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Agrobacterium-mediated transformation in medicinal trees

S. Mohana Priya, S. Indhu, R. Kalaiselvi and Rekha R. Warriar♦

Institute of Forest Genetics and Tree Breeding (IFGTB), P.B.No.1061, R.S.Puram, Coimbatore-641002, T.N., India

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Plant transformation relies on the bacteria *Agrobacterium* which has the potential to transfer genes of interest into a desired plant. The most commonly used species are *A. tumefaciens* and *A. rhizogenes*. This approach is convenient, especially in tree species, as conventional breeding takes a long time. It also helps to overcome other associated problems such as juvenility, heterozygosity, and incompatibility. The present review brings together research on *Agrobacterium* mediated genetic transformation in various medicinally important trees for production of secondary metabolites. Mostly the *Agrobacterium* mediated transformation aims at production of secondary metabolites in medicinal trees like *Azadirachta indica*, *Semecarpus anacardium* and *Gmelina arborea*, etc. More recent technologies are available with greater potential for gene manipulation. But they require an efficient transient transformation phase and successful regeneration protocols which is the key to future application of advanced technologies for improvement of medicinal trees.

Abbreviations: A_{600} -Absorbance at 600 nm; ACS-Acetosyringone; ATMT-*A. tumefaciens* mediated transformation; BA-Butyric Acid; DKW1-Driver and Kuniyuki medium; GFP-Green Fluorescent Protein; IAA-Indole Acetic acid; IBA-Indole Butyric acid; KT-Kinetin; LB-Luria Bertani medium; NAA-Naphthalene Acetic Acid; *npt II*-neomycin phosphotransferase gene; *hpt II*-Hygromycin phosphotransferase gene; OD-Optical Density; rpm-revolutions per minute; RT-PCR-Real Time-Polymerase Chain Reaction; RT- qPCR-Real Time-quantitative Polymerase Chain Reaction; WPM-Woody Plant Medium; YEB-Yeast Extract Beef medium; YFP-Yellow Fluorescent Protein; YMB-Yeast Mannitol Broth; 2YT-Yeast extract and Tryptone media.

1. Introduction

Agrobacterium is a gram negative bacterium commonly found in the soil, non spore forming and causing rapid proliferation of cells in plants. The genus *Agrobacterium* belongs to the plant-associated bacteria, *Rhizobiaceae*, which also include nitrogen-fixing symbiotic bacteria. Initially, three species were described: *A. tumefaciens*, which induces crown galls on dicots; *A. rhizogenes*, which induces hairy root disease; and *A. radiobacter*, the avirulent strain. Some species-specific *Agrobacteria*, namely; *A. vitis* for grapevine, *A. rubi* for *Rubiaceae* members and *A. larrymoorei* from *Ficus benjamina* have also been isolated.

The *Agrobacterium* transfers a particular DNA segment, the T-DNA (transferred-DNA), into plant cells, which integrates into the plant chromosome (Figure 1a). Once, it is integrated stably into the plant genome, it triggers production of plant hormones to develop tumors

or multiple roots. It also produces opines which are sugar-amino acid conjugates that *Agrobacterium* catabolizes. This way, the bacterium creates its own niche for growth and proliferation. *Agrobacteria* catabolize a variety of metabolites and hence various strains are available. These opine stimulate bacterial conjugation thereby spreading the plasmid.

A. tumefaciens is the most commonly studied and used species in this genus. It is a rod shaped and soil borne bacteria causing infection through wounds in roots or crown in graft union regions. *A. tumefaciens* is widely exploited for foreign genes introduction into plants and has facilitated the development of many transgenic tree species (Harfouche *et al.*, 2011). In order to obtain successful transgenic plants, *via*, *A. tumefaciens*, the main requirement is a robust organ regeneration and transformation system in the species (Mahender *et al.*, 2012). *A. tumefaciens*-mediated transformation (ATMT) has many advantages such as flexibility in choosing the starting materials (cells, tissues, protoplasts, hyphae, spores or blocks of mycelia), high transformation efficiency and high frequency of single-copy T-DNA insertions (Abuodeh *et al.*, 2000; de Groot *et al.*, 1998). It can also be used to tag genes to explore cell type-specific gene expression and function, mutations, pathogenicity in bacteria and fungi (Betts *et al.*, 2007).

Agrobacterium rhizogenes, similar to *A. tumefaciens*, is also used for introducing exogenous genes into plants. *A. rhizogenes* recognizes signal molecules expressed by wounded plant cells and produces a chemotactic response. The rhizogenic strains which contain a single copy of the Ri plasmid (Figure 1b) gets integrated into the plant chromosome leading to the formation of neoplastic, plagiotropic roots (hairy roots) caused by root-inducing genes *rol A to D* (Chilton *et al.*, 1982). Hairy roots have the unique character of being able to grow *in vitro* in the absence of exogenous plant growth regulators (Collier *et al.*, 2005). In addition, *A. rhizogenes* strains are also implied as genetic sources to produce new genotypes and phenotypes with high branching, wrinkled leaves, shortened

Corresponding author: Dr. Rekha R. Warriar

Scientist-F, Institute of Forest Genetics and Tree Breeding (IFGTB), P.B.No. 1061, R.S. Puram, Coimbatore-641002, Tamil Nadu, India

E-mail: rekhawarrier@gmail.com

Tel.: +91-9442918647; +91-4222484167

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Email: ukaaz@yahoo.com; Website: www.ukaazpublications.com

internodes and modified flowering (Tepfer 1984). The wild-type *A. rhizogenes* strains have been used to improve rooting ability in

woody species as T-DNA genes on Ri plasmid influence root system development (Tepfer, 1983).

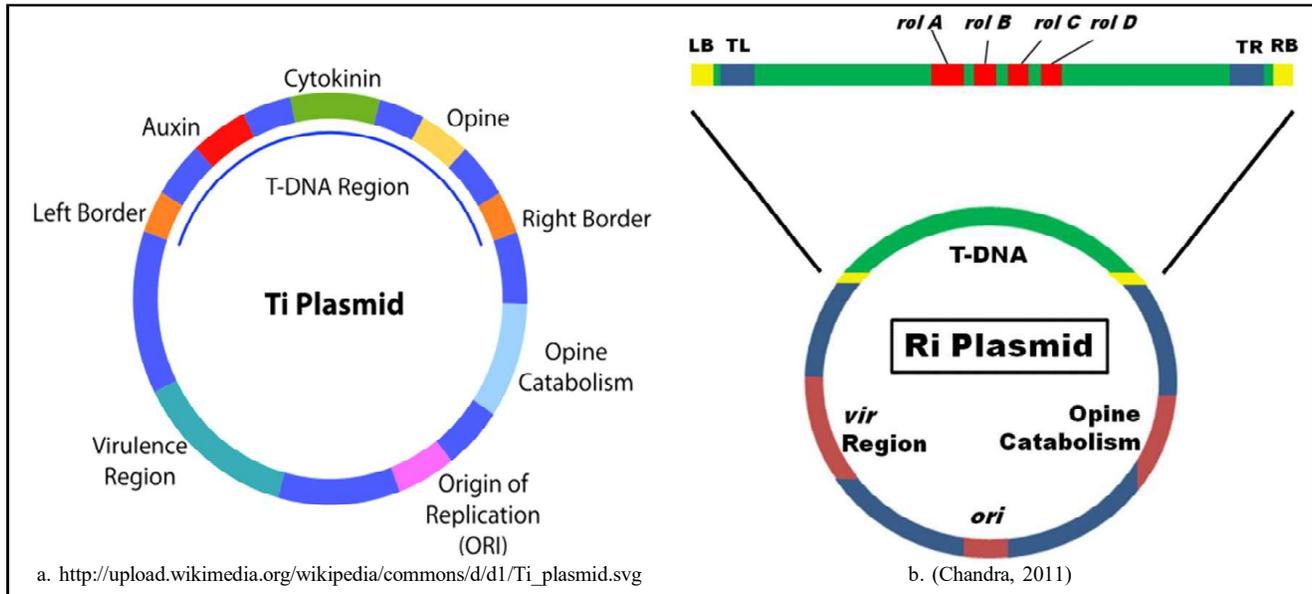


Figure 1: a. Ti and b. Ri Plasmids.

Many perennial trees have yielded therapeutic molecules for the treatment of many dreaded diseases like Taxol from *Taxus* spp. (for cancer treatment), quinine from *Cinchona* spp. (for malarial treatment) and aspirin from Willow (as analgesic). The high-value secondary metabolites in these trees are generally synthesized during the later stage of life and are produced in very less amount, not sufficient to meet the commercial demand. The continuously increasing demand of these metabolites has posed a great threat to the existence of many of these trees and has pushed many of them under different threat status. In the search for alternatives to production of desirable medicinal compounds from plants, biotechnological approaches, particularly, plant tissue cultures, are found to have great potential as a supplement to traditional agriculture (Rao and Ravishankar, 2002). Inconsistent yields coupled with instability of undifferentiated cell cultures initiated the efforts for development of new stable methods for enhanced

production of secondary products, which led to hairy root cultures. Desired production of defined chemical products within carefully regulated, highly controlled microenvironment suspension cultures (Charlwood and Charlwood, 1991) were identified as the advantages of this technology. This method has become popular in the last two decades as the most popular method of producing plant secondary metabolites. *In vitro* regeneration of medicinal plants from various explants to enhance the production of secondary metabolites has led to high-quality plant based medicine (Birch, 1997). Today genetic transformation is used as a powerful tool for enhancing the production of novel secondary metabolites; especially by *Agrobacterium rhizogenes* induced hairy roots (Leena and Jaindra, 2003). Hairy roots have been found to be suitable for the production of secondary metabolites because of their stability and high productivity in hormone-free culture conditions. Some commonly used strains of *A. rhizogenes* are presented in Table 1.

Table 1: Commonly used strains of *A. rhizogenes*

Strain name	Plasmid	Opines	Medium	Storage condition	Growth conditions	Reference
8196	pRi8196	Mannopine	Nutrient agar or nutrient broth	Frozen: -80°C or colder Freeze-Dried: 2°C to 8°C	Temperature: 26°C Aerobic condition	Dommissie <i>et al.</i> (1990)
ATCC 15834	pRi15834	Agropine				(Dommissie <i>et al.</i> (1990)
TR 105	Uncharacterised Ri Plasmid	Agropine				(Dommissie <i>et al.</i> (1990)
ATCC 43057	-	Agropine				Tepfer D <i>et al.</i> (1984)
AR1000	ATCC 15834 containing pARII	Agropine	YEB agar medium			(Dommissie <i>et al.</i> (1990)
LBA 9402	pRi1855	Agropine				Nguyen <i>et al.</i> (1992)
A4	Agropine type strain	Agropine				Vervliet <i>et al.</i> (1975)
1855	pRI-1855	Mannopine				Spano <i>et al.</i> (1982)

2. Agrobacterium mediated transformation in medicinal trees

Reports on induction of hairy roots and production of secondary metabolites are detailed below.

2.1 *Fagara zanthoxyloides*

Hairy roots of *Fagara zanthoxyloides* were obtained with the *A. rhizogenes* 9402 and a reduction of opine synthesis was observed. The infection produced numerous lateral branches, in both solid and liquid medium. The transformed roots produced the potent antileukemic fagaronine and the protein kinase C inhibitor chelerythrine, suggesting normal root metabolism. Bioefficacy was confirmed by studying the differentiation and growth inhibition of the K562 human erythroleukemic cells. Tryptophan and tyrosine regulated the chorismate mutase enzyme activity in the hairy roots (Couillerot *et al.*, 2000).

2.2 Henna (*Lawsonia inermis*)

Lawsonia inermis (Lythraceae) hairy roots were developed using *A. rhizogenes* NCIB 8196 by Bakkali *et al.* (1997) using leaf segments in half strength MS media containing 0.5 g/l of Claforan maintained at 25°C in the dark for 4 weeks. The pigment accumulation in hairy root cultures provides the possibility of independently studying morphological differentiation and biochemical differentiation. It is worth mentioning here, that root from whole plants were essentially lawsone-free. The hairy root cultures also accumulated (+)-catechin (1), 1, 2, 3, 6-tetra-O-galloyl-β-D-glucose (2) and 1, 2, 3, 4, 6-penta-O-galloyl-β-D-glucose (3) and galloylglucoses.

2.3 *Taxus* (Yew)

Kim *et al.* (2009) found that the hairy root cultures were established in *Taxus cuspidata* (Korean yew) using R1000, A4, and 15384. The hairy root line RC 11106 was found to be the most appropriate. Methyl jasmonate induction accumulated 52.5 mg/l of taxol over 2 weeks of incubation at a 20-L culture scale (Han *et al.*, 2000). Root formation in *Taxus mairei* induced by *Agrobacterium rhizogenes* (R1600, R1601, and R15834) revealed high 10-deacetylbaicatin (10-DAB) content in the roots (Chen *et al.*, 2004).

2.4 *Cinchona officinalis*

Cinchona officinalis (Rubiaceae) leaves cocultivated with strain LBA 9402 produced quinine for one year (Geerlings *et al.*, 1999). They also reported that hairy root lines of *C. officinalis* showed stable growth and morphology, and capacity of alkaloid production upto one year.

2.5 *Rauvolfia serpentina*

Ray *et al.* (2014) reported retention of stable phenotype of Ri-transformed root lines of *R. serpentina* over 3 years of *in vitro* culture. The phenotype of transformed root lines included creamish roots with a high degree of branching, plagiotropic growth, and devoid of extensive root hairs. Long-term stability of hairy root cultures of *R. serpentina* is also reported by Pandey *et al.* (2014) recording more than 6 years of *in vitro* culture. *R. serpentina* hairy roots (Ri-A4 transformed roots) produce considerably higher reserpine content than the normal roots after 10 weeks of growth. Ray *et al.* (2014) reported higher reserpine (approximately 3 mg/g

DW) content in the LBA 9402 root lines. Pandey *et al.* (2014) reported the production of all three major terpene alkaloid in *R. serpentina*, namely reserpine, ajmaline, yohimbine; yohimbine concentration being highest followed by ajmaline and reserpine.

2.6 *Morus alba*

Three *Agrobacterium rhizogenes* strains were tested for the secondary metabolites (betulin and betulinic acid) production and transformation capacity of *Morus alba* L. All the tested strains of *A. rhizogenes* (R1601, LBA9402 and R1000) were able to induce hairy root formation in leaf tissue explants (Park *et al.*, 2017). Result shows that betulin and betulinic acid production in hairy roots was found highest (167.8 ± 14.5 mg/flask) with strain LBA9402. Maximum production of betulin (8.7 ± 0.4 mg/g) and betulinic acid (4.1 ± 0.2 mg/g) was achieved with yeast extract and silver nitrate induction, respectively.

2.7 *Camptotheca acuminata*

Lorence *et al.* (2004) experimented the establishment of Hairy root cultures of *C. acuminata* by transformation of tissue with *Agrobacterium rhizogenes* strains ATCC 15834 and R-1000. The presence of rol genes in the plant genome that responsible for the hairy root phenotype was detected by southern blotting techniques. The hairy roots produced and secreted camptothecin (CPT) as well as the more potent and less toxic natural derivative, 10-hydroxycamptothecin (HCPT), into the medium. Remarkably, the cultures were able to synthesize the alkaloids at levels equal to, and sometimes greater than, the roots *in planta*, i.e., 1.0 and 0.15 mg/g dry weight for CPT and the HCPT, respectively.

2.8 *Nothapodytes nimmoniana*

Chang *et al.* (2014) studied the hairy root induction in *Nothapodytes nimmoniana* by cocultivating te stem and leaf segments with AR281, AR1600, and ATCC15834. Results showed variation among *A. rhizogenes* strains and between *N. nimmoniana* clones *in vitro*. Strain ATCC15834 had the highest transformation ability. Three fast-growing hairy root lines were used for growth and alkaloid production. The growth index of hairy roots was 3 and camptothecin (CPT) produced was 0.0537 - 0.1555% dry weight (DW), after 40 days of culturing. Growth was faster in liquid than in solid medium (9.8 mg/l). Approximately 93.9% (9.2 mg/l) of the total CPT produced was excreted into the culture medium.

2.9 *Azadirachta indica*

Azadirachta indica was transformed using *Agrobacterium rhizogenes* strain LBA920 (Srivastava, 2011). Hairy root inoculum (3 g/l dry weight) produced 13.3 g/l after 25 days with an azadirachtin yield of 3.3 mg/g root biomass in the shakeflask experiments and in the batch cultivation the biomass production was 9.8g/l dry weight accumulating 2.8 mg/g azadirachtin biomass equal to volumetric productivity of 1.09 mg/l azadirachtin per day.

2.10 *Gmelina arborea*

Dhakulkar *et al.* (2005) experimented hairy root induction using the *Agrobacterium* strain ATTCC 15834. Six different hairy root lines with 32 per cent success were produced with unique transformation sequences.

2.11 *Semecarpus anacardium*

In this species, optimization of the culture conditions and transformation frequency were studied by Panda *et al.* (2017) using *Agrobacterium rhizogenes* strain A4, ATCC15834 and LBA9402. The transformation resulted in hairy root initiation and 60 per cent transformation frequency was achieved in ATCC15834 strain. The other strains A4 and LBA9402 showed only 49 and 36 per cent transformation efficiency. PCR analysis confirmed the presence of gene specific primers *rolA*, *rolB*, *rolC* genes.

3. Conclusion

Genetic transformation can provide increased and efficient system for *in-vitro* production of secondary metabolites. This technique can be commercially exploited for secondary metabolites production through improved systems. The paper collates information on medicinal trees where this technology has been adopted for the production of secondary metabolites. Low success rate and recalcitrance of most species to transformation remains a major challenge for adoption of this technology in trees for production of the active principles. Once this challenge is overcome, it would be easier to adopt advanced technologies like trans-grafting, accelerated breeding, cis and intra genesis to explore production of new metabolites, and genome editing more frequently for enhancing metabolite production and providing quality medicine worldwide.

Conflict of interest

The authors declare that there are no conflicts of interest in the course of conducting the research. All the authors had final decision regarding the manuscript and decision to submit the findings for publication.

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