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INDUCING TRANSIENT GENE EXPRESSION IN *Nicotiana tabacum* PLANT BY AGROINFILTRATION METHOD

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Abstract

In this study I will demonstrate an Agrobacterium-mediated transient gene expression method, into Nicotiana tabacum leaves. By efficient infiltration of Agrobacterium cells carrying appropriate gene constructs into tabacum leaves, transient expression assays can be performed within several days without using expensive instruments or complicated procedures. By syringe infiltration, I will demonstrate a simple, efficient and scalable methodology to induce a target-gene into plant tissue. In addition, DNA, RNA and protein can be extracted from agroinfected leaves and used for reverse transcription-polymerase chain reaction (RT-PCR), polimerase chain reaction (PCR), Southern blot, Northern blot, Western blot, immunoprecipitation, and enzyme assays.

Key words*: Agrobacterium, gene, transfer.*

INTRODUCTION

Agrobacterium tumefaciens is a Gram-negative, non-sporing, which can cause crown gall disease (large tumour-like swellings that typically occur at the crown of the plant) of a wide range of dicotyledonous plants (Tzfira T., Citovsky V., 2008). According to Tzfira T. and Citovsky V., *Agrobacterium* is the only cellular organism that is naturally capable of transferring genetic material between the kingdoms of life and genetically transform, under laboratory conditions, a large number of plant species.

Transient gene expression systems is widely used for the functional characterization of genes and recombinant protein production in plant cells (Kapila et al., 1997). *Agrobacterium*, mediated transient gene expression (agroinfiltration), is based on infiltration of *Agrobacterium tumefaciens* culture, transformed to express the genes of interest, into intact plant leaves. The *Agrobacterium* assay, subsequently transfers a DNA segment, called transfer-DNA or T-DNA, into the target plant cells, thus inactivating the gene and mutation can then be analyzed. The T-DNA carries effector genes that allow transient gene expression (Hamilton, 1997; Vezina et al., 2009). This method has many advantages, including simple, rapid, and effective transformation compared with stable genetic transformations. Leaves of *Nicotiana tabacum* show reliable and high transformation efficiency for this specific method. In a syringe infiltration, an *Agrobacterium* suspension is infiltrated into the abaxial side of the leaves using a syringe *via* a needle hole, but without a needle. Using this method, the ability of various constructs can be simultaneously assessed on the same leaf. In such an assay, target gene is cloned into a T-DNA expression cassette driven by the CaMV 35S promoter, in a binary vector, into an *Agrobacterium tumefaciens* strain (Matoba et al., 2011; Whaley et al., 2011; Gleba et al., 2014; Zheng et al., 2012).

The binary vectors containing the T-DNA can carry inserts of up to over 100 kbp (Hamilton, 1997). T-DNA transferred to a plant cell will relocate to the nucleus, where its genes can be transcribed and expressed (Kapila et al., 1997). The majority of the plant cells in the infiltrated region express the transgene and the highest expression level, in 2-3 days after infiltration (Naomichi et al., 2016).

The main advantage of syringe infiltration is that different genes, either alone or in combination, can be expressed together in a single leaf (Liu et al., 2010). This assay has the

potential to identify inserts, that affect the production of an autonomous replicating gene that can spread systemically, through a plant and subjected to several analyses as polimerase chain reaction (PCR), Southern blot, Northern blot, Western blot, immunoprecipitation and enzyme assays.

MATERIALS AND METHODS

Preparation of *Agrobacterium tumefaciens* competent cells

A single *Agrobacterium tumefaciens* strain LBA4404 colony was incubated in 5 ml LB overnight at 28°C. Two ml of the overnight culture was added to 50 ml of LB broth in a 250 ml flask and shacked vigorously (250 RPM) at 28°C until the culture grew to an OD_{600} of 0.5-1.0. The culture was then chilled on ice and the cells were collected by centrifugation at 2700 RPM for 5 minutes at 4°C. Then, the supernatant was discarded and the cells were resuspended in 1 ml of 20 mM $CaCl_2$ ice-cold solution. The mixture was kept on ice and 0.1 ml aliquots were prepared in pre-chilled Eppendorf test tubes. After being flash frozen by immediate immersion in liquid nitrogen, the *Agrobacterium* competent cells were kept at -80ºC for use.

Direct *Agrobacterium* transformation

Direct *Agrobacterium* transformation by freeze-thaw method was used to transform LBA4404 competent *Agrobacterium* cells strain carrying pART27-ERI-1 hairpin construct into new *Agrobacterium* competent cells strain C81C1. 6 *µg* of pART27 ERI-1 construct DNA were added to 100*µl* of the competent *Agrobacterium* and gently mixed. The mixture was first freezed in liquid nitrogen for 5 minutes. The cells were then thawed by incubating at 37°C water bath for 25 minutes. After adding 1 ml of LB medium, the mixture was incubated at 28°C for 3 hours with gentle shaking. The whole mixture of the transformed *Agrobacterium* suspension was spread on LB agar plate containing 100 mg/ml spectomycin and 50 mg/ml rifampicilin final concentration and incubated for 2 days at 28°C. Grown colonies were then picked up and inoculated in 5 ml of LB with the same concentration of the above mentioned antibiotics. Culture tubes were kept at 28°C over night under vigorous shaking. Small scale DNA preparation was performed as described written below in *Isolation of Plasmid DNA with Mini-Prep Method*. The collected plasmid was then back-transformed onto *E.coli.* DH5α strain as described, as follows, in *Transformation of E.coli competent cells (heat-shock method)*. This step was done in order to verify the presence of the pART27 ERI-1 construct in the selected transformed *Agrobacterium.*

Isolation of plasmid DNA with Mini-Prep method

The mini-prep method was used for small-scale preparations extracting DNA for restriction digests and to check the cloned inserts before attempting a higher scale extraction. The integrity of the plasmid DNA was checked by gel electrophoresis and ethidium bromide staining.

1.5 ml of overnight bacterial culture was transferred to an eppendorf tube and the bacterial cells were collected by centrifugation at 13200RPM for 2 min in a microcentrifuge. Bacterial pellet was resuspended in 100 *µl* of Solution I (25 mM Tris-HCl, pH 8, 10 mM EDTA, pH 8, and 50 mM glucose) and cells were vortex vigorously and incubated at room temperature for 5 min. 200 *µl* of fresh Solution II (0.2 N NaOH and 1% SDS) was added followed by a further incubation for 5 min at 4°C in order to lyses the bacterial cells and release the plasmids. After thorough mixing, 150 *µl* of Solution III (5 M potassium acetate, pH 5.5) were added in the tube, mixed and left for 5 min on ice to let the genomic DNA and cellular debris precipitate. The solution was centrifuged for 10 min at 13200RPM in a microcentrifuge and the supernatant was transferred to a new sterile tube. The mixture was then washed with neutral phenol and extracted with chloroform/isoamylalcohol (24:1). The nucleic acids were precipitated at room temperature for 2 min by the addition of 2 volumes of 100% ethanol. The DNA was pelleted after 10 min of centrifugation at 13200RPM and the pellet was washed with 70% ethanol. After air-dry the pellet was resuspended in 50 *µl* sterile deionised water and stored at -20ºC, the efficiency yield is 3-5 *µg* of DNA/ ml of bacterial culture.

Transformation of *E.coli* competent cells

Competent bacterial cells were transformed by a simple heat-shock procedure. An aliquot of competent cells was thawed on ice and ligation prep. was added, and the tube was gently shaked to mix the contents. The mixture was stored on ice for 20 min and then the tubes were transferred to a water bath at 42°C and left for exactly 90 seconds. After, the tubes were rapidly transferred to an ice bath and allowed to chill for 2 min. 400 *μl* of LB was added and cells were incubated at 37°C for one and a half hour. During this time the bacteria can recover and express the antibiotic resistance marker encoded by the plasmid. To maximize the efficiency of transformation, the cells could be gently agitated. The cells were concentrated by centrifugation for 1 min 13200 RPM and gently resuspended in about 30 *μl* LB and 30 *μl* X-gal (5-bromo-4-chloro-3-indolil-β-D-galactoside) which is converted by β-galactosidase into a blue compound. The transformed cells were spread over the surface of the LB plates containing the appropriate antibiotics (ampicillin) by use of a sterile bent glass rod grown overnight at 37°C. The recombinant cells were selected by their ability to grow on LB ampicillin plates, under a white/blue selection, where the white colonies were the recombinant ones.

RESULTS AND DISCUSSIONS

In this research direct *Agrobacterium (A. tumefaciens)* cells strain carrying the desired gene (pART27-ERI-1 hairpin construct) was transferred to plant cells.

Experimental part as well as pART27-ERI-1 hairpin constructruction, were performed in Plant Molecular Biology Laboratory of the Institute for Molecular Biology and Biotechnology (IMBB-FORTH, Greece), during my PhD research.

Greenhouse grown *Nicotiana tabacum* wild type was used for agrobacterium injection. Plants were grown in the greenhouse with air-conditioning system. 10-15 *μl* of the transformed *A. tumefaciens* contains the expression vector pART27 ERI-1 were incubated in 5 ml LB supplemented with 100*μg/ml* spectomycin and 100 *μg/ml* rifampicilin over night.

Figure 1. *Agrobacterium* injection. A sterile needle tip was used to wound the lower surface of the leaf prior *Agrobacterium* suspension to be infiltrated into the abaxial side of the leaves using a needleless syringe

Once the *A. tumefaciens* is inside the leaf, gene of interest will transform to a portion of the plant cells and the gene is then transiently expressed over the plant. Towards the preparation of *Agrobacterium* infiltration, the transformed cells were harvested at OD_{600} of 0.8-1.0 by centrifuging at 2800 RPM at 4°C. The pellet was resuspended with 5ml of MMA medium (MS salts, 10 mM MES pH 5-6), and 5 *μl* of 200 mM acetosyringone was added. The pellet was resuspended by gentle pipetting and incubated for more than one and a half hour at 28°C with gentle shaking. The cells were then collected by centrifuging at 2800 RPM at 4°C and washed twice with 5 ml cold 10 mM $MgCl_2$ .The pellet was then resuspended in 5 ml cold 10 mM $MgCl_2$ to give an absorbance at 600 nm of 0.2-0.3.

***Agrobacterium* injection**

Suspensions of preinfiltrated *Agrobaterium* in a blunt-tipped plastic syringe were forced into intact leaves still attached to the plant. A sterile needle tip was used to wound the lower surface of the leaf prior injection and by pressing the tip of the syringe against the surface of the leaf, an *Agrobacterium* suspension was forced against the wounded area of the leaf (Figure 1). After being agroinfiltrated, plants were kept in the lab at room temperature conditions. Any possible effect that can be observed from the phenotype of the plant will then be subsequent for plant DNA isolation. Confirmation of DNA integration can be done by performing polymerase chain (PCR) reaction or Northern Blot assay.

Nicotiana benthamiana and *Nicotiana tabacum* (tobacco) are preferably used as favourite model plants. Especially for a short life cycle,

carries relatively large and easily infiltratable leaves that produce recombinant proteins at high levels and the leaf does not show necrosis upon infiltration with most *Agrobacterium* strains. In this experiment I used the green fluorescent protein gene (*GFP*)-transgenic *Nicotiana tabacum* plants, elaborated in Plant Molecular Biology Laboratory of the Institute for Molecular Biology and Biotechnology (IMBB-FORTH, Greece). The *GFP* is expressed temporary over 2-5 days, in the infiltrated area and *GFP* is visualized as bright green fluorescence under UV light. Following syringe infiltration, inducing ERI-1 gene, either alone or in combination, it is shown that can be expressed together in a single leaf (Figure 2).

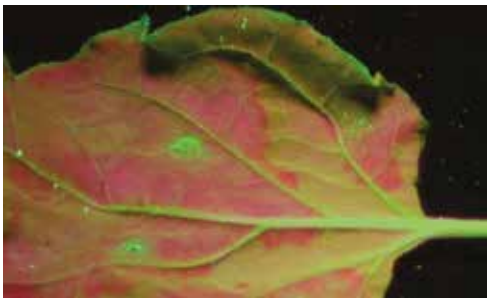

Figure 2. Inducing green fluorescent protein gene (*GFP*) and ERI-1 gene expression, in the infiltrated leaf area of transgenic *tabbaco* plant. *GFP* is visualized as bright green fluorescence under UV light

CONCLUSIONS

Most of the pART27 ERI-1 insert have been identified based on their ability to interfere with the local and systemic silencing of the green fluorescent protein gene (*GFP*) in GFP-transgenic *Nicotiana tabacum* plants in an agroinfiltration assay. The *GFP* is expressed temporary over 2-5 days, in the infiltrated area and *GFP* is visualized as bright green fluorescence under UV light. Following syringe infiltration, inducing ERI-1 gene, either alone or in combination, it is shown that can be expressed together in a single leaf. This assay has the potential to identify ERI-1 gene expression, that affect the production of an autonomous replicating gene that can spread systemically, through a plant and subjected to several analyses as polimerase chain reaction (PCR) for identification.

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