Insertion of an Antimicrobial Gene into *Agrobacterium* and its Further Use in Transforming Tobacco

Ekrem GÜREL

Abant Izzet Baysal University, Faculty of Science and Arts, Department of Biology, 14280 Bolu - TURKEY

Abstract: In the present work, we first describe the transfer of a novel gene, designated 5W, into the LBA4404 *Agrobacterium tumefaciens* strain using pPCV91 plasmid through a triparental mating system. The 5W gene was recently isolated from macadamia, a native nut tree species of Australia, and has been shown to have an antimicrobial effect against certain fungi *in vitro*. Secondly, this gene was tranferred into tobacco (*Nicotiana tabacum* cv. *Xhantii*) using leaf explants to develop a system which could then be used for transferring this antimicrobial peptide into sunflower cultivars.

Key Words: Macademia, tobacco, triparental mating, gene transfer, leaf explants

Antimikrobiyal Bir Genin *Agrobacterium*'a Yerleştirilmesi ve Tütünün Transformasyonunda Kullanılması

Özet: Bu çalışmada, evvela 5W olarak adlandırılan yeni bir genin, pPCV91 plasmidini kullanarak üçebeveynli eşleştirme (triparental mating) yöntemi ile *Agrobacterium tumefaciens* LBA4404 suşuna aktarılması tanımlanmıştır. 5W geni, Avustralya'ya özgü bir fındıksı ağaç türü olan makademiyadan izole edilmiş ve *in vitro* şartlarında belli funguslara karşı antimikrobiyal etki gösterdiği belirlenmiştir. Daha sonra ise, *Agrobacterium* aracılığı ile bu genin tütün (*Nicotiana tabacum* cv. *Xhantii*) yaprak explantlarına transferi tanımlanmıştır. Bu çalışmada oluşturulan sistemin, 5W geninin ayçiçeği çeşitlerine aktarılmasında kullanılması amaçlanmıştır.

Anahtar Sözcükler: Makademiya, tütün, üçebeveynli eşleştirme, gen aktarımı, yaprak eksplantları

Introduction

Despite the fact that all plants, and animals likewise, are exposed to numerous pathogenic microorganisms throughout their life cycle, microbial infections threatening their survival do not often occur. This suggests that such higher organisms have certain defence mechanisms against invading microorganisms. In fact, almost all plant species synthesize some proteins with antimicrobial properties and the demonstration of the role of such substances in inhibiting bacterial and fungal growth dates back to as early as the 1940s (Stuart and Harris, 1942; Fernandez et al., 1972). To date, a great number of antimicrobial peptides have been isolated from numerous plants species (Terras et al., 1995; Broekaert et al., 1997) and many of these peptides are today widely exploited in obtaining disease resistant varieties by first purifying such peptides from cultivated or wild plant species, and then cloning and transferring the corresponding genes into a plant genome, mainly through *Agrobacterium tumefaciens* or microprojectile bombardment-mediated gene transfer techniques (Carmona et al., 1993; Birch, 1997).

Because of the excellent response of tobacco (*Nicotiana tabacum*) cut leaf tissues to *A. tumefaciens* infection and further regeneration, the transformation of tobacco has become a model system for the expression of genes following *Agrobacterium*-mediated transformation (Draper and Scott, 1991; Florack et al., 1994). In the present work, we firstly describe the mobilization of a binary plasmid (pPCV91-5W) carrying a gene encoding an

antimicrobial peptide, designated 5W, which has been recently isolated from macadamia, a native tree (nut) species of Australia, into the LBA4404 *A. tumefaciens* strain through a triparental mating system. We secondly describe the introduction of this novel gene into the tobacco genome via *Agrobacterium*-mediated gene transfer. Our initial optimization studies for regeneration (Gürel and Kazan, 1998) and transformation (Gürel and Kazan, 1999) of several sunflower (*Heliatnhus annuus* L.) genotypes using LBA4404/pTOK233 and Agl-1/pKIWI strain/plamsid combinations have previously been carried out.

Materials and Methods

Plant Material and Culture Conditions

Tobacco (*Nicotiana tabacum* cv. *Xhantii*) plants were grown in a controlled-environment room at 25ºC in a 16/8 hours light/dark regime under fluorescent light. The fully expanded leaves were removed and cut into four equal pieces and then rinsed in sterile distilled water for 5 min before surface sterilization in 70% ethanol for 1 min and then in 1.0% sodium hypochlorite for 10 min. After rinsing in sterile distilled water 3-4 times, the sterilized leaf tissues were cut into approximately 1 cm^2 pieces, avoiding the major lamina veins, and then cultured in 9 cm diameter plastic Petri dishes containing ca. 20 ml of MSC medium which contained MS (Murashige and Skoog, 1962) salts and vitamins, 1.0 mg/l BAP, 0.1 mg/l NAA, 30 g/l sucrose and 8 g/l bacto-agar (Difco) at pH 5.7. A total of 180 explants were used per treatment, and half of the explants were preconditioned on the MSC medium for 24 hours and the other half for 48 hours before inoculations with bacterial suspensions.

After inoculation, leaf explants were transferred back to the MSC medium, and Petri dishes were sealed and incubated under continuous dim light for 3 days at 24ºC. At the end of this co-cultivation period, explants were transferred onto MSR medium without any washing and cultured at 25/20ºC day/light temperature in a 16/8 light/dark regime under white fluorescent light. The MSR medium was the same as the MSC medium supplemented with 500 mg/l cefotaxime and 100 mg/l hygromycin. Explants were subcultured onto fresh MSR medium at two week intervals for shoot regeneration, which was scored after 25 and 40 days' culture.

Plasmid Transfer by Triparental Mating

The binary plasmid pPCV91-5W used in this work was first constructed in the *Escherichia coli* strain DH5-α (Figure 1). To transfer this plasmid into an *A. tumefaciens* strain by triparental mating (Ditta et al., 1980; also see Figure 2), the LBA4404 strain was grown for 24 hours in liquid YEP medium (An et al., 1988) containing 50 mg/l rifampicin and 25 mg/l kanamycin in darkness at 28ºC on a shaker at 180 rpm. *E. coli* strains DH5-α harbouring the plasmid of interest (pPCV91-5W) and HA101 harbouring a helper plasmid (pRK2013) were grown overnight in liquid LB medium (Sambrook et al., 1989) containing 50 mg/l carbenicillin and 25 mg/l kanamycin, respectively, and cultures were shaken at 160 rpm. Both *A. tumefaciens* and *E. coli* cultures were spun down at 4000g at 25ºC for 10 mins. After decanting the supernatant, the pellet was resuspended in liquid YEP medium with no antibiotics. Then, 100 µl from each bacterial culture was taken and mixed in a sterile Eppendorf tube, and 100 µl of solution from this mixture was plated onto solid YEP medium again with no antibiotics. Plates were then incubated for 24 hours in darkness at 28ºC and the next day, this culture was streaked out onto solid YEP medium containing 25 mg/l kanamaycin, 50 mg/l rifampicin and 125 mg/l carbenicillin and incubated in darkness at 28ºC for another 2 days. Finally, liquid YEP cultures containing 25
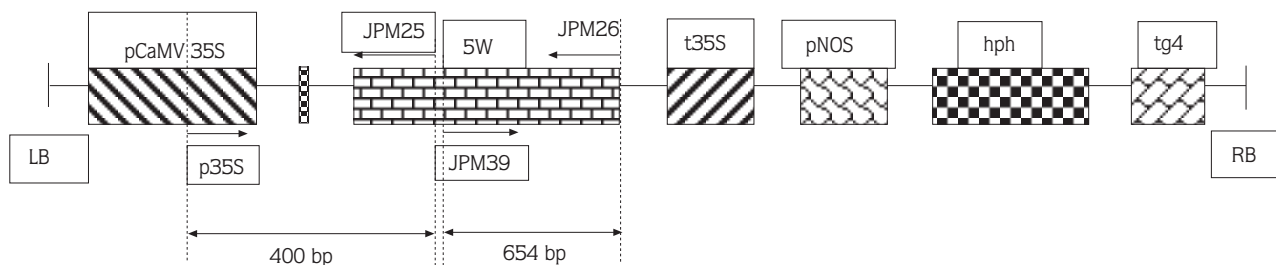

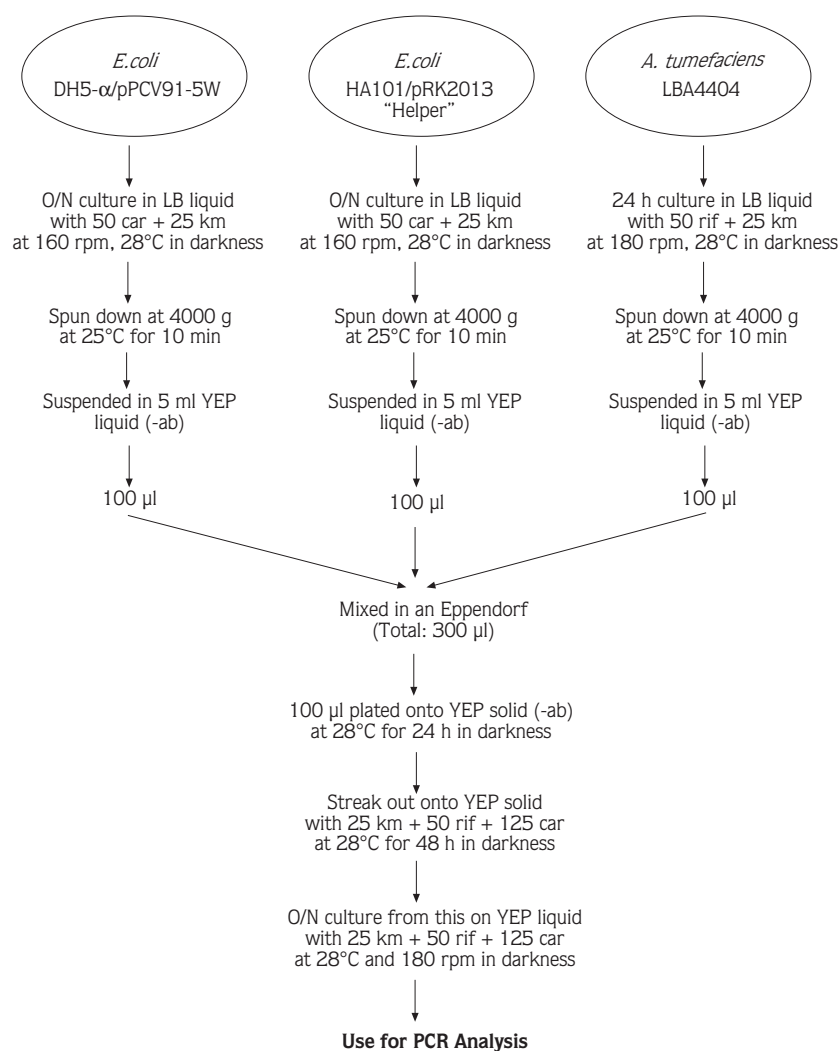Figure 1.   A diagramatic representation of the T-DNA construct of the plasmid pPCV91-5W.

Figure 2. A flow diagram of the process followed for the transfer of the 5W gene from *Escherchia coli* into *Agrobacterium tumefaciens* through triparental mating system. O/N: overnight, -ab: with no antibiotics. Concentrations of the antibiotics are given in mg/l.

mg/l kanamaycin, 50 mg/l rifampicin and 125 mg/l carbenicillin were inoculated with the bacteria growing on the selection plates. Further confirmation of plasmid transfer from *A. tumefaciens* to *E. coli* was done by PCR analysis.

Bacterial Culture and Inoculation

A. tumefaciens strain LBA4404, an octopine type disarmed strain derived from Ach5 (Hoekama et al., 1983) harbouring the pPCV91-5W plasmid, was grown overnight in liquid YEP medium containing 125 mg/l carbenicillin and 50 mg/l rifampicin. The overnight culture (OD_{600}= 1.65) was spun down at 4000g for 10 min at 25ºC, resuspended in sterile full-strength liquid MS medium without plant growth regulators and finally diluted 10 times with the same MS liquid medium. Sterile

tobacco leaf explants were then inoculated by immersing in the diluted bacterial suspension for 15 min, shaking gently, and then transferred back to the MSC medium without any dry blotting.

PCR Assay

Two sets of primers, JPM39 with JPM26 and JPM25 with p35S, were used (Figure 1). The PCR reaction mixture contained 2.5 μl of 10x PCR buffer (670 mM Tris-HCl pH 8.8, 166 mM $[NH_4]SO_4$, 4.5% Triton X-100, 2 mg/ml Gelatin), 4.0 μl of 4 mM $MgCl_2$, 2.5 μl of 125 μM dNTPs, 2.0 μl of 20 μM of each primer, 2 μl of overnight *A. tumefaciens* culture containing the binary plasmid vector and lastly 10 μl of distilled water containing 1 unit of Taq polymerase (Biotech International Limited) in a total of 25 μl reaction mixture.

For each primer set, a positive control with pure plasmid (pPCV91-5W) DNA was also used with the negative controls that contained no DNA. The PCR tubes containing the total reaction mixture were placed in a thermal cycler (MiniCycler, MJ Research) and overlaid with a drop of paraffin oil, and run for 30 cycles after an initial denaturation for 4 mins at 94ºC as follows: 94ºC for 1 min, 50ºC for 1 min and 72ºC for 1 min. After PCR amplification, 3 µl stopping (blue) solution was added each tube and the mixture was then loaded into 1% TAE (tris-acetate) agarose gel at pH 8.0. After adding 3 µl of ethidium bromide to the electrophoresis buffer, the gel was run at 115 mV for 30 mins and then observed under UV light and photographed. The PCR amplification and gel running were performed twice.

Results and Discussion

Triparental Mating

The major difficulties of plant transformation technology in achieving a high proportion of plants showing predictable transgene expression are the development of gene constructs and transfer methods. In this work, we studied the transfer of a new construct carrying a novel gene into *Agrobacterium* and then transferred it into tobacco leaf explants. In the initial attempts to introduce the pPCV91-5W plasmid into *Agrobacterium*, we used the strain GV3101 but, although the process was repeated several times, the introduction did not occur when selected on medium containing 25 mg/l kanamycin, 50 mg/l rifampicin and 125 mg/l carbenicillin for 2 or 3 days. This might be due to the resistance of GV3101 to the selective antibiotics used to select for binary plasmids. Our inability to transfer this plasmid into GV3101 may also be due to natural mutations in the bacterial genes functioning in the transfer and stable replication of received plasmid in the bacteria. However, when we used the same procedure for the strain LBA4404, the introduction was readily achieved. Both positive and negative controls of the PCR analysis confirmed the presence of the plasmid in both primer sets (Figure 3, see lanes 1, 2, 4 and 5). Primers JPM39 and JPM26 anneal to regions within the gene and amplify a DNA fragment of 654 bp while primers p35S and JPM25 anneal to the 35S promoter and the gene, and amplify a DNA fragment of 400 bp (Figure 1). Our construct contained both KnR, AmpR and HygR and this new construct carrying the hygromycin resistance gene was then used for our transformation studies.

Transformation and Shoot Regeneration

To examine the effect of the preconditioning culture on the efficiency of bacterial infection and eventually the transformation efficiency in tobacco leaf tissue, we divided the explants into two lots, one lot receiving a 24 hour treatment and the other lot 48 hours on MSC medium without antibiotics. Following inoculation and co-cultivation, the colour change in the explant tissue and the degree of tissue damage (presumably caused by wounding and bacteria) during the incubation period on
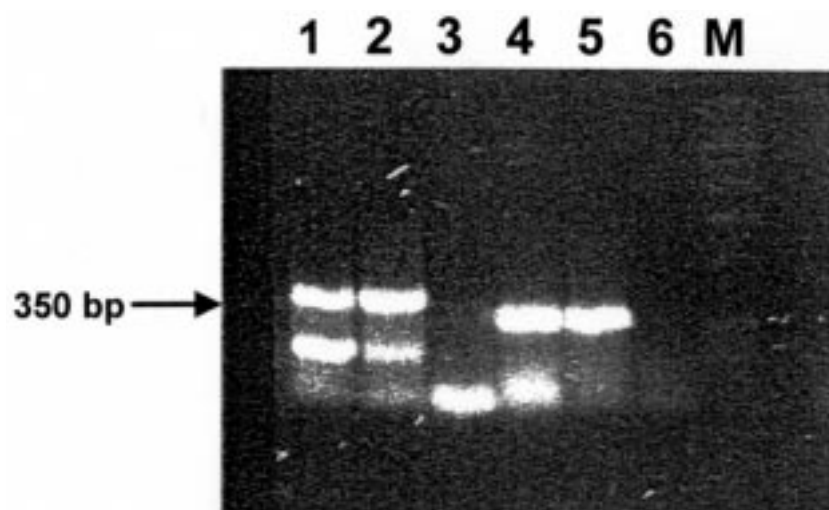

Figure 3. PCR gel assay showing the presence of the plasmid in both primer sets. Lane1: Bacterial DNA with primers JPM39 and JPM26, Lane 2: Pure DNA with primers JPM39 and JPM26 (positive control), Lane 3: No DNA with primers JPM39 and JPM26 (negative control), Lane 4: Bacterial DNA with primers p35S and JPM25, Lane 5: Pure DNA with primers p35S and JPM25 (positive control), Lane 6: No DNA with primers p35S and JPM25 (negative control), Lane 7: DNA size marker.

the regeneration medium (MSR) were observed. Those explants that were preconditioned for 48 hours were generally greener and looked less damaged than those preconditioned for 24 hours. As the incubation proceeded, most of the 24 hour preconditioned explants turned yellow and eventually died whereas many of the 48 hour preconditioned explants remained green and consequently produced shoots (Figure 4).

When the two treatments were compared in terms of shoot-producing ability, we observed that none of the 24 hour treated explants produced shoots at all and most of the explants died by 25 days' culture on the regeneration medium while a high proportion (12.2%) of the 48 hour treated explants produced shoots (Table 1). The mean number of shoots produced per explant was 0.44, which corresponded to a mean of 3.59 shoots per shoot-producing explant (i.e., without taking into account those explants that formed no shoots). In a following assessment of the 48 hour treated explants at 40 days' culture, only a slight increase could be observed in the mean number of shoots per explant (0.44 shoots at 25 days compared to 0.47 shoots at 40 days) but not in the number of explants producing shoots (i.e., % of explants producing shoots). This may indicate that those explants which had already produced shoots by 25 days' incubation had formed only a few more new shoots during the following 15 days of additional culture on the regeneration medium, despite the fact that they were sub-cultured onto fresh medium every two weeks. However, during this additional culture period, only 3 (out of 180) of the 24 hour treated explants produced very few tiny shoots.

Incubation of leaf explants on medium prior to bacterial inoculations is a common practice in *Agrobacterium*-mediated transformation of tobacco
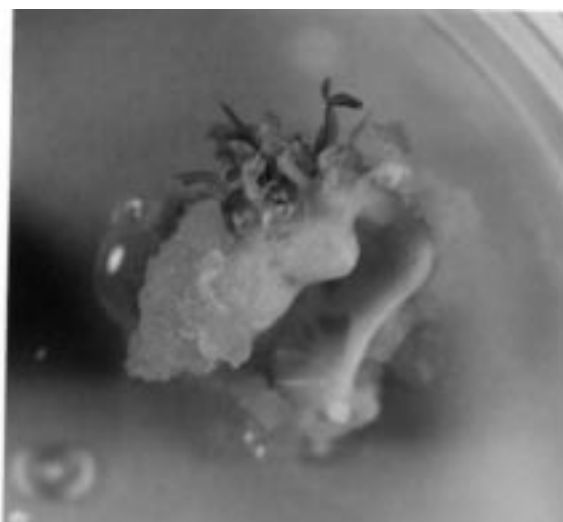

Figure 4.   Multiple shoot development from a leaf explant of tobacco on MSR medium containing 1.0 mg/l BAP, 0.1 mg/l NAA, 500 mg/l cefotaxime and 100 mg/l hygromycin.

tissues (Horsch et al., 1988), presumably allowing an initial growth and eliminating those that were damaged during sterilization or handling, as well as letting the wounded leaf tissue release a sufficient amount of wound-induced compounds like phenolics, sugars, amino acids and secondary plant metabolites. These compounds are known to stimulate chemotaxis of the bacteria to the wound site and thus activate the molecular events leading to the transfer of T-DNA material into the plant cells (Walden, 1997). It appears that the relatively more prolonged culture of the explants before inoculating with *Agrobacterium* helped the tissue to become more competent for the transformation by simply allowing more time to produce the necessary wound-induced compounds. Those explants which apparently did not produce a sufficient amount of such compounds,

Parameters	Preconditioning for 24 hours		Preconditioning for 48 hours	
	Scoring after		Scoring after	
	25 days	40 days	25 days	40 days
% of explants producing shoots	0.00	1.67	12.2	12.2
Mean number of shoots/explant	0.00	0.02	0.44	0.47
Mean number of shoots/shooted explant	0.00	1.34	3.59	3.86

Table 1.   Comparison of two preconditioning periods for shoot regeneration from tobacco leaf tissue which was inoculated with *Agrobacterium tumefaciens* LBA4404 strain harbouring the pPCV91-5W plasmid carrying the antimicrobial gene 5W.

therefore, might have failed to be infected by the bacteria and consequently became more vulnerable (as a result of being non-transformed) to the heavy selection of hygromycin as well as cefotaxime.

Hygromycin is an aminoglycosidic antibiotic produced by *Streptomyces hygroscopicus* and highly efficient in killing plant cells, bacteria and fungi by inhibiting protein synthesis (Lin et al., 1996). In transformation studies, hygromycin selection is based on the use of the gene aph IV isolated from *E. coli*, which encodes for the hygromycin phosphotransferase (hpt II) enzyme. Transgenic cells expressing this gene, therefore, confer resistance by inactivating hygromycin. The frequency of transformation with hygromycin selection is known to be lower in petunia and tobacco than with kanamycin selection (Hinchee et al., 1994). In a recent work, it was reported that the optimal concentration of hygromycin for the selection of transgenic tobacco plants was 50 mg/l (Lin et al., 1996). However, we decided to use an even higher concentration (100 mg/l) to reduce the number of escape shoots to a minimum. On the other hand, this does not necessarily imply that the shoots formed under this heavy selection are 100% transgenic, and further analysis of these putative transgenic plants will be necessary.

5W peptide is highly effective on several fungal pathogens including (their IC50 values are given in parantheses as µg/µl) *C. nicotianae* (5), *F. oxysporum* (10), *C. elegans* (2), *S. sclerotiorum* (20), *L. maculans* (10), *V. dahliae* (5), *B. cinerae* (>50), *P. cryptogea* (10), *P. parasitica* (25), *C. michiganensis* (50) and *P. syringae* (>50). Charaterization of transgenic tobacco plants transformed with this gene under the control of a constitutive 35S promoter will show whether this gene can confer any resistance to tobacco pathogens such as *Phytophthora nicotianae* cv. *parasitica, Cercospora nicotianae* or *Chalara elegans*. Once this is demonstrated, the gene can then be transferred to agronomically important crop species such as canola, sunflower and peanuts.

Acknowledgement

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