Research Article

Establishing *Agrobacterium* mediated transient gene expression assay in *Nicotiana tabacum*

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Citation

Muhammad Rafique Rind, Aneela Yasmin, Muharam Ali and Saboochi Raza. Establishing *Agrobacterium* based transient gene expression assay in *Nicotiana tabacum*. Pure and Applied Biology. Vol. 7, Issue 4, pp 1286-1293.
<http://dx.doi.org/10.19045/bspab.2018.700149>

Received: 25/05/2018

Revised: 03/09/2018

Accepted: 03/09/2018

Online First: 06/09/2018

Abstract

Preliminary functional characterization of a gene within a short time period is only possible through transient gene expression assays. Transient gene expression assays are simple, cost effective and easy to perform as compared to generation of stable transformants. Here we report an Agro-infiltration based transient gene expression assay, optimized in *Nicotiana tabacum* using β -glucuronidase (GUS) intron gene as an indicator to evaluate various factors effecting its expression. The factors evaluated include bacterial concentration and its growth mode, infiltration medium, concentration of acetosyringone and surfactant, co-cultivation time of *Agrobacterium* and host cells and temperature during co-cultivation. When *Agrobacteria* were grown in/ on YEP liquid and solid media, both mode of growth did not affect the transformation efficiency of bacteria and it was recorded 100% in both cases. However, the transient gene expression efficiency found optimal when fully expended true leaves of tobacco infiltrated with *Agrobacterium* strain EHA-101 carrying GUS intron marker gene in 0.3% glucose medium with an optical density (OD₆₀₀) of 0.3 without acetosyringone and surfactant. After infiltration samples were stored at 22°C for two days and evaluations were carried out after histochemical staining of agro-infiltrated discs by visual inspection. This optimized protocol gave the highest level of GUS expression- 3 in 66.7% of samples, followed by 33.0% (GUS expression level – 2) and 0% (GUS expression level – 1) of samples. The simplicity of this method suggests its utility for the production of pharmaceutical proteins in native plants of Pakistan and can help to promote biotechnology awareness at schools and colleges.

Keywords: *Agrobacterium* mediated transient expression; β -glucuronidase; *Nicotiana tabacum*

Introduction

Genetic transformation is a powerful tool to introduce new traits in elite germplasm and functionally characterize new genes. During this process usually exotic altered DNA is

introduced into a eukaryotic or prokaryotic cell followed by assessing the function and effect of its products on cellular level or on organism level [1]. Nevertheless, isolating a gene then transporting it into a cell without

destroying the cell is also a challenge for molecular scientists. The delivery of gene of interest (GOI) in a cell can be mediated by biolistic gun, microinjection, agrobacterium, electroporation, silicon carbide fiber, liposome, calcium phosphate co-precipitation, polybrene spermidine treatment etc. [2, 3]. Depending on the objective genetic transformation could be stable or transient. Stable transgenic are generated by introducing GOI into the cells that need to be integrated into the genome of the host. In reality only a small portion of targeted cells get GOI integrated in their genomes. The resultant stable transformants should be having GOI as a part of its genome that can replicate and transfer the expressing GOI to offspring [4]. Although stable transgenic express the gene of interest stably, it requires long term screening, laborious analysis and vigilant handling of potential transgenic for months. The expression variability is common in stable transgenic lines due to the copy number, silencing or insertion location of GOI in the host genome [5]. In addition to GOI a reporter gene is fused with it to know if the cells/tissues are transformed or not [6]. Thus, reporter genes report the presence of GOI in the transformants.

In contrast, ectopic transient gene expression can be used to study the function of GOI in short time, within days [7]. This is due to the expression of various copies of non-integrated GOI present in the host nucleus for first several days after the delivery of GOI into the host cells [8]. As these non-integrated copies of GOI cannot replicate, these will be lost with time not due to the degradation of non-integrated T-DNA copies but also as silencing process. It is demonstrated by infiltration of T-DNA carrying a GOI and a viral silencing suppressor gene that extended transient expression for as many as seven days post infiltration [9, 10]. According to [5], the

transient expression could be thousand times more powerful than in stable transgenics. There are different methodologies available for ectopic gene expression studies in plants namely electroporation or, polyethylene glycol (PEG) based transformation of protoplasts, biolistics and agro-infiltration [2, 3]. Among these methods electroporation or, polyethylene glycol (PEG) based transformation of protoplasts are optimized for few plant species [11]. In this regard, initial preparations like generating protoplasts is also time consuming whereas biolistic machines and consumables are expensive. However, agro-infiltration is a less expensive, less laborious and an ideal method for many plant species [4]. Transient gene expression has been utilized for a wide range of applications like demonstrating promoter activity, function of newly identified genes and proteins, evaluating virulence capacity of wild strains of *agrobacterium*, protein interactions in vivo and mass production of pharmaceuticals [12-15].

Agroinfiltration is a simple and popular tool to effectively study the transgenes' expression into a heterologous system like in tobacco. However, despite its popularity, there is little work done on the improvement of its efficiency to further increase the level of gene expression or protein yields. Keeping in view the usability and simplicity of transient gene expression we report here an efficient, reproducible, and relatively simple methodology for transient gene expression of GUS gene in a locally cultivated plant species of tobacco, namely *Nicotiana tabacum*. This optimized assay can be utilized for ectopic expression studies of newly identified genes. Further implications include genetic complementation, expression of resistance genes, RNAi experimentations, Protein trafficking and production. To our knowledge this is the first report on utilization of *Nicotiana tabacum* based

transient gene expression assay in Sindh, Pakistan.

Materials and methods

Tobacco, (*Nicotiana tabacum*) plants were grown for 6-weeks and counting from the upper side of plant 1-4 fully expended true leaves were used in present study. *Agrobacterium* strain used in present study was EHA 101 (pIG121-Hm) carrying β -glucuronidase (GUS) gene.

Bacteria were grown and used for agro-infiltration as described by [7, 8], with minor modifications. Sterile YEP (10 g L⁻¹ yeast extract, 5 g L⁻¹ sodium chloride, 10 g L⁻¹ peptone, pH 7.5, agar 1.5%) solid and liquid media were used to grow bacteria. Liquid YEP media was inoculated by a single colony of bacteria and were allowed to grow until bacterial density reached the OD (600nm) 1.0-1.5 at 180 rpm. Then the bacteria were harvested by centrifugation at 4500 rpm for 10 min, washed in distilled sterile water at RT and bacterial suspensions were prepared for infiltration, accordingly. In addition bacteria were grown on YEP agar plates until complete bacteria lawn is formed on plate then these bacteria were removed from plates, washed in distilled sterile water at RT, centrifuged at 4500 rpm for 10 min and bacterial suspensions were prepared for infiltration, accordingly. All leaves were infiltrated by a 1 ml needle without syringe and samples were kept in dark at 22°C. Histochemical staining was performed as described by [16]. Leaf discs were merged in staining solution and samples were incubated at 37°C in dark, overnight. Next day discs were washed in 75% ethanol to remove the green chlorophyll.

The parameters studied to optimize GUS gene expression in tobacco were bacterial growth mode (solid and liquid), bacterial density (OD₆₀₀ 0.0, 0.3, 0.5, 1.0), infiltration medium (0, 0.3%, or 0.5% glucose medium), the effect of acetosyringone (0, 100 μ M), surfactant (Tween 20- 0.0, 0.01%) and co-

cultivation time of *Agrobacterium* and host cells (0, 2, 5- days). Each experiment was conducted as randomized complete block design with three replications each containing at least 10 leaf discs. The effect of different parameters was recorded in the form of percent transformation efficiency (GUS expression showing discs/total discs inoculated * 100) and level of gene expression. Level of gene expression was rated by visual inspection of inoculated discs after staining and rating for observed blue colour (GUS expression) on a scale mentioned in (Figure 1).

Results and discussion

An Agro-infiltration based transient gene expression assay was optimized in model plant *Nicotiana tabacum*. *Agrobacterium* strain EHA101 was harboring β -glucuronidase (GUS) intron gene that was used as an indicator to evaluate various factors effecting its expression. Among evaluated factors first bacterial concentration, infiltration medium, and co-cultivation time was optimized. Then holding these factors constant effects of bacterial growth mode, surfactant and acetosyringone on GUS expression were evaluated.

Density of bacterial suspension is an important factor to show gene expression [7]. All the tested bacterial concentrations (OD₆₀₀ 0.3, 0.5, 1.0) displayed 100% transformation efficiency however the level of expression was variable (Table 1, Figure 3A). It is interesting to notify that the bacterial densities OD₆₀₀ 0.3 showed the highest level of GUS expression that is 3 in 66.7% (Fig.3A) of samples followed by OD₆₀₀ 0.5 (23.3%; Figure 3A) and OD₆₀₀ 1.0 (10.0%; Figure 3A) thus we finalize OD₆₀₀ 0.3 as optimal concentration of bacteria for GUS expression in tobacco. It is due to the fact that *Agrobacterium* is a plant pathogen that transfers its T-DNA to the host genome and induces plant defenses in *Arabidopsis* [17, 18], grapevine [6], suppresses plant defenses

in tobacco cell cultures [19]. In present study as the bacterial concentration was increased to OD₆₀₀ 1.0 the texture of leaf was effected negatively and wilting was observed furthermore the expression level was also affected negatively (Figure 3A). The observed levels of GUS expression i.e. GUS 1, 2 and 3 at OD₆₀₀ 1.0 were 40%, 50% and 10%, respectively. In addition to bacterial strain, bacterial concentration or density can also affect the infection process and thus the transformation efficiencies. Low amount of bacteria in a suspension will result in low bacteria and host cell ratio and low transformation frequencies whereas high bacterial concentration will damage the tissues and revealing low transformation frequencies [20].

Previously it was shown that different infiltration media [21], effect the expression positively. In present study we also observed the positive effect of glucose as infiltration media (Figure 3B). Glucose at the concentration of 0.3% improved the expression level of all tobacco discs from 2 to 3 and none of the evaluated discs revealed 1st level of GUS expression. Thus infiltration medium was found critical for improving gene expression in present study. Although the transformation efficiency was 100% when bacterial suspension was prepared in distilled sterile water also, the highest GUS expression level was recorded as 01 (Figure 3A; 50%) that was increased to 3 (Figure 3A; 66%) when 0.3% glucose solution was used as infiltration medium. As the concentration of glucose in infiltration medium was increased to 0.5% the highest expression level 3 is also decreased to 50%. However, there are reports demonstrating no effect of infiltration media on the gene expression but

these finding are in different plants like rose [7], *N. benthamiana*, *Arabidopsis*, tomato and lettuce [4]. Moreover addition of acetosyringone [8].or surfactants [22] is also reported to improve the transient gene expression significantly. Acetosyringone is added to infiltration media to induce virulence genes of *Agrobacterium* [23] whereas; surfactants facilitate the infiltration of bacterial suspension into the host tissues [22]. In the present study neither acetosyringone (100µM) nor surfactant (Tween-20; concentration 0.01%) improved the transient GUS gene expression in *N. tabacum* significantly (Figure 3C, 3D). Same observations recorded in *Arabidopsis* and lettuce [4]. Agroinfiltration is a tool to remarkably facilitate the studies of functional characterization of newly identified genes meanwhile *Agrobacterium* is actually a plant pathogen and elicits immune responses in host also [24].

When *Agrobacteria* were grown in/ on YEP liquid and solid media, both mode of growth did not affect the transformation efficiency of bacterial that was 100% in both cases and the highest GUS expression level was found as 3 on visual scale (Figure 3E; 66.7%). This finding will provide scientists, a choice as that they can use any medium for the growth of bacteria for agro-infiltration studies [25]. Nonetheless, the co-cultivation of host and *Agrobacterium* was evaluated on day 0, 2 and 5. The day 0 was the same day of infiltration. Within first hour of infiltration of *Agrobacterium* suspension in the host did not revealed any gene expression however, gene expression level was found non-significantly different when staining was carried out after day 2 or day 5 (Figure 3F).

Figure 1. A visual scale to categorize different levels of GUS expression in tobacco

Bacterial Density	OD ₆₀₀ = 0.0	OD ₆₀₀ = 0.3	OD ₆₀₀ = 0.5
Infiltration Medium			
0.0%			
0.3%			
0.5%			

Figure 2. Effect of different parameters on transient GUS expression

Table 1. Percent transformation efficiency of GUS infiltrated tobacco discs

100% transformation efficiency	0% transformation efficiency
Bacterial density (O.D ₆₀₀ -0.3, 0.5, 1.0) Infiltration medium (0, 0.3, 0.5%) Co-cultivation time of host and bacteria (2, 5 days) Surfactant (Tween-20: 0, 0.01%) Acetosyringone (0, 100μM) Bacterial growth mode (solid, liquid)	Bacterial density (O.D ₆₀₀ -0) Co-cultivation time of host and bacteria (same days post infiltration)

Note: Percentage represents three replications each containing at least 10 leaf discs

Figure 3. X-Axis and Y-Axis here represent percentage of tobacco discs revealed GUS expression and different levels of GUS expression based on a visual scale provided in Fig. 1, respectively. All graphs reveal the effect of different parameters on the level of GUS expression; A: effect of bacterial concentration; B: Effect of different concentrations of glucose as infiltration medium; C: Effect of acetosyringone; D: Effect of surfactant; E: Effect of bacterial growth mode; E: Effect of bacteria and host co-cultivation period

Conclusion

In present study the transient GUS gene expression was optimized. The optimal GUS expression was detected, when bacterial concentration maintained as 0.3 OD₆₀₀ in 0.3% glucose solution without additives like acetosyringone and surfactant in *N. tabacum*. The samples were evaluated after 2-days of infiltration that resulted the highest level of GUS expression level – 3 in 66.7% of samples, followed by 33.0% (GUS expression level – 2) and 0% (GUS expression level – 1) of samples. This syringe based agro-infiltration method is a simple, less expensive and efficient method for transient expression of genes and its protein that can revolutionize the production of pharmaceutical proteins in a local plant adapted to our environmental conditions. Additionally, the simplicity of this method also suggests its utility to promote biotechnology education at schools and colleges.

Authors' contributions

Conceived and designed the experiments: A Yasmin; Performed the experiments: MR Rind; Analyzed the data: A Yasmin; Wrote the paper: MR Rind & A Yasmin.

Acknowledgments

The authors are thankful to Higher Education Commission of Pakistan (HEC) for the award of indigenous scholarship to M. Rafique for Ph.D.

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