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A Simple Method for Transient Expression of Reporter Gene in Brinjal Leaves through Agroinfiltration

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A B S T R A C T

Brinjal (*Solanum melongena* L.) is one of the most important vegetable crops in India. In spite of the docility of brinjal towards genetic transformation through *Agrobacterium*, development of stable transgenic lines in brinjal is a cost, labour as well as time consuming process. On the other hand, the agro infiltration process has been optimized in different crops for the rapid analysis of transgene through transient expression. The process has also gained importance in synthetic biological applications for rapid production of therapeutic biomolecules in plants. Here, we have evaluated the agroinfiltration process in brinjal leaves through transient expression of a reporter gene driven by a constitutive promoter. Following optimization of the incubation period, proper infiltration medium and the optical density of the *Agrobacterium* suspension, we advocate the usefulness of the process for gene function analysis and other possible downstream applications through transient expression of a transgene in brinjal.

Introduction

The soil-borne bacterium, *Agrobacterium tumefaciens* is called the natural genetic engineer, because it has the capacity to transfer and integrate a part of its tumour inducing (*Ti*) plasmid DNA (the *transfer DNA* or *T-DNA*) into the plant genome. Through exploring this natural system, plant genetic engineers place their gene of interest in the engineered *Ti* plasmid and deliver the gene of interest through *Agrobacterium*. The robustness of the system has allowed the generation of numerous transgenic plants in a huge number of crop plants. However, *Agrobacterium*-mediated plant transformation for obtaining stable transgenic line is a time-consuming process and depends on several factors. First of all, an efficient *in vitro* plant regeneration system (through tissue culture) for the target plant species appears as a prerequisite for *Agrobacterium*-mediated plant transformation. Most importantly, the site of integration of the transgene delivered by *Agrobacterium* is random in nature. Naturally, effect of the transgene in the transgenic plant is highly influenced by this 'position effect'. For this reason, a tissue-culture independent system for initial evaluation of the transgene effect becomes very much pertinent in this regard.

The interaction between *Agrobacterium* and plant cell involves the transfer of the single

stranded *T-DNA* containing the transgene, into the plant cell nucleus, where, integration of this transgene into the plant genome is the ultimate requirement for achieving a stable transgenic line.

Interestingly, the delivered but non-integrated transgene molecules remain transiently present in the nucleus. These molecules are capable of being transcribed and further processed to express the transgene in a transient manner (Kapila *et al.,* 1997). More interestingly, expression given by these non-integrated transgene molecules (i.e., the transient expression) is often ~1000 fold higher than the stable expression of the integrated transgene (Janssen and Gardner, 1989). Apart from this quantitative advantage, the transient expression system provides several other advantages, including, less requirement of time to analyse the effect of the transgene and freedom from the biased position effect (Kapila *et al.,* 1997).

The transient expression system has been mostly used *in planta* through the process of syringe agroinfiltration, where a needleless syringe is used to deliver the *Agrobacterium* suspension through the lower surface of leaves in the intercellular spaces of the leaf. This method has been optimized in different crop species (Wroblewski *et al.,* 2005)and has been successfully explored for diverse objectives, which include the study of gene silencing (Schöb *et al.,* 1997; Johansen and Carrington, 2001), plant promoter analysis (Yang *et al.,* 2000), plant *R* and *Avr* gene interactions (Van der Hoorn *et al.,* 2000), recombinant protein production (Joh *et al.,* 2005), rapid production of therapeutic agents in plant systems (Huang *et al.,* 2006; Chen *et al.,* 2013), gene function analysis (Hoffmann *et al.,* 2006; Santos-Rosa *et al.,* 2008), analysis of transcription factors (Berger *et al.,* 2007), plant-based synthetic biology applications (Sainsbury and Lomonossoff, 2014) and many more.

In this study, we have analysed the utility of the agroinfiltration process in brinjal (*Solanum melongena* L.), one of the most important vegetable crops in India. Through *Agrobacterium*-mediated delivery of the *β-glucuronidase* (*uidA/gusA*) reporter gene under the transcriptional regulation of a constitutive promoter, we have analyzed the optimum incubation time, suitable infiltration medium and optimum bacterial concentration in order to achieve sufficient transient expression of transgene through agroinfiltration in brinjal leaves.

Materials and Methods

Plant material

Fully expanded leaves of 28–35 days' old brinjal (cv. Pant Rituraj) seedlings were used for agroinfiltration in the present study.

Plasmid and bacterial strain

The *pCAM: 2X-gusA* recombinant plasmid (Chattopadhyay *et al.,* 2011) was used in the present study. This recombinant plasmid is derived from the pCAMBIA1391z promoter-probe vector, which contains the *β-glucuronidase* (*uidA/gusA*) reporter gene downstream of the multiple cloning site (MCS). The engineered *uidA*/*gusA* gene of this vector contains a catalase intron, which ensures that reporter gene expression is seen only after eukaryotic processing and not from the prevailing bacterial cells, if any. The *pCAM:2X-gusA* recombinant plasmid contains the enhanced (*2X*) cauliflower mosaic virus (CaMV) 35S promoter, cloned in MCS region of the plasmid to drive the constitutive expression of the *uidA*/*gusA* reporter gene. This recombinant plasmid was mobilized into chemically competent *Agrobacterium tumefaciens* strain LBA 4404/*virG*N54D (Fits *et al.,* 2000) cells. The positive *Agrobacterium* clones harbouring the

recombinant plasmid were selected on Luria-Bertani (LB) agar plates containing 20 mgl^{-1} rifampicin, 75 mgl^{-1} chloramphenicol and 50 mgl^{-1} kanamycin antibiotics.

Agroinfiltration procedure

Loop-full culture of *Agrobacterium* clone harbouring the recombinant plasmid (*pCAM:2X-gusA*) was inoculated in 3 ml of LB medium, containing 20 mgl^{-1} rifampicin, 75 mgl^{-1} chloramphenicol and 50 mgl^{-1} kanamycin antibiotics and allowed for overnight growth in a shaker at 28 °C. Next day, 100 µl of the saturated *Agrobacterium* culture was again inoculated in 5 ml of LB media, containing 20 mgl^{-1} rifampicin, 75 mgl^{-1} chloramphenicol and 50 mgl^{-1} kanamycin antibiotics and allowed for overnight growth in a shaker at 28°C. The optical density at 600 nm wavelength (OD_{600}) of this culture was checked through spectrophotometry, and the cells of the culture were precipitated through centrifugation (6000 RPM for 5 min) at room temperature. The harvested cell pellet was resuspended in appropriate volume of infiltration medium [i.e., sterile water or 5% (w/v) sucrose or ½ strength Murashige and Skoog (MS) liquid medium (pH 5.2) or ½ strength MS liquid medium (pH 5.2) containing 20 mM $CaCl_2$] to achieve different OD_{600} values of this final suspension.

The final *Agrobacterium* suspension was used for agroinfiltration of the brinjal leaves. For this purpose, the suspension was infiltrated in the lower surface of the brinjal leaves through a needle-less 2 ml syringe. Infiltration of the *Agrobacterium* suspension was clearly indicated by dark green colouration of the infiltrated leaf part.

Histochemical assay

Histochemical assay for β-glucuronidase (GUS) activity was carried out as per standard method (Jefferson *et al.,* 1987). Leaf discs from the infiltrated parts were punched through a punching machine and collected in 0.5 ml microcentrifuge tubes. The leaf discs were submerged in the assay buffer [50 mM sodium phosphate buffer, pH 7.2, 0.2% (v/v) triton X-100, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide and 2 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc)] and kept at 37 °C incubator. After appearance of the characteristic blue colour conferred by the reporter gene expression, the leaf discs were dechlorophyllized in 70% ethanol and imaged.

Results and Discussion

Effect of incubation period

Transient expression of the *β-glucuronidase* (*uidA/gusA*) reporter gene was first analyzed after different time intervals from the agroinfiltration process to optimize the proper incubation period. The transient expression of the reporter gene, as revealed by histochemical assay of the agroinfiltrated leaf parts, was found to be the maximum after 3 days of infiltration (Fig. 1). The expression was observed till 5 days after agroinfiltration, which ultimately reduced to become almost invisible after 7 days of agroinfiltration. This result is quite expected, as, in most of the cases, generally, the transient expression of transgene has been reported to be evaluated 4-5 days after infiltration (Wroblewski *et al.,* 2005).

Effect of different infiltration medium

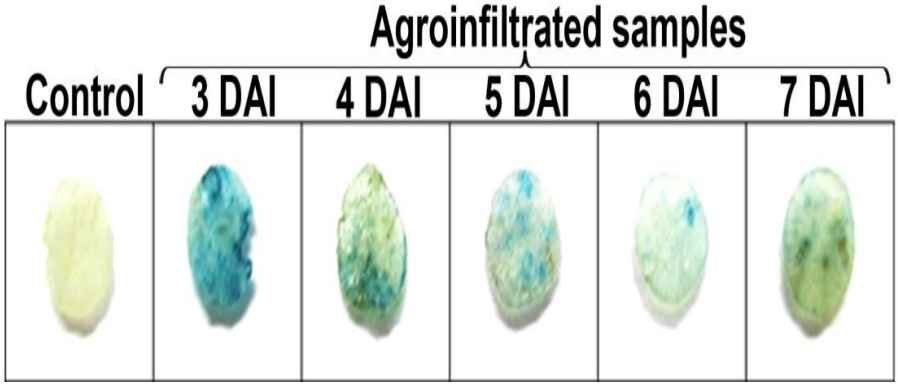
Selection of a suitable infiltration medium has been documented to be an important parameter for gene delivery in plant systems (McIntosh *et al.,* 2004; Tague and Mantis, 2006; Du *et al.,* 2010). Hence, in the present study, the effect of different media used for agroinfiltration was further analyzed through histochemical assay of the agroinfiltrated leaf

parts after 3 days of infiltration. It was found that even sterile water, when used as the infiltration medium, was sufficient to produce transient gene expression. However, 5% (w/v) sucrose solution as well as ½ strength MS liquid medium might be considered better, if uniformity of transient expression is considered (Fig. 2). Use of 5% (w/v) solution has also been found to be suitable for transient gene expression assay in *Anthurium* (Hosein *et al.,* 2012). Supplementation of 20 mM $CaCl_2$ in ½ strength MS medium did not improve the level of transient expression.

Effect of optical density of the Agrobacterium suspension

The concentration of the *Agrobacterium* suspension used for gene delivery has been regarded as a significant factor in plant transformation, as reported earlier (Santos-Rosa *et al.,* 2008; Kim *et al.,* 2009; Fitch *et al.,* 2011). So, the effect of the final optical density (OD_{600}) of the *Agrobacterium* suspension in 5% (w/v) sucrose solution used for agroinfiltration was further analyzed in a similar manner after 3 days of agroinfiltration. For this purpose, different *Agrobacterium* suspensions with a wide range of OD_{600} values (i.e., 0.2 to 3.2) were used and transient expression of the reporter gene was evaluated. It was observed that, even the *Agrobacterium* suspension with OD_{600} ~ 0.2 was sufficient for transient expression of the reporter gene. However, better uniform expression of reporter gene was recorded when *Agrobacterium* suspension with OD_{600} ~ 1.6 was used (Fig. 3). This result is in corroboration with previous similar studies in *Anthurium* (Hosein *et al.,* 2012) and tomato (Gao *et al.,* 2009), where *Agrobacterium* suspension with OD_{600} ~ 1.5 has been found to be most efficient. The *Agrobacterium* suspension with OD_{600}> 1.6 was not found to further improve the transient expression of the reporter gene, as observed in the present study.

Fig.1 Histochemical assay for transient expression of *gusA* reporter gene in non-infiltrated (control) leaf disc and agroinfiltrated leaf discs of brinjal after different incubation periods



DAI = days after infiltration

Fig.2 Histochemical assay for transient expression of *gusA* reporter gene in agroinfiltrated leaf discs of brinjal, where different infiltration medium was used. S1, S2, S3 = three independent leaf discs from different agroinfiltrated zones of the leaves

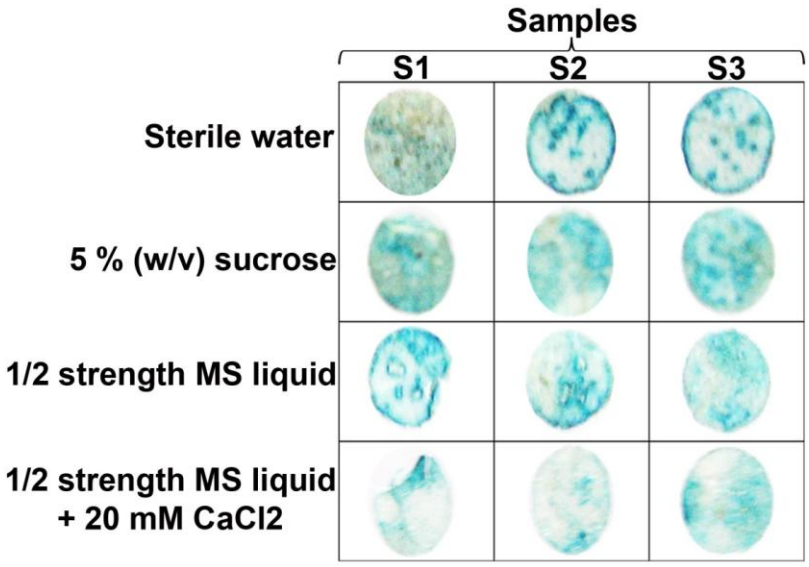
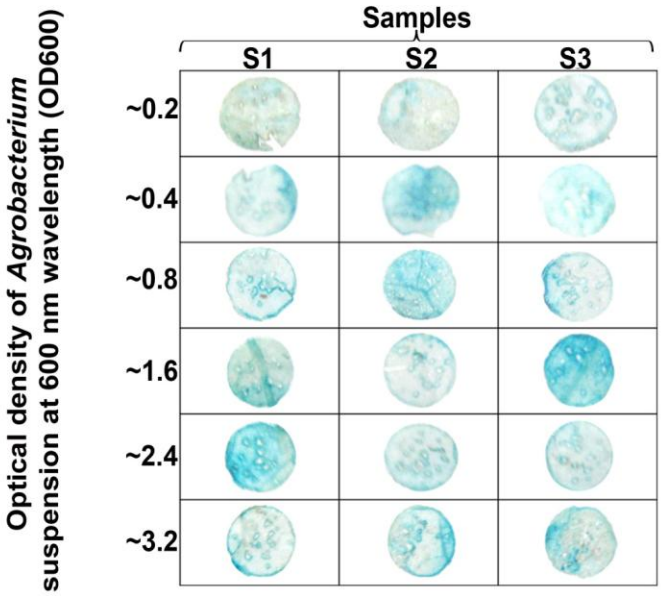


Fig.3 Histochemical assay for transient expression of *gusA* reporter gene in agroinfiltrated leaf discs of brinjal, where Agrobacterium suspension of different concentrations (i.e., with different OD_{600} values) were used. S1, S2, S3 = three independent leaf discs from different agroinfiltrated zones of the leaves



In this study, we have reported a simple method that allows transient expression of a transgene in brinjal leaves, following agroinfiltration. Being an important vegetable crop, brinjal demands attention of the researchers involved in the field of plant molecular biology. The method reported here should be rendered very much useful for rapid functional analysis of

different genes of brinjal and may indicate the possibility of heterologous gene expression in brinjal system. For example, the efficacy of an uncharacterized gene towards insect resistance in brinjal might be analyzed through agroinfiltration (as described in this study) of a suitable gene construct followed by insect bio-assay with the infiltrated leaf part. It is important to mention here that, agroinfiltration in tomato has been reported to induce tissue necrosis (Van der Hoorn *et al.,* 2000) and a similar type of necrotic response has been predicted for other solanaceous crops (Wroblewski *et al.,* 2005). However, in the present study, no significant tissue necrosis following agroinfiltration was noticed. Hence, we advocate the agroinfiltration method, reported in this study, to be adopted for analysis of transient gene expression in brinjal plant system. Furthermore, owing to the simplicity and robustness of the agroinfiltration technique, the method might be evaluated for other possible downstream applications through transient expression of a transgene in brinjal.

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