP24 amplification products from transgenic plants. Lane M GeneRuler 1 kb DNA Ladder 250 to 10,000 bp (Thermo scientific); lanes 1-7 transgenic tomato plants, lane 7 untransformed tomao.

Transformation efficiency improved by adding appropriate amount of hormones to co-cultivation medium which leads to increase cell division of explants while keep the cell activity, finally improve cell growth after transformation. PCR results indicated that the AP24 gene also had been integrated into the genome of T1 progeny of tomato and could be effective in resistance to fungi pathogens. Finally for determination of stability of AP24 gene in transgenic tomatoes, 7 lines of T1 progenies were analyzed by PCR and the result showed 48-88% transformation efficiencies.

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