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REVIEW

Natural plant genetic engineer *Agrobacterium rhizogenes*: role of T-DNA in plant secondary metabolism

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Abstract *Agrobacterium rhizogenes* is a natural plant genetic engineer. It is a gram-negative soil bacterium that induces hairy root formation. Success has been obtained in exploring the molecular mechanisms of transferred DNA (T-DNA) transfer, interaction with host plant proteins, plant defense signaling and integration to plant genome for successful plant genetic transformation. T-DNA and corresponding expression of *rol* genes alter morphology and plant host secondary metabolism. During transformation, there is a differential loss of a few T-DNA genes. Loss of a few ORFs drastically affect the growth and morphological patterns of hairy roots, expression pattern of biosynthetic pathway genes and accumulation of specific secondary metabolites.

Keywords *Agrobacterium* · Plant secondary metabolism · Ri Plasmid · *Rol* genes · T-DNA · Virulence proteins

Introduction

Plant genetic transformation refers to the introduction and integration of "foreign" DNA in the plant cells and subsequent regeneration of transgenic plants. Tremendous progress in the development and production of transgenic plants and releasing them in the market has been achieved through major achievements in the DNA delivery systems by *Agrobacterium* and direct DNA transfer methods in various crop plant species. Research is now focused on stable integration of delivered DNA and successful expression of transgene. With this technology, the construction of organisms with novel genes has become possible. Transgenic organisms allow scientists to cross the physical and genetic barriers that separate pools of genes among organisms. Genetically-modified crops are in use commercially all over the world for more than a decade to improve various agricultural, nutritional and food processing traits, such as insect, herbicide, and virus resistance, vitamin enrichment, and controlled fruit ripening (James 2006). With genome sequences for plant species becoming rapidly available by the use of functional genomics approaches, efficient plant transformation systems are essential for the application of genomic sciences to understand physiological, biochemical, and molecular mechanisms of metabolic pathways. The technology of plant transformation is only moderately successful in many agronomically important crops, which is a major limiting factor for the biotechnological exploitation of economically important plant species and the wider application of genomic sciences (Dan et al. 2009).

However success has been obtained in the field of plant transformation methods in recent years through

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various gene transfer methods like particle bombardment, electroporation and by the use of a natural plant genetic engineer i.e. *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. Particle bombardment and electroporation transformation procedures are very effective for transient expression. They do not show any plant host range problems and bear high DNA delivery rate. But the disadvantage is that copy number of DNA insertions can be high and may lead to gene silencing/co-suppression, where as utilizing *Agrobacterium* based plant transformation methods, it is very effective, low cost and simple to use and can be used for germ line transformation. The copy number of DNA insertion is often low. Its only disadvantage is that host range may be limited by the plant hypersensitive response (Lessard et al. 2002).

Agrobacterium genus and host range

Agrobacterium genus has a number of species, *A. radiobacter* is an "avirulent" species, *A. rhizogenes* causes hairy root disease, *A. tumefaciens* causes crown gall disease, and *A. rubi* causes cane gall disease. *Agrobacterium vitis* causes galls on grape and a few other plant species (Miranda et al. 1992). Depending upon the type of *Agrobacterium* plasmid present within a particular strain, the properties develop accordingly. Curing and replacing the original plasmid with another property-bearing plasmid can alter the disease symptoms. For example, infection of plants with *A. tumefaciens* C58, containing the nopaline-type Ti plasmid pTiC58, results in the formation of crown gall teratomas. When this plasmid is cured, the strain becomes non-virulent. Introducing Ri (root inducing) plasmids into the cured strain converts the bacterium into a rhizogenic strain (Lam et al. 1984). *Agrobacterium* has a wide range of host infection including transfer of DNA to human cells (Kunik et al. 2001), sea urchin embryos (Bulgakov et al. 2006), angiosperms, gymnosperms, fungi, including yeasts, ascomycetes and basidiomycetes (Gelvin 2003). Several monocotyledonous plants like rice, corn, wheat, sugarcane, *Linum flavum* have been transformed successfully. Reports are also available for efficient transformation in *Typha latifolia* (Nandakumar et al. 2005), *Alstroemeria* (Akutsu et al. 2004) and Banana (Matsumoto et al. 2009). Often the regeneration rates are poor with monocot plants which is further reduced under selection during transformation. Problems and possibilities of monocot transformation have been recently reviewed by Sood et al. (2011). The poor regeneration in monocot species has led to the development of germ line transformation strategies. Germ line transformation is supposed to serve as a means to overcome this limitation by directly transforming germ cells. Success has been achieved in *Arabidopsis thaliana* in which flowers were dipped into solution containing *Agrobacterium* in the presence of surfactants (Tague 2001). Transgenic seeds were produced directly but at a low frequency.

The discovery of hairy roots

The term 'hairy root' was mentioned for the first time by Stewart et al. (1900) as far back as, cited by Hildebrandt (1934). The distinctive symptom of hairy root disease is the formation of a mass of roots. A large number of small roots protrude as fine hairs directly from the infection site in response to *A. rhizogenes* infection, a phenomenon that gave rise to the term 'hairy root'. Riker et al. (1930) and his associates described and named the hairy root causing organism *Phytomonas rhizogenes*, which was renamed *Agrobacterium rhizogenes* by the same group.

Ri plasmid of *Agrobacterium rhizogenes*

Virulent strains of *Agrobacterium* contain tumor-inducing (Ti) or Ri plasmids (Gelvin 2009). *A. rhizogenes* contain Ri plasmid possessing different gene segments (Fig. 1). The transferred DNA (T-DNA) is referred to as the T-region when located on the Ti or Ri plasmid. During infection with *Agrobacterium*, a piece of DNA is transferred from the bacterium to the plant cell (Fig. 2). This piece of DNA is a copy of segment called T-DNA (Chilton et al. 1982). T-DNA is part of the approximately 200 kb Ti/Ri plasmid present in *Agrobacterium* which encodes functions for Ti/Ri plasmid conjugation, opine synthesis and catabolism and the initiation, transfer and integration of the T-DNA. T-regions on native Ti and Ri plasmids are approximately 10–30 kbp in size. T-regions are defined by T-DNA border sequences. These borders are 25 bp in length and highly homologous in sequence. They flank the T-region in a directly repeated orientation (Gelvin 2003).

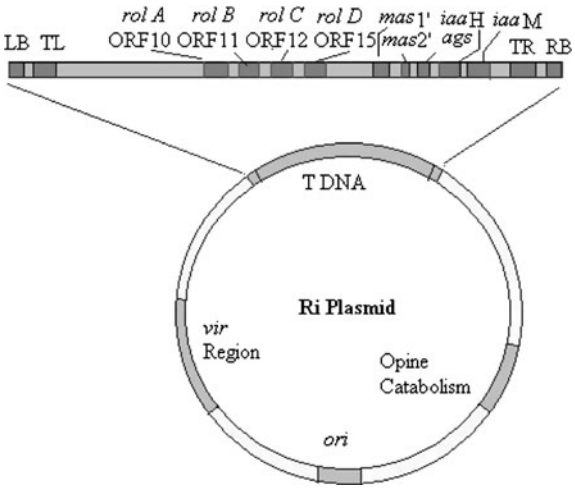

Fig. 1 Schematic representation of Ri Plasmid of *A. rhizogenes*

When T-DNA is integrated into the plant cell genome, the T-DNA expresses enzymes that direct the production of opines, which are synthesized and excreted by the transformed cells and consumed by *Agrobacterium* as nutrient source (Dessaux et al. 1992). Ri plasmids can be classified according to the opines produced. In nopaline Ti plasmid, mannopine and cucumopine Ri plasmid types, a single T-DNA has been found, whereas in octopine (*pTi*) and agropine (*pRi*) types (Fig. 1), two regions (TL-DNA and TR-DNA) have been identified (Trovato and Linhares 1999). Two T-DNAs are separated from each other by about 15 kb of non-transferred DNA. The right T-DNA (TR) contains genes homologous to the T-DNA from Ti plasmids (Huffman et al. 1984; Jouanin 1984). The left T-DNA (TL) of agropine Ri-plasmid A4b is about 20 kb in length (Huffman et al. 1984). Mannopine and cucumopine T-DNAs share with the agropine type TL-DNA two strongly conserved segments which flank only partially homologous central region (Filetici et al. 1987; Brevet and Tempe 1988). In the central, less conserved region of the TL-DNA of agropine T-DNA, the root-inducing (*rol*) genes are located. TR-DNA contains two genes, *iaaM* and *iaaH*, responsible for the biosynthesis of auxins (De Paolis et al. 1985) and the genes responsible for the synthesis of the opines mannopine (*mas1′* and *mas2′*) and agropine (*ags*) (Fig. 1). Both TL-DNA and TR-DNA are transferred and integrated independently into the host plant genome, but the transfer of TL-DNA is essential for induction of the hairy root syndrome, and transfer of TR-DNA does not provoke formation of roots from transformed cultures (Nilsson and Olsson 1997; Sevon and Oksman-Caldentey 2002). Genes encoded in T-DNA are of bacterial origin but have eukaryotic regulatory sequences enabling their expression in infected plant cells (Mishra and Ranjan 2008). Molecular biology of Ri plasmid has been reviewed by Sinkar et al. (1987). Detailed information regarding the mechanism involved in the genetic transfer can be referred in literatures by White et al. (1985); Gelvin (2000) and Tzfira et al. (2004).

Virulence proteins and T-DNA transfer

For successful transfer of T-DNA, *Agrobacterium* first attaches to the plant cell walls. This is facilitated by the products of *Agrobacterium* chromosomal genes *chvA* and *chvB* genes. *chvB* gene encodes a protein involved in the formation of a cyclic β-1,2 glucan into the periplasm i.e. (space between the cell wall and plasma membrane), which helps in the attachment of *Agrobacterium* to the plant cell. Several plant proteins are required for the attachment of bacterium to the plant cells. Details can be found in a recent review by Gelvin (2010). Generation of single strand T-strand molecules (ssT-strand) and T-strand transport to the plant cells occurs mainly due to the activity of *Agrobacterium* virulence (vir) proteins. VirD1 and VirD2 nick the Ti/Ri plasmid at T-DNA border repeat sequences, following which VirD2 covalently binds to the 5′- end of the resulting T-strand. VirD2/T-strand leaves the bacterium through a Type IV protein secretion system (T4SS), which is made up of 11 Vir B proteins and VirD4. T4SS includes a membrane transport channel (Fig. 2) and a T-pilus. VirB2 is a major pilin protein involved in the transfer of T DNA and vir proteins to plant cells. Role of VirB5 has not been explored in any detail. After getting inside the cytoplasm, virulence effector proteins and TDNA strand target the nucleus. Virulence effector proteins contain nuclear localization signal (NLS) sequences which are important for nuclear targeting. Each T-strand is coated with several VirE2 molecules in the plant cell and they help in targeting the nucleus. T-strands with associated vir proteins interact with plant proteins and form super T complexes. Importin α protein (plant protein) helps in nuclear targeting. VirE2 and VirE3 protein interact with importin α protein and help in nuclear targeting. Histone proteins

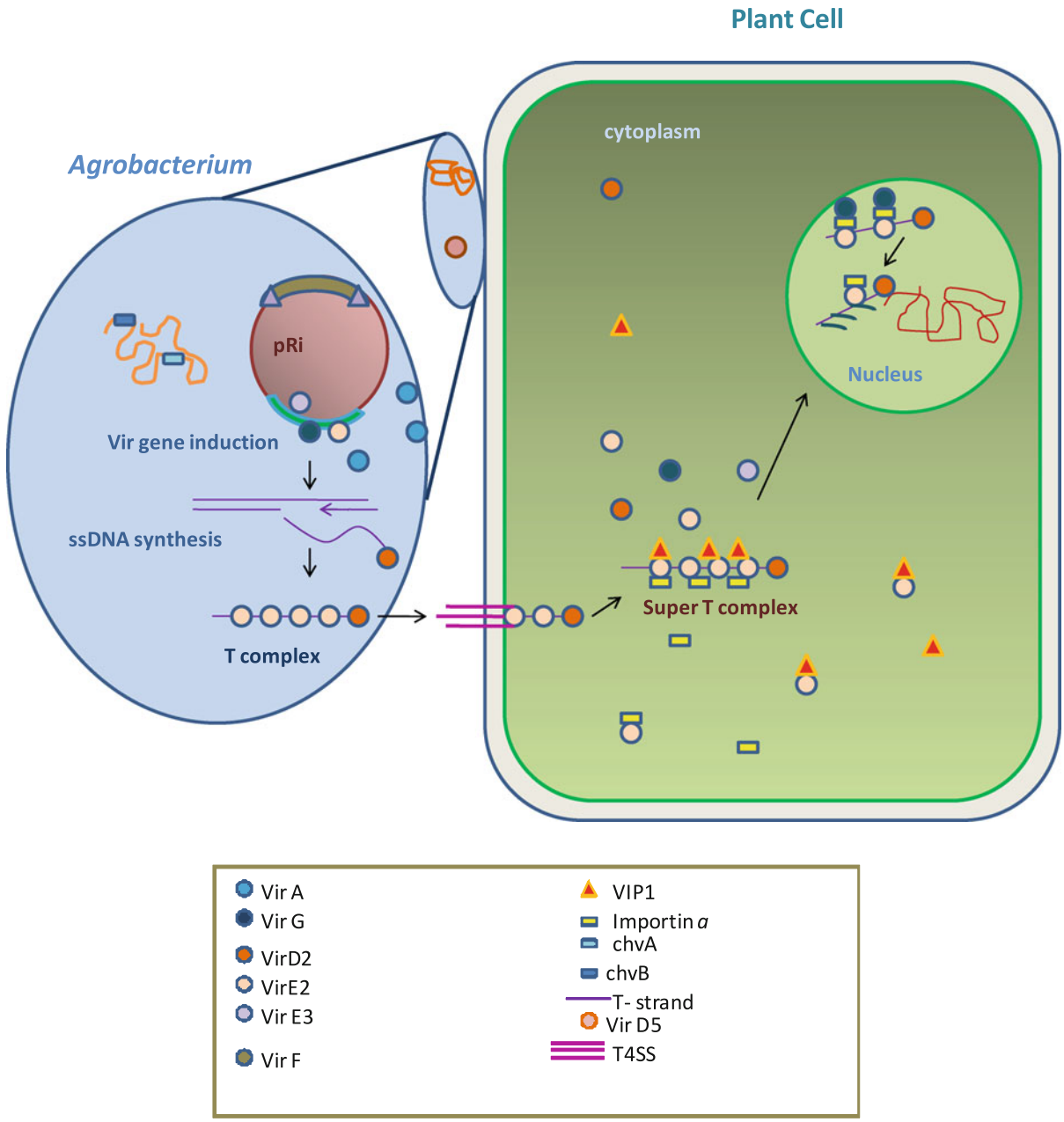

Fig. 2 *Agrobacterium* Ri plasmid based T-DNA transfer for plant genetic transformation. Names of virulence effector proteins and plant proteins are shown in *box*

also help in protecting the transferred T-DNA strand. During integration of T-strand to host plant genome both vir effector proteins and plant proteins are removed from T-strands. VirF protein has an important role in removal of proteins from T complex. Some *Agrobacterium* strains lacking expression of VirF proteins are weakly virulent on some plant species.

Agrobacterium and plant defense signaling

Hairy roots are produced as a response to integration of the wild-type T-DNA of *A. rhizogenes* into the plant genome. Hairy-root cultures, derived from various plants species, stably produce high amounts of secondary metabolites (Giri and Narasu 2000;

Table 1 Secondary metabolites produced using hairy root cultures

Metabolites	Plant species	Family	References
Ajmalicine, Ajmaline	*Rauvolfia micrantha*	Apocynaceae	Sudha et al. (2003)
Anthraquinones	*Rubia cordifolia*	Rubiaceae	Shin and Kim (1996)
Camptothecin	*Camptotheca acuminata*	Nyssaceae	Lorence et al. (2004)
Ginkgolides	*Ginkgo biloba*	Ginkgoaceae	Ayadi and Tremouillaux-Guiller (2003)
Ginsenosides	*Panax ginseng*	Araliaceae	Kunshi et al. (1998)
Morphine, codeine	*Papaver somniferum*	Papaveraceae	Bonhomme et al. (2004)
Isoflavones	*Psoralea corylifolia*	Fabaceae	Shinde et al. (2009)
Glycyrhizin	*Glycyrrhiza glabra*	Fabaceae	Mehrotra et al. (2008)
Plumbagin	*Plumbago rosea*	Plumbaginaceae	Satheeshkumar et al. (2009)
Podophyllotoxin	*Podophyllum hexandrum* Royle	Berberidacae	Li et al. (2009)
Tylophorine	*Tylophora indica*	Asclepediaceae	Chaudhuri et al. (2006)
Plumbagin	*Drosera burmanii*	Droseraceae	Putalun et al. (2010)
Tropane alkaloids	*Przewalskia tangutica*	Solanaceae	Lan and Quan (2010)

Sevon and Oksman-Caldentey 2002; Guillon et al. 2006; Georgiev et al. 2007; Srivastava and Srivastava 2007; Shilpa et al. 2010, Chandra and Chandra, 2011). Table 1 lists few secondary metabolites produced in hairy root cultures. For further details please see the recent review by Chandra and Chandra (2011).

The amount of secondary metabolites (phytoalexins) increases when the plant is damaged by pathogens. The general mechanism of the activation of plant defense involves: 1) detection of pathogen signal; 2) activation of H^+-ATPase; 3) increase in Ca^{2+} influx from an intercellular space into the cells; 4) activation of calcium dependant protein kinase (CDPK); 5) activation of NADPH oxidase. Active oxygen radicals generated by NADPH oxidase participate in the activation of MAP kinases, which leads to the increased expression level of certain protective genes, including the genes of biosynthesis of secondary metabolites (Bulgakov et al. 2003). Other important role in the activation of secondary metabolite synthesis is also played by the jasmonic acid signaling pathway (Blechert et al. 1995) and in some cases by signaling pathways of salicylic acid and ethylene (Cho et al. 1988). Bulgakov et al. (2003) have illustrated that *rolB* and *rolC* genes in *Rubia cordifolia* strains activate the synthesis of anthraquinones, typical plant phytoalexins (Bulgakov et al. 2002). Ethylene did not affect anthraquinone synthesis either in the transformed or in non transformed cultures. Jasmonic and salicylic acids increased the content of anthraquinones in both transgenic and normal strains with a similar pattern. This indicates that pathways of ethylene, jasmonic, and salicylic acid are not involved in the activator function of *rol* genes (Bulgakov et al. 2002). *Agrobacterium* attachment inhibits plant defense signaling (Anand et al. 2008). At AGP17 also plays role in suppression of host defense response as demonstrated in *Arabidopsis* with decreased level of Salicylic acid (SA) content (Gelvin 2010). Plant defense responses play role in early stages of transformation. Plants with reduced defense response by inactivation of the SA signaling pathway shows hypersusceptibility towards *Agrobacterium* mediated transformation, whereas plants with elicited defense response shows resistant to transformation. *Rol* genes mediate uncommon signal transduction pathways in plants. They act on phytoalexin production independently of plant defense hormones and the calcium-dependent NADPH oxidase pathway (Bulgakov 2008). In some cases, *rol* genes transformation provoked a biphasic effect with initial suppression and subsequent activation of biosynthesis for particular groups of secondary metabolites (Bulgakov 2008; Bulgakov et al. 2005, 2008).

Effect of T-DNA on plant secondary metabolism

Agrobacterium rhizogenes rolA, rolB and *rolC* oncogenes have always been considered to be modulators of plant growth and cell differentiation. These *rol*

genes are potential activators of secondary metabolism in transformed cells from the Solanaceae, Araliaceae, Rubiaceae, Vitaceae and Rosaceae families (Bulgakov 2008). Shkryl et al. (2008) studied activity of *rol* genes individually and their combined action on secondary metabolism. Individual *rolA*, *rolB*, and *rolC* genes are capable of increasing biosynthesis of anthraquinones (AQs) in transformed calli of *R. cordifolia*. Investigations revealed that the *rolA*, *rolB*, and *rolC* genes, integrated into the DNA of *R. cordifolia* cells individually or as the *rolABC* combination had a stimulatory effect on AQ production. Activation of AQ production in *rol*-transformed cells of *R. cordifolia* seems to be caused by the activation of isochorismate synthase (*ICS*) gene expression, because high correlation was found between the expression of the *rolB* and *rolC* genes and expression of the *ICS* gene.

In transformed plant cell cultures, the *rolC* gene alone can stimulate production of tropane alkaloids (Bonhomme et al. 2000a), pyridine alkaloids (Palazon et al. 1998a), indole alkaloids (Palazon et al. 1998b), ginsenosides (Bulgakov et al. 1998), and anthraquinones (Bulgakov et al. 2002, 2003). The *rolB* gene activates production of anthraquinones (Bulgakov et al. 2002, 2003) and stilbenes (Kiselev et al. 2007). The stimulatory effect of the *rolA* gene on nicotine production was also observed by Palazon et al. (1997). However, *rolA* and *rolB* failed to stimulate ginsenoside production in transformed ginseng calli (Bulgakov et al. 1998) and, similarly, the production of caffeic acid metabolites was clearly reduced in *rolC* transformed callus cultures of *Eritrichium sericeum* and *Lithospermum erythrorhizon* (Bulgakov et al. 2005).

Bonhomme et al. (2000a) studied the effect of *rol* genes in secondary metabolites production. Two series of *Atropa belladonna* hairy root lines were obtained: the first transformed via *A. tumefaciens* harboring *rolC* and *npt II* genes, and the other transformed with *rolABC* and *npt II* genes. Hyoscyamine and scopolamine production was measured after 3 and 4 weeks of culture to evaluate the possible role of *rolC* gene in tropane alkaloid formation. The *rolC* gene alone played a significant role (17-fold increase) in the hairy root growth rate. However the *rolABC* genes together led to a much higher (75-fold increase) increase in hairy root growth rate. In contrast, the *rolC* gene alone was as efficient as the *rolABC* genes together (mean value of total alkaloids: 0.36% dry weight, i.e., 12-fold more than in untransformed roots) to stimulate the biosynthesis of tropane alkaloids in *A. belladonna* hairy root cultures. A correlation exists between the expression of the *rol*C gene and tropane alkaloids (Pinol et al. 1996; Bonhomme et al. 2000a, b), *Catharanthus roseus* alkaloids (Palazon et al. 1998b) and ginsenoside production (Bulgakov et al. 1998).

Moyano et al. (1999) showed that the inoculation of leaf sections of tobacco, *Duboisia* hybrid and *Datura metel* plants with the A4 strain of *A. rhizogenes* induced transformed roots with the capacity to produce alkaloids such as nicotine, hyoscyamine and scopolamine. The obtained hairy roots showed two morphologies: typical hairy roots with a high capacity to produce alkaloids, and callus-like roots with faster growth and lower alkaloid production. The *aux*1 gene located in the TR-DNA of *A. rhizogenes* was detected in all roots showing callus-like morphology. However, this gene was only detected in 25–60% of the established root cultures showing typical hairy morphology. These studies demonstrated a significant role of *aux* genes in the morphology of transformed roots and the importance of typical hairy root morphology in the production of scopolamine. The studies with *Panax ginseng* hairy roots also support the effects of the genes located in the TR-DNA on root morphology and secondary metabolism (Mallol et al. 2001).

Effect of loss of T-DNA genes on plant secondary metabolism

During transformation, there is a differential loss of a few T-DNA genes. To decipher the effect of loss of T-DNA genes on the various aspects of hairy roots in *Catharanthus* hairy root cultures, ten hairy root clones were analyzed for the presence or absence of T-DNA genes and its implications. The loss of a few ORFs drastically affected the growth and morphological patterns of hairy roots. The absence of TR-DNA from hairy roots revealed increased transcript accumulation and higher alkaloid concentrations, whereas callusing among hairy root lines led to decreased transcript and alkaloid accumulation. Not only is the integration of T-DNA at certain regions of host plant genome important but also the presence or absence of important ORFs that affects the expression patterns of biosynthetic pathway genes, regulators, and accumulation of specific alkaloids (Taneja et al. 2010).

Limitations

Although *A. rhizogenes*-mediated transformation has several applications it still has certain limitations. These include the genotype, wounding of plant tissue, synthesis of phenolic *vir* gene inducers by the plant, bacterial attachment, T-DNA transfer into the plant cytoplasm, T-DNA nuclear translocation and T-DNA integration (Gelvin 2000), the density of the bacterial suspension (Park and Facchini 2000).

Temperature also influences the rate of transfer of T-DNA (Salas et al. 2001): 25°C appears beneficial for plant cell susceptibility to infection and for stable T-DNA insertion into the plant chromosomes. A number of chemicals may also promote these processes, e.g., acetosyringone (Joubert et al. 2002). Ultrasonication can deliver and foreign DNA into protoplasts (Kumar et al. 2006). High salt media favor hairy root formation in some plants. Low salt medium favours excessive bacterial multiplication in the medium and so the explant needs to be subcultured several times to fresh antibiotic containing medium before incubating it (Mishra and Ranjan 2008).

Conclusion and future prospects

To date rapid success has been obtained in exploring the molecular mechanisms of T-DNA transfer, interaction with host plant proteins, their role in plant defense signaling and integration to plant genome for stable gene transfer for successful plant genetic transformation. T-DNA and corresponding expression of *rol* genes alter morphology and plant host secondary metabolism. From being a research curiosity, plant transformation technology has now reached a platform of commercial reality. T-DNA and *rol* genes have effect on plant secondary metabolism. There are still certain practical limitations in cases of recalcitrant species. There is scope for improvement in foreign gene transfer and expression methods. Removal of extra DNA that is not necessary in the final product i.e. selectable marker genes is the key area for future development. Advances in molecular biology and genetic techniques will help scientists in exploring more about bacterial and host proteins interaction and also efficient transformation in recalcitrant species. Still many questions need to be answered about the manipulation of host metabolism by the *Agrobacterium* for its advantage, interaction of *Agrobacteria* with other organisms in the rhizosphere, manipulating both *Agrobacteria* and host genome for high expression of transgene, effect of *Agrobacteria* on horizontal gene transfer among different plant communities.

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