Genetically modified medicinal plants. II. Transfer and expression of a marker kanamycine resistance gene in *Atropa belladonna* plants

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Abstract

An indirect, mediated genetic transformation of Atropa belladona vitroplants has been performed using the disarmed Agrobacterium tumefaciens pTi carrying the nptII gene for neomycinephosphotransferase. Its expression into the plant host genome conferred the kanamycin resistance trait which has been easily detected by both in vitro and molecular-genotype (PCR) and phenotype (protein) - assays.

Keywords: *Atropa belladonna*, *Agrobacterium tumefaciens*, transgenic plant, marker *nptII* gene, NPTII-protein neomycin phosphotransferase II, PCR-polymerase chain reaction

Introduction

Plant genetic transformation technology has become a versatile platform for cultivar improvement as well as for studying gene function in plants. The success in this domain represents the culmination of many years of efforts in tissue culture and plant genetic engineering techniques improvement (HANSEN et al, 1999).

Plants are a tremendous source for the discovery of new products of medicinal value which nowadays are considered less risky then those of microbial or animal origin (SHANKS et al, 1999). The beneficial medicinal effects of plant material typically result from the endogenous combination of their secondary products. The progress of the genetic engineering domain with medicinal plants has been ascribed however to the increase yield in pharmaceutically important secondary metabolites (BRISKIN, 2000; DANIELL et al, 2001).

One of the most extensively studied medicinal plant, the *Atropa belladonna*, a member of the *Solanaceae* family is abundant in tropane alkaloids, actually used as antimicrobial compounds in pharmaceutical drugs (BONHOMME et al., 2000,). The general aim of the genome manipulation domain with such plants is referred to as developing techniques for interest gene transfer into the plant genome in order to improve the biosynthetic rate of the interest compounds. An essential concern in such strategies regards therefore the choice of the correct marker genes for a good assistance of the genetic transformation steps, as the generation and moreover recovery of transgenic plants using only the genes of interest are still not generally feasible.

One of the initially used selectable marker for such purposes is a microbial gene for antibiotic resistance isolated from the Tn5 transposon of *E.coli*, named neomycin phosphotransferase or *nptII*. It has been elected from the bacterial domain as a good marker due to its poor interferences with plant endogenous kinases.

This paper represents a continuation of the previously reported model for the study of the marker *nptII* gene transfer and expression in potato (CUCU, 2002) and also of the already initiated study of the transformation system for *A.belladona* plants (NEGOIANU-TENEA et al., 2002). The genotype (*nptII gene*) and phenotype (NPTII protein) assays for the marker gene detection and expression, respectively, are proposed for a further standardization of the OGM analysis methodology in medicinal plants.

Materials and Methods

Obtaining of Atropa belladonna transformant plants

The techniques for obtaining the transformant *Atropa belladonna* vitroplants and maintaining as an "in vitro" culture has been performed by an indirect (*Agrobacterium* mediated) method described in a previously published paper (NEGOIANU-TENEA et al, 2002).

The *Agrobacterium tumefaciens* GV 3010 strain, kindly provided by the Faculty of Biotechnology, USAMV, Bucharest, has been used. Its binary plasmid vector pMP90 contained a marker gene, *nptII*, and a reporter gene, *gus* , both controlled by the P_{NOS} promoter.

Atropa belladonna var. *nigra* vitroplantlets were obtained by micropropagation of plantlets derived from sterilized seeds, kindly provided by the Cluj Botanical Gardens.

The regeneration of putative transformed shoots from the infected tissue has been performed as described earlier (NEGOIANU- TENEA et al, 2002).

The analysis of the putative plant transformant material

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 marker *nptII* gene in the entire vitroplants.

The primer pair had the following sequences:
5'-GCATACGCTTGATCCGGCTACC-3'/5'-TGATATTCGGCAAGCAGGCAT-3'
(MATSUMOTO, FUKUI, 1998). A 231bp amplicon has been considered as the amplified *nptII* DNA fragment. The PCR reaction mixture (25μl) consisted in: 50mM of each dNTP, $0.2gl^{-1}$ of genomic DNA, 1mM 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 in comparison 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.

Phenotype analysis

The expressed NPTII protein has been detected as a 59kDa fraction of the total plant protein extract (COLBERE-GARAPIN et al, 1981, L.HERRERA-ESTRELLA et.al, 1994),

electroforesed in a SDS-PAGE system described elsewhere (CUCU, 2002). A protein mixture marker representing the domain of 29-205 kDa has been used. The plant tissue used for this assay was represented by regenerated and selected shoots.

The enzyme NPTII activity has been estimated by the "in vitro" method for the selection of the transformed plant tissues on kanamycin containing media (CALIN et al., 1996). The selective medium ($500gl^{-1}$kanamycin containing) has been used and the explants were further cultured under 16h/light and 8h/dark regime, in a clean room, at 22^{o}C and every two week transferred to fresh stepwise diluted (400, 300 and $200mgl^{-1}$) selective medium. The rooting capacity criterium on selective media has been used for the estimation of the NPTII detoxification capacity.

Results and Discussions

This work was aimed at developing an optimized experimental model for the analysis of the modified *A.belladonna* vitroplants by the *A.tumefaciens* pTi mediated marker *nptII or neo* gene transfer.

The analysis of the modified *A.belladonna* plants implied the detection of both the *nptII* gene at the genomic DNA level and its expressed NPT II polypeptide. As certain reports suspected eventual posttranslational modification of the protein folding which might have had dramatic effects upon its catalytical activity, we also examined the NPTII "in vitro" detoxifying ability of a kanamycin containing medium. The choice of the marker *nptII* gene was based in fact on its corresponding "in vitro" easy and rapid method of analysis consisting in estimation of the plant tissues resistance on the so-called *selective media*.

NptII is one of the first widely used dominant selectable marker in eukaryotes. It encodes nemycin phosphotransferase conferring resistance to amynoglycosides (Fig.1). This family of antibiotics, which includes kanamycin, neomycin and gentamycin, interact in the cytosol of prokaryotes and eukaryotes with at least three ribosomal proteins and with specific bases within the decoding region of the smaller ribosomal RNA subunit, resulting in inhibition of protein synthesis and increased frequency of induced translational errors. These activities determine in fact the observed *toxicity* of the selective media upon the viability of the plant tissues.

On the other hand, the mechanism of *resistance* is determined by the phosphotransferase activity of NPT II protein, which blocks the active –OH group on the antibiotic for the ribosome binding (**Figure 1**). The marker gene has been called therefore also aminoglycoside phosphotransferase (*aph*) and its corresponding protein, APH. Of the seven major groups of APHs that have been distinguished on the basis of their substrate specificities, one is widely used for the expression of a dominant marker selection in eukaryotes: APH (3')-II isolated from *E.coli* Tn5.

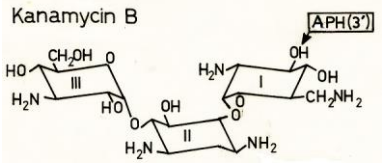

Figure 1. The amynoglycoside structure of the antibiotic kanamycin B; the 3' -OH group is pointed with the arrow.

This enzyme inactivate kanamycine by transferring the γ-phosphate of ATP to the hydroxyl group in the 3' position of the pseudosaccharide. The active enzyme is a 59 kDa dimer, based on a 29 kDa monomer, which may electrophoretically detected in tissues which are not showing kanamycin resistance, yet being transformed with the marker gene.

The obtained *genotype and phenotype profiles* may confirm both the integration and the expression of the *nptII* gene into the *A.belladonna* genome; the further study of the concordance between those two types of results need to be performed in different environmental conditions and in different tissue types in order to complete the whole image of the gene expression.

Both the marker gene and its corresponding protein sequence are important to be known for such analysis, i.e. in order to choose the right primer sequence for the PCR amplification reaction of the *nptII* gene and the right protein marker domain for the comparison of the NPTII fraction.

Genotype analysis

The electrophoretic behavior of the DNA amplified fragment by PCR, as compared to the DNA weight marker, indicated an amplicon of 231bp (Fig.2). This is reported as the correct amplified DNA fragment by using the above mentioned specific primers (MATSUMOTO et al., 1998).

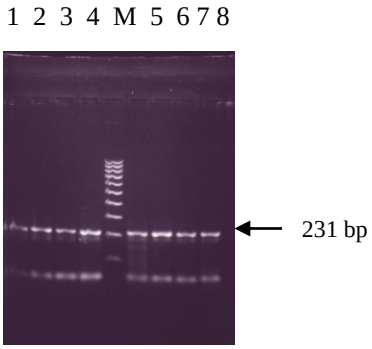

Figure 2. Amplicons obtained by PCR amplification of *nptII* DNA fragments on modified *A.belladonna* genomic DNA (1-8 transformant samples; M-molecular weight DNA).

The 231 bp amplicon represents a part of the coding *nptII* sequence. An alternative for the detection of the same gene using the PCR reaction for the amplification of its promoter sequence has been reported earlier (CUCU, 2002). This approach is often advantageous in the case a study of the correlation between the promoter DNA methylation level and the gene expression is needed.

Phenotype analysis

The expression of the *nptII* marker gene as its corresponding NPT II polypeptide has been estimated by the detection of the native 59 kDa dimer into the total protein extract obtained from the regenerated shoots (Fig.3). The transformed ($T_{1,2}$) shoots actively expressed the NPT II fraction, as compared to those nontransformed and control ones (not shown). Usually this method is applied for different tissue types (shoots, callus, roots or tubers) in order to detect certain monomerisation processes which may have important effects upon the enzyme activity. Therefore, both the "in vitro" and the molecular approaches have to be used for a correct analysis in order to assure a real assessment of the transformation efficiency.

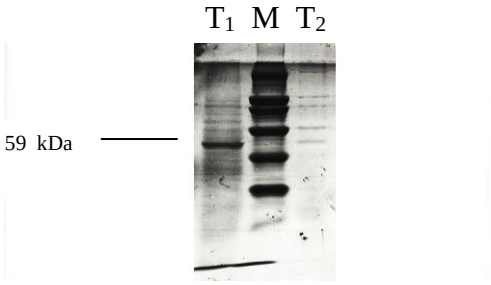

Figure 3. The electrophoretic pattern of the 59 kDa protein fraction corrsponding to the NPTII polypeptide.

The enzyme NPT II activity has been estimated by the "in vitro" assay of the regenerated plant shoots ability to detoxify the kanamycin containing medium. The *rooting capacity* of the "in vitro" prelevated shoots developed by the initially infected explant stands for the efficient phosphotransferase activity of the NPTII protein. Usually, the direct correlation between the high rooting capacity and the positive protein electrophoretic results have been obtained.

Conclusions

The most adopted selectable marker gene is *nptII*, derived from the transposon Tn 5, which encodes neomycin phosphotranspherase (NPTII). This enzyme conferres resistance to a group of aminoglycoside antibiotics which include neomycin, kanamycin, G418, by catalyzing their inactivation by phosphorylation.

This paper presented the experimental and analytical conditions for an efficient *Agrobacterium* mediated gene transfer into the *A. belladonna* genome which included the molecular analysis of selectable marker *nptII* gene. Both the genotype and phenotype approach confirmed the transfer, integration and expression of the marker antibiotic resistance gene into the host genome. The "in vitro" approach completed also the analysis by confirming the phosphotransferase activity of the expressed NPTII.

However, the R0 line regenerants analysed here corresponds to a *transient transformation*. The descendants need to be further obtained through the "ex vitro" accommodation and analysed by both approaches, in order to confirm the stable integration and expression of the foreign gene.

Acknowledgements

The research has been supported by the Grant A5/569/2000-2002 and represent part of the GN-T activity during the Ph.D training programme. The authors are thankful to Drd. A. Călin for her advice in the „*in vitro*" setting up of the experimental transgenesis model.

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