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ORIGINAL PAPER

Genetic Modified Medicinal Plants. I. *Agrobacterium tumefaciens* Mediated Reporter *gus* Gene Transfer and Expression in *Atropa belladonna* Plants

G. NEGOIANU-TENEA, A. CALIN, N. CUCU

University of Bucharest, Institute of Genetics, Transgenesis and Epigenetics Laboratory
1-3 Aleea Portocalelor, sector 6, Bucharest 76258, Romania, e-mail: centragen@elt.ro

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Abstract

An optimized experimental model for genetic modification of Atropa belladonna plants has been established by an Agrobacterium tumefaciens mediated gene transfer technique. The reporter GUS system has been studied into the transformed plant material both at the DNA and protein levels.The PCR analysis confirmed the integration of the gus gene and its expression as β-glucuronidase has been detected by a sensitive enzyme assay on a fluorogenic substrate.

Keywords: *Atropa belladonna, Agrobacterium tumefaciens,* transgenic plant, reporter *gus* gene, β-glucuronidase, PCR-polymerase chain reaction

Introduction

Plants constitute a major source of natural products for the pharmaceutical, agrochemical, cosmetics and food industry interest. In the past few decades, efforts have been made to produce valuable plant metabolites using different biotechnological tools, such as plant "in vitro" culture techniques which enable the product synthesis and accumulation not only in the whole plant, but mainly in plant tissues, organs or undifferentiated cells (ALFERMANN and PETERSEN 1995; YEOMAN and YEOMAN 1996). Genetically modified organisms offer, even in this field, interesting solutions towards the fullfilment of this tasks by improving the biosynthetic rate of the secondary metabolites with economic interest into the genetically transformed "hairy roots" (DANIELL ET AL., 2001).

A worldwide used medicinal plant *Atropa belladonna* (deadly nightshade), a poisonous herbaceous perennial plant species, which is native to Eurasia (ZARATE R, 1999), is a member of the *Solanaceae* family. Its significance lies in the fact that its species are abundant in tropane alkaloids, proved to exhibit antiviral (GRUTERS et al, 1987), antimetastatic (HUMPHRIES et al, 1988), antihyperglycemic and immunostimulatory (MOLYNEUX et al, 1993) activities in pharmaceutical drugs (GOLDMANN etal, 1990; BONHOMME ET AL, 2000).

This paper represents so far the first Romanian approach to establishing the experimental and analytical conditions for an efficient *Agrobacterium* mediated gene transfer into the *A.belladonna* genome. The methods described here represent an early step in the standardisation of the technologies aiming at the genetic transformation and analysis of *Atropa belladonna* plants, in particular, and of medicinal plants, in general.

Materials and Methods

2.1. *Bacterial strains*

The *Agrobacterium tumefaciens* GV 3010 strain, kindly provided from the Faculty of Biotechnology, USAMV, Bucharest, has been used. Its binary plasmid vector pMP90 contained a marker gene for kanamycine resistance, neomicynephosphotransferase or *nptII*, and a reporter gene for β-glucuronidase or *gus*, both controlled by the P_{NOS} promoter derived from nopalinesynthase gene. The *Agrobacterium* was grown on a liquid LB (Luria Bertani) medium containing 50mgl^{-1} rifampycin in the dark at 28^{o}C for two days until the optical density (OD) at 600nm reached the value of 0,5 (corresponding to the long-phase status).

2.2. *Plant material*

Atropa belladonna var. *nigra* vitroplantlets were obtained by micropropagation of plantlets derived from sterilized seeds, which had been kindly provided from the Cluj Botanical Gardens. They were subcultured on a Linsmaier and Skoog (LS) solid medium (LINSMAIER and SKOOG 1965) supplemented with 3% sucrose. This culture was maintained at 22^{o}C on a daily 16h/light and 8h/dark regime.

Gene transfer and "in vitro" bacterial and plant tissue selection have been performed according to the mediated transgenesis in plants described by CALIN et al (1998).

2.3. *A.tumefaciens mediated gene transfer in A.belladonna vitroplants*

The gene transfer has been performed via bacterial infection and further co-culture of plant leaf explants (0,8-1cm diameter) in *Agrobacterium* suspension, for 2-5 minutes. After gentle manual shaking, the leaves were dried on sterile Whatman paper to remove excess bacterial suspension and plated on solid LS medium.

2.4. *Regeneration and R0 transformant line obtaining*

a) Bacterial and plant selection

After two days of co-culture, explants were first transferred to a fresh LS medium supplemented with 500mgl^{-1} cefotaxime for bacterial selection.

A selective medium (500gl^{-1}kanamycin containing) has been used for the early transformed tissue plant selection. The explants were further cultured under 16h/light and 8h/dark regime, in a clean room at 22^{o}C and every two week they were transfered to a fresh stepwise diluted (400, 300 and 200mgl^{-1}) selective medium .

b) Callus initiation and proliferation

A S2 (LS supplemented with 2mg/l zeatin) medium has been used for callus initiation and proliferation in *A.belladonna* culture. When adventive shoots developed and more than two leaves emerged a transfer to LS solid medium has been performed. Rooted shoots were excised and repeatedly selected on the specific medium.

2.5. *The analysis of the putative plant transformant material*

2.5.1. The genotype analysis

DNA was isolated from leaves in liquid nitrogen by a CTAB method as described by DOYLE and DOYLE (1990). The polymerase chain reaction (PCR) was used to confirm the presence of the reporter *gus* gene in the entire vitroplants.

The primer pair had the following sequences:
5'-GAACAACGAACTGAACTGGCA-3'/5'-CTAGTGCCTTGTCCAGTTGC-3'
(MATSUMOTO, FUKUI, 1998). A 130 bp amplicon has been considered to be the amplified *gus* DNA fragment. The PCR reaction mixture (25µl) consisted of: 50mM of each dNTP,

$0.2gl^{-1}$ of genomic DNA, 1μM of each primer, Red Taq DNA polymerase and PCR buffer. A termal cycler (Perkin Elmer Gene Amp PCR system 2400) has been used for the amplification reaction with the following cycle set up: 5 min denaturation / 95 ^{0}C, followed by 35 cycles comprising - 45 sec denaturation/95^{o}C, 50 sec annealing / 56 ^{o}C and 2 min extension / 72^{o}C. DNA amplified fragments were detected on a 2%(w/v) agarose gel, stained with (1μg/ml) ethidium bromide. The amplicon samples were run comparatively with a DNA weight marker PCR Low Ledder. The electrophoresis gel has been visualised and photographed in a dark chamber UVP Jencons-PLS, at 302nm.

2.5.2. The GUS fluorogenic assay

The fluorogenic assay is based on the ability of β-glucuronidase (GUS) to cleave the substrate 4-methylumbelliferyl-β-glucuronide (MUG). The resultant fluorescent 4-methylumbelliferone (4MU), stands for the *gus* expression in the transformant plant. A transiluminator (as a component of the above - mentioned dark chamber) has been used for the excitation at 365nm of the MU compound; the resultant 455 nm fluorescence was visualized and photographed with a Polaroid Gel Camera. For the determination of the GUS activity the plant tissues were grounded on ice and the total protein was extracted using the following buffer: 50mM NaH_2PO_4,10mM EDTA, 0,1% Triton X-100, 0,1%SDS, 10mM β-mercaptoethanol. After centrifugation, the clear supernatant was treated with an activated charcoal powder for the adsorbtion of the endogenous β-glucuronidases. The revelation buffer (1mM MUG in 0.2M Na bicarbonate) was added to the subsequent supernatant and the final solution was incubated at 37^{o}C for 120 minutes; the visualisation has been performed directly into the tubes, on the transiluminator, at 365nm (JEFFERSON, 1987).

All reactives were purchased from Sigma and were of a.p. grade.

Results and Discussions

This work was aimed at developing an optimized experimental model for the obtaining and analysis of the modified *A.belladonna* vitroplants by the *A.tumefaciens* mediated gene transfer. It represents an early step into a larger program focused on the medicinal plants transgenesis which is currently run in our laboratory. At this level, the transfer and expression of the *gus* or *uidA* gene for β-glucuronidase, as a reporter activity in *A.belladonna* plants has been studied. An adaptation of the Jefferson method for the the study of this reporter bacterial gene is described.

The proposed model first comprised the introduction of the *A.belladonna* plants into an "in vitro" culture (**Figure 1**) which has been reported in a previous paper (Negoianu-Tenea et al, 2000). The subsequent *gus* reporter gene transfer mediated by the vector pMP90 of the *A.tumefaciens* strain GV 3010 represents so far a first approach of the transgenesis domain for medicinal plants in our country.

The GUS system is represented by the protein β-glucuronidase and its corresponding *gus* gene. This system is widely used in plant transgenesis as a reporter one, due to the rapid and sensitive enzyme cleavage of a fluorogenic or chromogenic substrate. X-gluc or 5-bromo-4chloro-3-indolyl-β-D-glucuronide is usually used as chromogenic substrate as indicative for histochemical analysis. Certain shortcomes derive from the possible interference with the plant endogenous β-glucuronidase, which may be solved at the protein level only by specific treatments with methanol and also from the required numerous and time consuming steps of the biochemical reaction. 4-MUG is an alternative, more sensitive and rapid approach on a fluorogenic substrate. However this needed again specific measures to avoid the above mentioned desadvantages, such as the treatment of total protein with carbon powder.

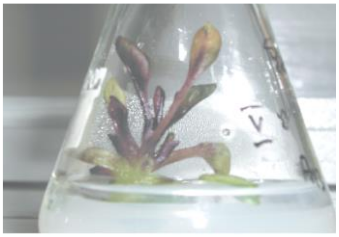

Figure 1. A vitroplant of *Atropa belladonna*

The molecular analysis of the modified *A.belladonna* plants was based on two major approaches: at the DNA and protein levels, respectively, which are proposed in a previous paper regarding the molecular analysis of genetically modified plants (CUCU, 2002). The corresponding genotype and phenotype analysis confirmed both the integration and the expression of the *gus* gene into the *A.belladonna* genome.

Genotype analysis

The electrophoretic behavior of the PCR amplified DNA fragment, as compared with the DNA weight - marker, indicated an amplicon of 130bp (**Figure 2**). This is reported as the correct amplified DNA fragment by using the above mentioned specific primers (MATSUMOTO, FUKUI, 1998). However, our electrophoresis revealed also an extra DNA fragment as amplicon of lesser weight

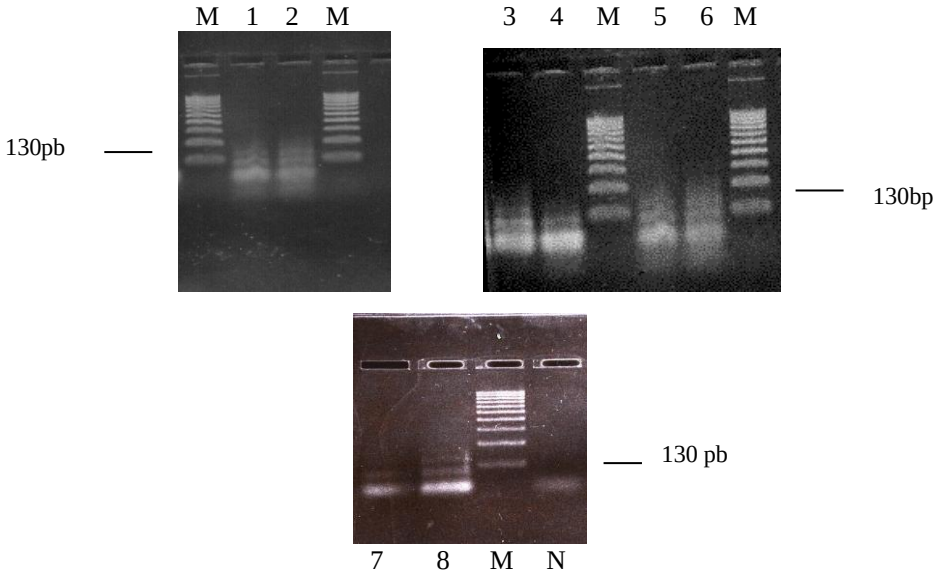

Figure 2. Amplicons obtained by PCR amplification of *gus* DNA fragments on modified *A.belladonna* genomic DNA (1-8 – transformed plant; N-untransformed plant; M- DNA molecular weight marker)

(samples 1-3,5,6,8), which might be ascribed to either the different plasmid vector (pBI 121) carrying the *gus* sequence used in the refference paper or the high concentration of primers in the corresponding PCR mixture used in our experiment. A study of such hypothesis is currently performed in our laboratory.

Phenotype analysis

Transformation was also confirmed by the GUS assay. This is based on the enzyme reaction developed by the β-glucuronidase (GUS), the expression of the integrated *gus* gene.

The GUS positives represented in (**Figure 3**) contained the transformed total protein solution which is able to cleave the fluorogenic MUG substrate, as compared with the nontransformed (GUS negative) samples. The previous treatment of the total protein solution with the carbon powder which adsorbed the endogenous β-glucuronidase, assured the correct estimation of the transformant state. A variable blue fluorescence has been observed which is explained by the different activities of GUS in various transformant plants.This may be further estimated by comparison with a dilution series of a 4-MU stock solution.

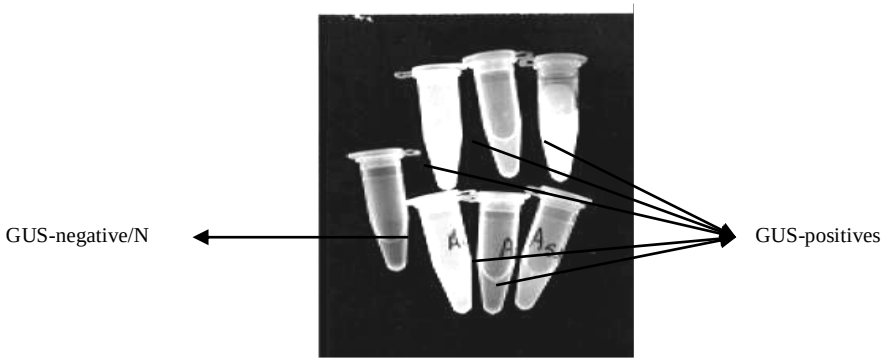

Figure 3. Eppendorf tubes containing the total plant extract whose GUS content determines the release of the MU fluorescent compound from its nonfluorescent glucuronide (MUG) as enzyme substrate.

Conclusions

This work was aimed at proving the efficiency of the mediated transgenesis experimental model of *Atropa belladonna* plants by a very sensitive analytical method.

The modified *Atropa belladonna* has been analysed by both genotype and phenotype assays. They confirmed both the integration and expression of the reporter *gus* gene into the plant host genome. However, the R0 line of regenerants, which has been considerred in our work, corresponds to a *transient* transformation. The descendants need to be further obtained and analysed also by both approaches, as meiose barrier and natural conditions are often known to suppress the foreign gene expression in transformed plants. Therefore the correspondence of the genotype and phenotype results should be periodically tested in order to consider a *stable* transgene integration and expression.

The analysed system considerred so far only the reporter *gus* gene, which is usually assisting the transfer of a *gene of interest* in plant. The molecular analysis of integration and expression of the other above mentioned marker gene, *nptII*, in medicinal *A.belladonna* plants, derived from the same vector, is currently under study in our laboratory.

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