T-DNA promoter tagging in *Nicotiana tabacum*

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Abstract

Plant development is primarily controlled at the level of gene expression. In order to analyse this regulation it is necessary to isolate genes which are involved in organ development through cellular and tissue determination or which respond to environmental signals. Promoter tagging was chosen in order to identify genes potentially associated with plant development by their spatial and temporal pattern of expression. The introduction of a promoterless reporter gene tag allows the expression patterns of plant genes to be readily characterised.

A new series of promoter tagging vectors were constructed from the plasmid pPCV604 (Koncz, 1989). The selectable kanamycin resistance marker gene from pBin6 (Bevan, 1984) was cloned into pPCV604 to create pGT. The hygromycin phosphotransferase gene in pGT was then replaced with a promoterless β-glucuronidase (*gus*) gene coupled with octopine synthase termination sequence subcloned from pKiwi101a (Janssen and Gardner, 1989) creating pGTG. This binary transformation vector required the helper pRK replication functions of *Agrobacterium tumefaciens* strain GV3101. In order to bypass this restriction, the vector sequence of pBin19 was combined with the T-DNA of pGTG to create pBin19-GTG. The latter plasmid was found to have a higher *Agrobacterium tumefaciens*-mediated *Nicotiana tabacum* transformation efficiency in strain LBA4404 than pGTG in strain GV3101.

In both the pGTG and pBin19-GTG promoter tagging vectors the promoterless *gus* gene has an initiation codon 62 base pairs inside the T-DNA. This sequence includes translation termination codons in all three reading frames. Therefore, insertion of the T-DNA into a plant gene could lead to activation of the *gus* gene, under the control of the plant gene promoter, via transcriptional fusion.

Nicotiana tabacum leaf segments were transformed with pGTG or pBin19-GTG and transgenic plants selected on kanamycin. A population of 87 transgenic tobacco plants were fluorometrically screened for GUS activity in leaf and root material; 37% were found to contain GUS activity, indicating a high frequency of promoter tagging.

Two transgenic plants with root specific *gus* expression were analysed histochemically.

Progeny after self-fertilisation lacked GUS activity, though this was restored in progeny of one plant with 5-azacytidine treatment, suggesting involvement of methylation in the gene silencing. Southern hybridisation, inverse PCR cloning of T-DNA flanking sequences and segregation on kanamycin indicated the presence of multiple T-DNA copies within the primary transformants. Furthermore, inverse PCR sequence from one plant indicated multiple and truncated T-DNA insertions at one or more loci.

A further population of transformed plants was generated with pBin19-GTG and histochemically screened for GUS activity in roots (14 positive from 147 tested), shoots (27 positive from 147) and floral organs (14 positive from 56). Overall, combining results from all plant organs tested, an average of 33% of plants were found with GUS activity in one or more organs. A diverse range of patterns of *gus* expression were observed and described including patterns involving root branching.

Forty four plants from this population were analysed for T-DNA copy number via Southern hybridisation with a *gus* probe (right border junction T-DNA) and *npt*II probe (central T-DNA). Multiple copies were frequently found with an average of 3.3 T-DNA copies per transgenic plant. Overall, an average of 11% of T-DNA insertions were found to be involved in *gus* activation.

Comparison of the fluorometric (37% positive, 87 plants tested) and histochemical (22% positive, 147 plants tested) screens for GUS activity in root and shoot material was discussed and it is suggested that further care is needed in assigning promoter tagging hits from fluorometric screening.

Variable expression was observed with promoter tagged genes. It is suggested that further research is required to determine whether this variation was due to silencing, perhaps by methylation, or was a result of the tagged promoters' normal expression patterns.

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Abbreviations

AB	*Agrobacterium* minimal media, Section 2.1.1
Ap	ampicillin
CTAB	hexadecyltrimethylammonium bromide
EDTA	ethylene diamine tetra acetic acid, sodium salt unless specified
Gm	gentamycin
gus	β-glucuronidase gene
GUS	β-glucuronidase enzyme
hpt	hygromycin B phosphotransferase
kb	kilobase pairs
Km	kanamycin
LB	left border of T-DNA, a 25 base pair conserved sequence
LB	Luria Bertani bacterial media, Section 2.1.1
MS	Murashige and Skoog plant media, Section 2.4.2
MU	methyl umbelliferone
MUG	methyl umbelliferone glucuronide
Nic	*Nicotiana* media, Section 2.4.2
nos	nopaline synthase gene from *Agrobacterium tumefaciens* T-DNA
npt	neomycin phosphotransferase gene, conferring kanamycin resistance
ocs	octopine synthase gene from *Agrobacterium tumefaciens* T-DNA
P_{35S}	promoter region of the 35S cauliflower mosaic virus transcription unit
PCR	polymerase chain reaction
P*mas*	promoter region of *mas* gene
P*nos*	promoter region of *nos* gene
RB	right border of T-DNA, a 25 base pair conserved sequence
Rf	rifampicin
Ri	root inducing, referring to *Agrobacterium rhizogenes* plasmid
rpm	revolutions per minute
SOB	bacterial media, Section 2.1.1
SOC	bacterial media, Section 2.1.1
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate (20xSSC: 3 M NaCl, 0.3 M trisodium citrate)

SUDS	an agarose gel loading buffer also containing detergent suitable for halting restriction endonuclease enzyme activity, Section 2.3.12
Tc	tetracycline
T-DNA	transferred DNA, essentially the section of DNA bounded by left and right border sequences that is inserted into the plant genome by *Agrobacterium*
TE	buffer, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0
Ti	tumour inducing, referring to *Agrobacterium tumefaciens* plasmid
Tp	trimethoprim
TY	Tryptone and yeast extract bacterial media, Section 2.1.1
vir	virulence genes on *Agrobacterium* Ti plasmid
XGal	5-bromo-4-chloro-3-indolyl β-D-galactoside
XGluc	5-bromo-4-chloro-3-indolyl β-D-glucuronide (cyclohexylammonium salt)

Other standard abbreviations are as used by Biochemical Journal (1992)