

Relative efficiencies of transient transformation of *Arabidopsis thaliana* and *Nicotiana tabacum* upon heterologous expression of membrane proteins

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A cyanobacterial gene encoding a protein with multiple transmembrane helices, when transformed into *Arabidopsis thaliana* and *Nicotiana tabacum*, demonstrated several differences in the relative responses of both the host species, particularly in efficiencies of regeneration of transformed tissue from leaf explants. The gene of interest (GOI) encoded an integral membrane protein containing 12 transmembrane helices and functioned as a sodium dependent inorganic ion transporter in a native cyanobacterium. *Agrobacterium tumefaciens*-mediated transformation was carried out by coculture method. The leaves of *N. tabacum* successfully regenerated into transformed callus tissue and gave positive results upon GUS assay; while the *A. thaliana* leaves did not give any transformed callus and, a few days post agro-infection, all the explants displayed tissue necrosis. The results obtained point towards *A. thaliana*'s recalcitrance to *Agrobacterium*-mediated transformation by coculture method and the difficulty of expressing hydrophobic membrane proteins in heterologous systems.

Keywords: *Agrobacterium tumefaciens*, *Arabidopsis thaliana*, hydrophobic membrane protein, *Nicotiana tabacum*, transformation efficiency

Introduction

Transient expression studies provide a base for analyzing success rate of transgene expression in a heterologous host system. *Arabidopsis thaliana* and a few *Nicotiana* species (*N. tabacum*, *N. benthamiana* & *N. attenuata*) are widely used by researchers as model host plants to study protein expression following various transformation methods. *A. thaliana*, in terms of its competency with different transformation methods, has been well studied using a variety of explants and its performance has been investigated in various transient transformation experiments, such as, young seedlings transformed by vacuum infiltration¹, leaves transformed by agroinfiltration² and coculture³ method. However, nowadays, these methods are rarely used due to the poor demonstration of *A. thaliana* plants in transient expression studies. Instead, *A. thaliana* is now most widely used to develop stable plants by floral dip method⁴, which has proved to be a highly successful and efficient transformation method. Several *Nicotiana* species, on the other hand, are relatively easy to transform by *Agrobacterium*-mediated coculture, agroinfiltration and particle bombardment methods, and are found to be effective and resilient for both transient as well as stable protein expression experiments⁵.

The biochemical nature of proteins also influences their expression in heterologous systems. Of various categories, the membrane proteins being of hydrophobic nature offer the greatest challenge. The membrane proteins play various important roles in the living cells and function as transporters, receptors, enzymes etc. In most organisms, they constitute about 20-30% to the total protein. High-level expression of membrane proteins remains a challenge to this day because of their low natural abundance and tendency to form aggregates⁶. These features also make them difficult targets for structural characterization. For optimized expression of hydrophobic proteins, various host systems are tested but it is still a major challenge. Possible reasons behind the lack of success can be that these proteins may have some lethal effects on the host or have undergone alterations at the molecular level upon expression. In addition, the host machinery may or may not allow proper folding and activation of the protein.

Researchers have been using genes from different organisms and have tried their expression in various bacterial, plant and other eukaryotic heterologous host systems but the success rate is always variable with respect to both the type and complexity of host and protein⁷. Specifically, in case of heterologous systems, we need to optimize all the parameters related to host growth and transformation according to the nature of the protein. Apart from the discussed aspects so far,

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few more crucial points in protein expression studies are there, which include selection of proficient transformation techniques and expression vectors. Not all the methods and vectors available are suitable for all the proteins and host types.

The present study involves the transformation of a hydrophobic membrane protein of cyanobacterial origin containing 12 transmembrane helices into *A. thaliana* and *N. tabacum* by *Agrobacterium*-mediated coculture method and analysis of the efficacy of the transformation protocol by histochemical GUS reporter gene assay. The protein under study, being huge in size and hydrophobic in nature, is difficult to express and hence we were interested in knowing the relative response of both the host plant species to this protein.

Materials and Methods

The transformation of plants were carried out by *Agrobacterium*-mediated coculture method⁸. The detailed methodology followed is given below.

Growth Conditions

A. thaliana plants were grown in the commercially purchased soilrite mixture in a plant growth chamber running at 22°C, while *N. tabacum* plants were germinated and maintained in a mixture of vermiculite, clay and sandy soil at 25-28°C temperature in a plant tissue culture room. Both the plants provided with 16 h light and 8 h dark period.

Preparation of Genetic Constructs

Genetic constructs were prepared by fusing appropriate targeting sequence upstream of the cyanobacterial gene in plant expression vector pRI 101-AN containing *gus* reporter gene.

Transformation of *Agrobacterium tumefaciens* Strain GV3101

Chimeric pRI constructs harboring gene of interest were transformed into chemically competent *Agrobacterium* GV3101 host cells. The transformed *Agrobacterium* colonies were grown into a primary culture and then into a secondary culture until OD reached 0.6-0.8 at 600 nm wavelength.

Plant Transformation

Once the constructs and plants were prepared, the plant leaves were excised and cut into small pieces. These leaf explants were inoculated with *Agrobacterium* culture and incubated for 10-20 min. Then the infected explants were co-cultivated on MS media [2,4-D (4 mg/L)] for 2 d in dark at appropriate temperature.

Regeneration of Transformed Explants

The co-cultivated explants were washed with cefotaxime solution (500 mg/L) to kill excess *Agrobacterium* and further transferred to callus inducing regeneration media [2,4-D (4 mg/L) + kanamycin (50 mg/L) + cefotaxime (250 mg/L)] for growth and selection of transformants.

Analysis of Transformants

The successfully transformed explants survived on antibiotic containing medium and developed into a callus, which was further analysed by GUS histochemical assay⁹ along with a negative control sample. The assay was performed by incubating some pieces of callus into GUS staining solution [2 M X-gluc, 20 mM sodium phosphate buffer pH 7.0, 1 mM K₃Fe(CN)₆, 0.1% Triton-X, 10 mM EDTA pH 8.0] at 37°C for 1-24 h. In this reaction, a chromogenic substrate was used, which turns blue after oxidation by GUS enzyme.

Results and Discussion

Both the plant species upon transformation with cyanobacterial membrane protein showed a remarkably contrasting response in terms of tissue survival and regeneration. After transformation with *Agrobacterium*, the leaves were incubated on regeneration medium for callus induction (Fig. 1) and transformed callus was subsequently used to perform GUS assay along with the control callus generated from non-transformed wild type plant leaves. The assay gave positive results only for the transformed callus as evidenced by the appearance of blue color (Figs 2A & B); only in case of *N. tabacum*. Stained callus tissues along with control callus were analyzed under compound microscope (Figs 2A-C). The results of GUS assay clearly indicate the successful transformation of cyanobacterial gene into *N. tabacum* plant. However, in case of *A. thaliana* plant, all the transformed explants started dying and decolorizing (Fig. 1C), which was followed by tissue darkening (Fig. 1D). In few of the explants, callus had initiated at the edges but those too could not grow further and ultimately turned into dead tissue. Any attempt to resolve this issue by carrying out frequent sub-culture did not make any significant difference in the tissue survival rate. Hence, due to unavailability of callus tissue, it was not possible to carry out GUS histochemical assay for this host species. This observation is in concurrence with earlier reports, where huge success has been obtained in transforming *N. tabacum* with this method, while only a few reports

are there for *A. thaliana*³. A summary of the experiments performed and the results obtained is provided in Table 1.

In consideration of the results obtained, it can be

said that the usage of *A. thaliana* leaves for transient studies is quite challenging. Usually, the explants are too fragile to cope up with the stress of *Agro*-infection

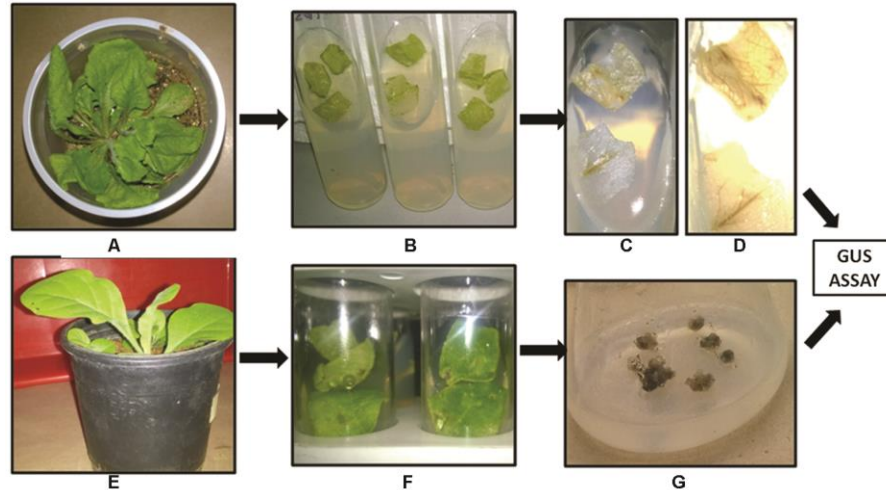


Fig. 1 (A-G) — Transformation of *A. thaliana* (A-D) and *N. tabacum* (E-G) by *Agrobacterium*-mediated coculture method. The fully-grown leaf explants were used for agro-infection followed by regeneration of transformed callus tissue. A. 6-8 wk-old *A. thaliana* plant; B. Agro-infected *A. thaliana* leaves growing on regeneration media; C. & D. 10-15 d post transformation leaves turning into dead tissue; E. 6-8 wk-old *N. tabacum* plant; F. Agro-infected *N. tabacum* leaves growing on regeneration medium; & G. 30 d post transformation leaves regenerating into callus tissues.

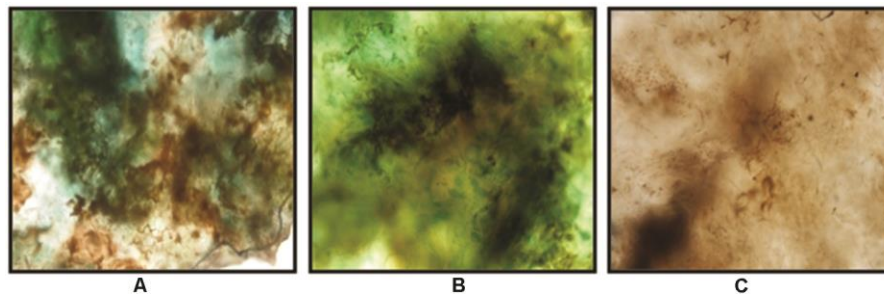


Fig. 2 (A-C) — GUS histochemical assay results were analysed by observing the transformed and non-transformed callus tissue of *N. tabacum* under the compound microscope (Olympus-bright field): A. & B. Callus tissue regenerated from agro-infected leaves; & C. Callus tissue regenerated from non agro-infected wild type leaves (Control).

Table 1 — Comparative analysis of various experimental parameters used while transformation of *A. thaliana* and *N. tabacum* with a heterologous membrane protein

No.	Experimental parameters	<i>A. thaliana</i>	<i>N. tabacum</i>
1	Age of plant used as a source of explants	6-8 wk	6-8 wk
2	Explant used for agro-infection	Leaves	Leaves
3	Duration of agro-infection	10 min	20 min
4	Response of agro-infected explants on regeneration media	Necrosis observed in the majority of explants 10-15 d post agro-infection. Tiny callus tissue obtained in few cases	Majority of explants showed callus initiation 10-15 d post agro-infection
5	Sub culturing of transformed tissue (To improve tissue survival)	Every 7-10 d	Every 12-15 d
6	Regeneration of transformed callus	Fully grown callus could not be obtained	Fully grown callus obtained 30 d post transformation
7	GUS histochemical assay	Could not be performed because no transformed tissue was obtained	Positive GUS assay

and, therefore, the time duration of infection for any given protein and any given plant type has to be standardized individually. The nature of targeted protein might adversely affect the host cells and also the agro-infection process possibly induces plant immune system to get hyperactive, thereby inhibiting further growth of explants. In certain cases, it is also possible that a cell type is capable of transformation but not of regeneration.

For the protein under study, the results obtained indicate the recalcitrance of *A. thaliana* leaves to the coculture method of transformation. This observation is also supported by other reports, which indicate that *A. thaliana* leaves are not suitable for transient expression studies². Certain modifications in the protocol, such as, reducing light intensity prior to transformation, optimizing wounding sites on leaves and replacing cefotaxime with other antibiotics to regenerate better quality of transformed shoots, have been attempted with varying success¹⁰.

The chimeric construct used here comprises of targeting signal, transporter gene and *gus* reporter gene, which along with being large in size is also majorly hydrophobic in nature. These features make it hard to transform and express. Therefore, one has to be extremely critical while selecting a plant expression host, expression vector and reporter gene *etc.* There are reports suggesting that with increasing protein size, the expression becomes unpredictable or there may not be any expression at all⁶. On the other hand, host genome can also contribute to the varied susceptibilities of different plants, which can be explained by the fact that plant genome itself contains genes involved in *Agrobacterium* infection¹¹. This also gives an evolutionary reason as to why some plants get easily transformed by *Agrobacterium*, while others do not and it also supports the argument that *Agrobacterium* virulence is dependent on the host plant¹².

It is important to make a well-informed decision during the choice of host plant species for transformation. Here we find an acute recalcitrance of *A. thaliana* to transient transformation with a heterologous membrane protein, which has not been the case with *N. tabacum*. While the underlying reason might vary from host plant's incompatibility with the transformation protocol to elicitation of stress responses in it, an oft-observed phenomenon must be archived and reported.

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Conflict of Interest

The authors declare no conflict of interest

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