Agrobacterium-mediated Transformation of Hevea Cells Derived from In Vitro and In Vivo Seedling Cultures

P. AROKIARAJ* AND WAN ABDUL RAHAMAN W.Y.*

Tumour tissue was produced on stems of in vitro and in vivo propagated rubber (Hevea brasiliensis) seedlings of PB 5/51, infected with a strain of Agrobacterium tumefaciens 541 (isolated by the Rubber Research Institute of Malaysia in 1971). The tumour tissues exhibited phytohormone-independent growth in tissue culture and produced octopine. In contrast, controls inoculated with distilled water on seedlings did not produce crown gall. Octopine-utilising Agrobacterium strain 541 was distinguished from non-utilisers on a solid medium containing octopine as the only added nitrogen source with bromothymolblue (BTB) as the pH indicator. On blue BTB plates, this strain formed orange-yellow colonies, while those formed by non-utilisers were translucent. This process of cell transformation may facilitate the transfer of genes of economic importance to rubber.

Crown gall is a neoplastic disease produced in most dicotyledonous plants by infection of the plant with the Gram-negative soil bacterium Agrobacterium tumefaciens. The virulent trait of A. tumefaciens is carried on diverse tumour-inducing (Ti) plasmids which range in size from about 200 kb to 250 kb. In the course of infection, a portion of the Ti plasmid, the T-DNA is stably transferred to the plant and causes two fundamental changes in the physiology of the plant cells. First, the cells become transformed. Whereas normal plant tissue grows in callus culture only when auxin and cytokinin are added to the medium, the growth of crown gall tissue is characteristically phytohormone-independent. Second, crown gall tissues characteristically synthesise opines, primarily octopine or nopaline, which are not synthesised by normal plant tissues. The particular opine produced is coded by the Ti plasmid. Thus, the A. tumefaciens – plant interaction is one in which a prokaryote ‘genetically engineers’ a eukaryote to synthesise a compound which can be used as a carbon, nitrogen and energy source by the bacterium.

Hevea brasiliensis Muell-Arg is a perennial cross-pollinating tree and hybridisation between good inbred lines will bring about heterotic vigour. Due to the long generation required for Hevea in selection, some important techniques effective in crop improvement could be applied to Hevea, thus, considerably shortening the process of selection for the desired characters. Genetic transformation would be greatly facilitated by the development of gene transfer systems (with selective markers and reporter gene) which may be adapted to allow genes to be introduced into cells using the tissue culture regeneration systems currently available for Hevea. The regeneration of plants from the transformed cells, facilitates the rapid improvement of commercially important cultivars. Engineered genes integrated stably into plant chromosomes can serve as powerful tools for studying the effects of new genetic material on plant development. To exploit the above potential for Hevea, of fundamental importance is the stable transformation of Hevea plants with the development of the Agrobacterium tumour-inducing plasmid (Ti) system.

Simple methods for gene transfer have been devised involving leaf disc transformation procedure with A. tumefaciens. In this report,
strain 541/71 of *A. tumefaciens*, was used for
the induction and *in vitro* culture of crown gall
tumours of *Hevea brasiliensis*.

**EXPERIMENTAL AND MATERIALS AND METHODS**

**Bacterial Strain and Culture Medium**

*A. tumefaciens* strain 541/71 was isolated
by the Rubber Research Institute of Malaysia
(RRIM) in 1971. This strain of *Agrobacterium*
was grown in tubes containing 2 ml of sterile
medium composed of 5 g/litre tryptone,
3 g/litre yeast extract and adjusted to pH 7.0
before autoclaving. The tubes were placed
on an orbital shaker rotating at 110 rev/min
and incubated in the dark at 28°C for 36-48 h
until the bacteria reached late log phase (> 10^9
bacteria/ml).

Bromothymolblue indicator medium^6* (BTB)
containing: K_2HPO_4, 0.09 g/litre; NaCl,
0.15 g/litre; MgSO_4·7H_2O, 0.50 g/litre;
CaCl_2·6H_2O, 0.1 g/litre; glucose, 2.0 g/litre,
BTB 0.15 g/litre, and octopine, 0.1 g/litre (as
the nitrogen source), unless otherwise stated.
The pH was adjusted with NaOH to 7.5. The
medium was solidified with 1.8% (w/v) Difco
bacto-agar.

**Plant Materials**

Seeds of *Hevea brasiliensis* PB 5/51 were
surface-sterilised with 20% (v/v) chlorox
(sodium hypochlorite; a.i. 5.25%) containing
two drops of Teepol/100 ml for 20 min and
washed several times in sterile distilled water.
They were then germinated on Murashige and
Skoog^7* (M&S) hormone-free medium,
solidified with 0.7% Difco-bacto agar and
incubated at 26°C ± 1°C, with a 12 h
photoperiod of 30µE (micro Einstein) m^{-2}s^{-1}
irradiance provided by fluorescent light.

Non-sterile plants were grown by sowing the
seeds in polybags containing sand and soil
(1:1 mixture) and incubated in the glasshouse.

**Crown Gall Induction**

The bacterial suspension was applied by
scratching/injecting the stems of two-week
old *in vitro* seedlings with a hypodermic
needle containing a thick bacterial suspension.
Inoculation was with small (5-10 ml) droplets.
A control was set up to measure the wounding
response by injecting the plants with sterile
distilled water. One-month-old seedlings of
PB 5/51 grown *in vivo* in the glasshouse
were used for infection with *A. tumefaciens*. The
stems of the seedlings were first swabbed with
ethanol (to remove foreign contaminants) in a
laminar flow chamber. After injection of the
bacterial suspension on the stems, (as described
for the *in vitro* plants) the infected areas were
wrapped with sterile non-absorbent cotton wool
before removal, three days later.

**In Vitro Culture of Tumours**

After three weeks, the tumours were excised
from the *in vitro* infected seedlings and
transferred to M&S basal medium (without
phytahormones) but containing an inhibitory
concentration of Ampicillin (1 mg/ml) to free
the isolated tumours of the infected bacteria.
The tissue was transferred to fresh medium
weekly. After a series of passages, with a
reduction in the concentration of Ampicillin
with every transfer of the tumour tissue, the
tissue was usually free of bacteria and was
further maintained on an antibiotic-free M&S
basal medium under the same conditions.

After six weeks, the tumours isolated from
the *in vivo* grown seedlings were surface
sterilised by immersion in 10% (v/v) chlorox
solution containing two drops of Teepol/100 ml
for 15 min and rinsed with sterile water. The
tumour tissues were then subjected to *in vitro*
culture as described earlier.

**Detection of Opines in Tumour Tissue**

The method of Christon *et al.^8* was used
to analyse the opines synthesised in the
transformed tissue. About 100 mg fresh weight
of tumour tissue was incubated overnight in
M&S basal liquid medium containing 5mM
L-arginine to enhance octopine production.
Tissues were macerated in a sterile Eppendorf
tube. After centrifugation for 10 min in a
Beckman microfuge 11 at 13 000 rev/min

COPYRIGHT © MALAYSIAN RUBBER BOARD
(10,000 g), 5 μl of the supernatants, 2 μl of octopine 0.02% (w/v), nopaline 0.02% (w/v) and 5 mM of L-arginine were spotted on Whatman k-5 silica gel plates. The plates were developed in 50 ml of TLC buffer containing methanol: 2-butanol: 0.1 M sodium acetate, at pH 4.6 (15:1:4). After 2 h, the plates were removed, dried and sprayed with a 1:1 fresh mixture of 10% NaOH (w/v) in 60% ethanol: 0.04% (w/v) phenenthrenequinone (Sigma Chemical Co.) in absolute ethanol and immediately visualised under UV light (254 nm) and photographed through a UV filter.

RESULTS AND DISCUSSION

Development of Tumours

Two weeks after inoculation, tumour formation was readily obtained from infected areas of in vitro propagated rubber plantlets. Four weeks after infection, the whitish yellow tumours on the infected areas reached a diameter of 1.5 cm (Figure 1b). Tumour formation was observed after three weeks in the wounded areas on in vivo plants. Though they were slow-growing, the brownish galls measured a diameter of 2 cm upon continued growth after eight weeks (Figure 1c).

Growth of In Vitro Cultured Tumour Tissues

Tumour tissues cultured in vitro grew independently in M&S basal medium lacking phytohormone, even at one-tenth concentration M&S basal medium, they showed prolific growth (Figure 1d). Attempts were made to differentiate the cells but they remained as fast-growing, undifferentiated cells.

Based on experiments, the strain 541 of A. tumefaciens is an octopine-utilising agrobacterium. When 10^9 bacteria (strain 541) were plated on BTB plates, this strain formed orange-yellow colonies indicating the octopine-degrading bacteria, while those of non-utilisers of octopine were translucent. Octopine as a nitrogen source acidified the medium whenever a strain could utilise the nitrogen source present. Strains were tested for utilisation of nopaline by substituting for octopine in the same medium (Table 1).

Octopine Synthesis in Tumour Tissue

All tumours except those from control tissue were octopine-positive (Figure 2).

Crown gall formation normally occurs at wound sites. Wounding may have three contributions: 1) to provide access for the bacteria to a cell surface recognition site; 2) to stimulate wound-associated cell divisions which make plant cells competent for transformation; and, 3) to stimulate the production of wound-associated compounds such as acetosyringone which both attract Agrobacterium and induce the vir genes required for T-DNA transfer. Thus, Agrobacteria do not penetrate the healthy plant cells but transform them into tumour cells by transferring a piece of DNA (T-DNA) into them. The T-DNA carry genes coding the formation of specific metabolites, the opines as well as those involved in auxin and cytokinin biosynthesis.

Crown gall tumour cell induced by A. tumefaciens strain 541 in rubber seedlings remained small on the plant but can easily produce sterile (bacteria-free cultures), fast-growing callus in culture medium lacking phytohormones because of the constitutive expression of the genes in the T-DNA that encodes for auxin and cytokinin. Normal plant cells required in addition the presence of plant hormones for growth in such a culture medium. As the T-DNA genes also code for the production of opines, the sterile tumour tissue was shown to contain octopine (TLC detection) induced by the octopine-utilising strain.

Agrobacterium strains carrying mutated Ti plasmids induce crown galls with an altered hormone balance and in some species, this result in the generation of a 'shooty' mutant tumour (if the genes for the tms1-tumour morphology shoot region 1, or tms2 are inactivated). These properties have been exploited in some laboratories, to regenerate shoot from semi-oncogenic 'shooty mutant' tissue.
a) Seedling inoculated with sterile distilled water did not develop tumours (Control)

b) Tumours induced by tumourigenic Agrobacterium strain 541 on stem of in vitro propagated rubber seedling.

Figure 1. Development of tumours.
c) Presence of crown gall on stem of in vivo grown rubber seedling, infected with A. tumefaciens 541.

d) Phytohormone-independent growth of tumour tissue.

Figure 1. Development of tumours (contd.).

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>Strain C58</th>
<th>Strain T37</th>
<th>Strain 541</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octopine</td>
<td>Translucent&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Translucent</td>
<td>Orange-yellow</td>
</tr>
<tr>
<td>Nopaline</td>
<td>Orange-yellow</td>
<td>Orange-yellow</td>
<td>Translucent</td>
</tr>
</tbody>
</table>

<sup>a</sup>Strains C58 and T37 utilise nopaline. 541 utilises octopine
<sup>b</sup>Orange-yellow colonies of spontaneous octopine-utilising mutants appeared

Since plasmid transfer is involved in the formation of crown galls, the system is amenable to manipulation for gene transfer in *Hevea*. Transformed tissues from *Hevea* could facilitate rapid improvement of commercially important clones.
Lane 0: Arginine
Lane 1: Untransformed tissue (Control)
Lane 2: Nopaline standard
Lane 3: A mixture of octopine and nopaline standards
Lane 4: Transformed calli from in vitro propagated PB 5/51 seedlings
Lane 5: Transformed calli from in vivo germinated PB 5/51 seedlings

Figure 2. TLC octopine detection in tumour tissue produced on stem of Hevea plants.
ACKNOWLEDGEMENT

The authors wish to thank Encik Zain Karim of the Polymer Research and Process Division, Rubber Research Institute of Malaysia for the supply of \textit{A. tumefaciens} strain 541 used in this study, Mr Tsan Fan Kui for photography and Cik Maimon binti Haji Hashim for typing the manuscript.

\textit{Date of receipt: September 1990  
Date of acceptance: December 1990}

REFERENCES


