Chapter 1
Agrobacterium rhizogenes-Mediated Transformation and Its Biotechnological Applications in Crops

Ibrahim Ilker Ozyigit, Ilhan Dogan and Ebru Artam Tarhan

Abstract The history of *Agrobacterium*-related plant biotechnology goes back for more than three decades with the discovery of molecular mechanisms of crown gall disease in plants. After 1980s, gene technologies began developing rapidly and today, related with the improved gene transfer methods, plant biotechnology has become one of the most important branches in science. Till now, the most important genes related with agricultural affairs have been utilized for cloning of plants with the deployment of different techniques used in genetic engineering. Especially, *Agrobacterium tumefaciens* was used extensively for transferring desired genetic materials to plants rapidly and effectively by the researchers to create transgenic plants. Recognition of the biology of *Agrobacterium* species and newly developed applications of their T-DNA systems has been a great step in plant biotechnology. This chapter provides the reader with extensive information on *A. rhizogenes* which is responsible for the development of hairy root disease in a wide range of dicotyledonous plants and its T-DNA system. This knowledge will be useful in improving utilization of crops and the formulation of new and up-graded transgenic based food products.

Introduction

The increase in demand for food is dramatic with an expanding population growth in the world. According to latest projections, continued increase at the current rate of the population is expected to reach between 7.5 and 10.5 billion by

I. I. Ozyigit (✉) · E. Artam Tarhan
Department of Biology, Faculty of Science & Arts, Marmara University,
Goztepe, 34722 Istanbul, Turkey
e-mail: ilkozyigit@marmara.edu.tr

I. Dogan
Department of Molecular Biology and Genetics, Faculty of Science,
Izmir Institute of Technology, 35430 Urla, Izmir, Turkey
e-mail: ilhandogan@iyte.edu.tr

E. Artam Tarhan
e-mail: eartamtarhan@gmail.com

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2050 (Census 2012). Climate changes in terms of shifting weather patterns will result in decreased water availability and in conjunction with this, providing food for this inevitable future population size will be a very hard task without adding new arable lands (Milly et al. 2005). To deal with this challenge one of the major solutions is plant breeding, which has been used since ancient times in order to create desired genotypes and phenotypes for specific objectives. The main goals of conventional plant breeding are improvement of crop yield and quality, agricultural convenience and resistance to the parasites. While the conventional plant breeding efforts used in the past were sufficient, nowadays with the increasing demand additional and supplementary technology necessities emerged (Gepts 2002). As a result of industrial revolution and its reflection to the biological and agricultural sciences, plant biotechnology reached spectacular success with understanding of how genes operate and function in plant. The first genetically modified crops were obtained in the early 1980s by using *Agrobacterium tumefaciens* following the plant regeneration systems, production of novel chimeric genes and transformation vectors. Multidisciplinary studies of academic institutions and agricultural seed companies took the leadership on genetic engineering and biotechnological progresses of crop plants (Özcan et al. 2004). Although, many political, regulatory, ethical and religious obstacles are still present, the adoption rate of crop biotechnology in the area of agriculture is high at global level. Crop biotechnology involves a different set of technologies such as industrial use of recombinant DNA, cell fusion and tissue engineering. *Agrobacterium*-mediated transformation has always been the most commonly used method for novel transgenic technologies. Till now, a number of commercially valuable crops like tomato, potato, rice, wheat, maize, cotton, soybean, alfalfa, barley, carrot, sugarcane, pepper and broccoli were obtained using *Agrobacterium*-mediated transformation (Ozyigit 2012).

Characteristics of *Agrobacterium rhizogenes*

Certain bacterial species are capable of transferring some of their genes to higher plants ending up with insertion and permanent integration in the nuclear genome (Broothaerts et al. 2005; Kumar et al. 2006). Members of genus *Agrobacterium* are widely known for their ability of forming a wide variety of different neoplastic diseases, including crown gall (*A. tumefaciens* and *A. vitis*), hairy root (*A. rhizogenes*) and cane gall (*A. rubi*) (Gelvin 2009; Ozyigit 2012). Among them, the first identified one was *A. rhizogenes* (formerly *Phytomonas rhizogenes*) in 1930s belonging to the family Rhizobiaceae in the alpha-2 subclass of Proteobacteria (Riker et al. 1930; Hildebrand 1934; Conn 1942; White 1972; Kersters and De Ley 1984; Woese et al. 1984; Willems and Collins 1993).

A. rhizogenes is a rod-shaped Gram-negative, non-spore forming (0.6–1 μm by 1.5–3.0 μm in size) soil bacterium that occurs singly or in pairs and is motile by means of one to six peritrichous flagella (Conn 1942; Meyer et al. 2000; Tzfira and

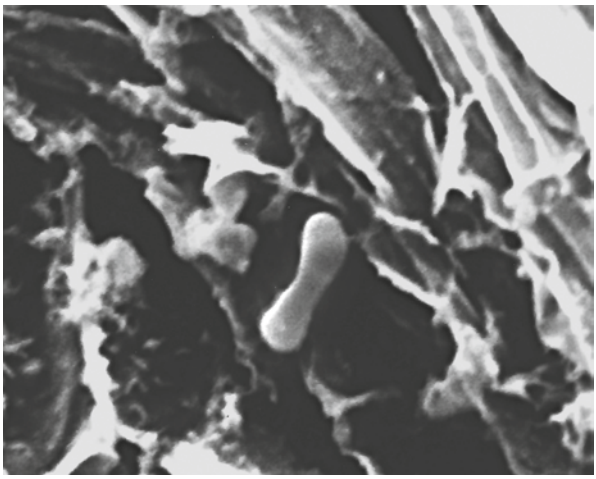

Fig. 1.1 Scanning electron micrograph of attachment of *Agrobacterium rhizogenes* strain R1000 to sunflower (*Helianthus annuus* L.) cotyledonary node cell

Citovsky 2000; Giri and Giri 2007; Murugesan et al. 2010) (Fig. 1.1). It is a close relative of the better known *A. tumefaciens*, which is the best-characterized species among the genus *Agrobacterium* (Rao 2009; Ozyigit 2012) (Fig. 1.1).

All *A. rhizogenes* strains are characterized by the presence of a large root inducing (Ri) plasmid containing a highly conserved "core" DNA region required for hairy root formation (Filetici et al. 1987; Gelvin 2003; Veena and Taylor 2007). Like the crown gall disease, which is caused by *A. tumefaciens* (Ream 2002; McCullen and Binns 2006; Ozyigit 2012) *A. rhizogenes* causes hairy root (root-mat) disease in infected plants through genetic transformation (Weller and Stead 2002; Weller et al. 2005).

Hairy Root Disease

The "hairy root" is the term first used in 1900 by Stewart et al. (as quoted by Hildebrandt 1934). The distinctive symptom of hairy root disease is the formation of a mass of roots. Following the *A. rhizogenes* infection, hairy root formation occurs as a result of protruding large numbers of small roots as fine hairs directly from the infection site (Chandra 2012) (Fig. 1.2). Besides the plagiotropic root growth, hairy-root disease is characterized as short internodes, a high degree of lateral branching, wrinkled leaves, reduced apical dominance, reduced fertility, profusion of root hairs, abnormal flower production, advanced flowering, increased number of flowers, enhanced growth rates and changed secondary metabolite accumulation (Ackermann 1977; Tepfer 1983; Balandrin et al. 1985; Charlwood and Charlwood 1991; Pellegrineschi et al. 1994; Flores et al. 1999; Lee et al. 2001; Keil 2002; Casanova et al. 2004; Veena and Taylor 2007) (Fig. 1.2).

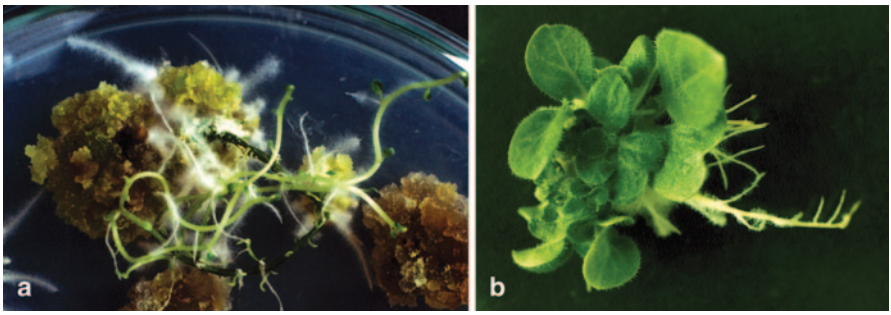

Fig. 1.2 Hairy root formation induced by *A. rhizogenes* strain 8196 in potato (*Solanum tuberosum* L.) callus cultures (**a**), regenerated tobacco (*Nicotiana tabacum* L.) plantlets (**b**). (From Arican)

In nature, when plants are suffering from wounds, phenolic compounds are released from wounded sides and that cause attraction for *A. rhizogenes*. The bacterium moves toward the wounded sites by chemotaxis and infect plant cells. Subsequent infection at wound site followed by transfer of a particular DNA segment (T-DNA) from the root-inducing (Ri) plasmid (pRi) of the bacteria (Kumar et al. 2006). *A. rhizogenes*-induced roots have the unique property of being able to grow *in vitro* without exogenous plant growth regulators (Lee et al. 2001; Rao and Ravishankar 2002). With this unique ability, by the utilization of *A. rhizogenes* strains in *in vitro* plant organ cultures, broad range difficulties were eliminated and as a result, fast growing organs with the capable of producing extensive branching and main metabolites even higher than the mother plant or new metabolites undetected in the mother plant or in other kinds of *in vitro* cultures were generated (Doran 2002; Nader et al. 2006; Bensaddek et al. 2008).

Over the three decades, hairy roots have been applied in a wide range of fundamental studies of plant biochemistry, molecular biology, and physiology, as well as for agricultural, horticultural, and large-scale tissue culture purposes (Doran 2002). In general, hairy root cultures have been used extensively in root nodule research (Diaz et al. 1989; Quandt et al. 1993; Diouf et al. 1995; Hu and Du 2006; Hirotaka and Hiroshi 2003; Aarrouf et al. 2012), production of artificial seeds (Uozumi and Kobayashi 1997), plant secondary metabolites and proteins (Aarrouf et al. 2012), plant breeding and plant improvement, experimental systems to study responses to chemicals (Downs et al. 1994; Mugnier 1997), plant morphology and development (Bandyopadhyay et al. 2007; Turgut-Kara and Ari 2008; Hasancebi et al. 2011; Aarrouf et al. 2012), detoxifing environmental pollutants (Rugh 2001), validate and analyze the functions of genes conferring resistance to root specific pathogens (Remeeus et al. 1998; Hwang et al. 2000; Alpizar et al. 2006; Aarrouf et al. 2012) and study interactions with other organisms such as nematodes (Kifle et al. 1999), mycorrhizal fungi and root pathogens (Mugnier 1997; Christey 2001). Besides these sights, enhanced rooting in plants helps establishment or surviving transplant shocks or abiotic stress like drought, salinity and heavy metal stress (Bulgakov, 2008; Li et al. 2011).

Fig. 1.3 Scanning Electron Micrograph of *A. rhizogenes* strain 8196 colonizing sunflower (*H. annuus* L.) cotyledonary node cell wall

The Mechanism of Hairy Root Formation

The overall process of hairy roots disease by *A. rhizogenes* wild strains is defined by the following four steps. Chimiotactism is the first step leading to induced movement of *Agrobacterium* towards to the plant cells. The following step is binding of *Agrobacterium* to the surface components of the cell wall (Fig. 1.3). After binding, transfer and integration of the transfer-DNA (T-DNA) into the plant genome is completed. The last step is subsequent induction of root formation and growth (Zupan et al. 1996). The information gained in the first three steps is better understood because of the similarities in biological processes and existing models of pathogenesis provided by extensive studies of *A. tumefaciens* stain C58 (Tomilov et al. 2007; Abarca-Grau et al. 2011). The compositions as well as structures are broadly similar for Ri and the Ti plasmids from *A. rhizogenes* and *A. tumefaciens*, respectively (Gelvin 2003; Ozyigit 2012) (Fig. 1.3).

Comparative studies showed a high degree of homology between Ri and Ti plasmids indicating that there are conserved regions between the two types of plasmids. This shows general mechanisms such as activation, processing, and movement of the T-DNA from the bacteria to the plant cell are highly sustained. A segment in both Ri and Ti plasmids called T-DNA consists of highly homologous 24-bp direct repeats known as border sequences (Yadav et al. 1982; Filichkin and Gelvin 1993; Ziemienowicz 2001; Veena and Taylor 2007; Chandra 2012). During infection with *Agrobacterium*, T-DNA is transferred from the bacterium to the plant cell (Rao et al. 2009). The wild-type T-DNA encodes oncogenes and opine catabolism genes, which cause neoplastic growth of tissues and the production of opines (Guyon et al. 1980, 1993; Costantino et al. 1994; Gaudin et al. 1994; Weising and Kahl 1996; Hong et al. 1997; Lee et al. 2001; Rao and Ravishankar 2002; Veena and Taylor 2007). Also, another segment known as the virulence (vir) region in the Ti-plasmid is involved in transferring of DNA into the plant genome (Bulga-

kov et al. 2004). Hairy roots are capable of growing in the absence of exogenous plant hormones on the plant cells due to the presence of T-DNA. *Agrobacterium* species are highly adapted for sophisticated parasitic relationship with host plants and thus found to establish a unique ecological niche by genetically engineering (Vilkar et al. 1987).

Gall Proteins

One of the similarities of Ri and Ti plasmid is that bearing nearly identical organization of the vir operons (Zhu et al. 2000). Only noticeable difference can be seen is neither genomes nor Ri plasmids of *A. rhizogenes* contains *vir*E1 and *vir*E2 genes (Moriguchi et al. 2001; Hodges et al. 2004). As known from studies about *A. tumafaciens* VirE2 is a single-stranded DNA binding protein and VirE1 acts as a chaperone of VirE2. The VirE2 covers single-stranded T-DNA (T-strands) from nuclease attack (Rossi et al. 1996; Ozyigit 2012) and involves nuclear import of T-DNA to the plant cells (Yusibov et al. 1994; Rossi et al. 1996; Zupan et al. 1996; Gelvin 1998). *vir*E genes play critical roles in pathogenesis of *A. tumefaciens* (Christie et al. 1988; Citovsky et al. 1992; Ward and Zambryski 2001; Duckely and Hohn 2003; Ozyigit 2012). However, the absence of *vir*E genes or no other homolog genes in the *A. rhizogenes* genome clearly shows that *vir*E genes are not necessary in the mechanism of hairy root induction (Moriguchi et al. 2001). Recent studies imply that despite sharing no homology, the *GALLS* gene located on the Ri plasmid can substitute VirE2 function in *A. tumefaciens* (Hodges et al. 2004). GALLS protein differs from VirE2 with ATP-binding and helicase motifs resembling to those in TraA protein involved in conjugation. Both GALLS and VirE2 contain nuclear localization sequences and a C-terminal type IV secretion signal. Mutations in these domains lead to loss of GALLS ability to substitute for VirE2 (Sinkar et al. 1988; Hodges et al. 2006). However, mechanism of GALLS protein in *A. rhizogenes* is still not fully known. All these facts reveal that in spite of differences in their virulence systems, the Ti and Ri plasmids are share a common ancestor. However, the way of T-DNA transfer and those other variations in T-DNA processing also show signs of independent evolution from each other. Current understanding of the molecular bases of the differences between hairy root and gall formation will be accelerated by further studies on genome sequencing and comparison of various *Agrobacterium* strains (Hodges et al. 2006).

Ri Plasmid

Ri plasmid in all *A. rhizogenes* strains has a region known as T-DNA which carries genes (*rol*-genes) involved in root initiation and development and genes essential for opine biosynthesis (Slightom et al. 1986; Hansen et al. 1994a). *Agrobacterium*

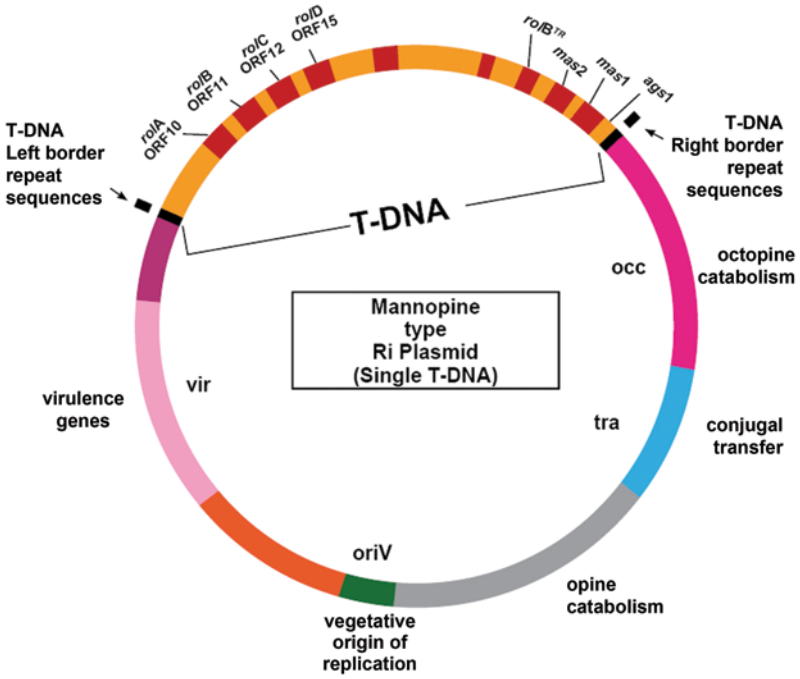

Fig. 1.4 Schematic representation of Mannopine type Ri plasmid of *A. rhizogenes*

T-DNA makes up a small region (approximately 200 kb) of Ti/Ri plasmids which are involved in functions not only for Ti/Ri plasmid conjugation, opine synthesis and catabolism, but also initiation, transfer and integration of the T-DNA (Ozyigit 2012). Although T-DNA contains genes with bacterial origin, these genes have eukaryotic regulatory sequences enabling their expression in infected plant cells (Giri and Narasu 2000). After integration of T-DNA into genomic DNA of the plant cell, T-DNA expresses enzymes that direct the synthesis of unusual amino acid sugar derivatives known as opines, which used by the *Agrobacterium* as nutrient source (Petit et al. 1983; Dessaux et al. 1992; Gartland 1995; Moyano et al. 1999; Navarrete et al. 2006; Bensaddek et al. 2008; Ozyigit 2012).

There are at least two classes of opines produced by *A. rhizogenes* strains. One such class is represented by opines of agropine group, and the other class being the agrocinopine group. Most of the *A. rhizogenes* strains are capable of producing agrocinopine type opines and all or a few strains of producing agropine type opines. The agropine-type opines including agropine, mannopine, agropinic acid and mannopinic acid are produced by the strains known as the agropine-type whereas all agropine-type opines excluding agropine are produced by the strains known as the mannopine-type (Figs. 1.4, 1.5) (White et al. 1982; Petit et al. 1983; Tempe et al. 1984; Savka et al. 1990; Gartland, 1995; Navarrete et al. 2006).

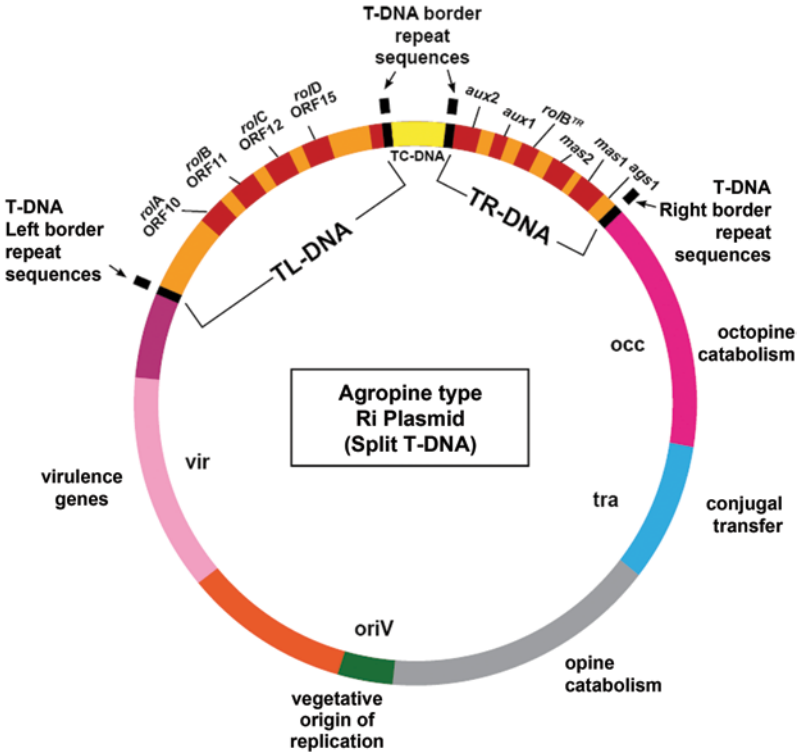

Fig. 1.5 Schematic representation of Agropine type Ri plasmid of *A. rhizogenes*

The most common *A. rhizogenes* strains which represented by Ri plasmids are agropine-type: pRiA4, pRi1855, pRiHRI, pRi15834, and pRiLBA9402, mannopine-type: pRi8196, cucumopine type: pRi2659 and mikimopine-type pRi1724. Although mikimopine and cucumopine are stereo-isomers, there is no homology between opine biosynthetic genes on the nucleotide level (Filetici et al. 1987; Davioud et al. 1988; Gartland 1995; Ouartsi et al. 2004; Veena and Taylor 2007) (Fig. 1.4).

Among the different known strains of *A. rhizogenes*, K47, K599 and HRI are hyper-virulent types known to be capable of infecting a broad range of plant hosts. More research on the virulence factors of these strains needs to be done for understanding of whether they are located on the chromosome(s), plasmid(s) or both (Petit et al. 1983; Isogai et al. 1988; Porter 1991; Suzuki et al. 2001). Also, there are differences between *A. rhizogenes* strains in terms of polarity of infection of the plant tissue. For example, root growth can be induced by some strains of *A. rhizogenes* only on the apical surfaces of carrot root discs and yield no detectable outgrowth on the basal surfaces, whereas root proliferation can be induced by others both inoculation of apical and basal surfaces (Cardarelli et al. 1985; Ryder et al. 1985; Capone et al. 1989; Limami et al. 1998). Based on these findings, various *A. rhizogenes* strains were further classified as polar and non-polar types. Agropine

type strains are non-polar whereas all other strains are polar. Agropine type strains give rise to the formation of the hairy roots regardless of the orientation of the disc and the strains other than agropine type form hairy roots when the disc is placed inverted orientation. The presence of second T-DNA encoding genes responsible for auxin production possibly causes observed variation in the polarity of infection in the plant cells transformed by the agropine-type Ri plasmid (Meyer et al. 2000; Veena and Taylor 2007) (Fig. 1.5).

Ri T-DNA Genes

Independent transformations of both left T-DNA (T_L-DNA) (about 15–20 kb) and right T-DNA (T_R-DNA) (about 8–20 kb) to the plant genome termed as "split" T-DNA are carried out by Agropine strains pRi, whereas mannopine strains only transfer a single T-DNA (T_L-DNA). T_L-DNA of pRi contains the four *rol* genes, designated as *rol*A, *rol*B, *rol*C and *rol*D (Schmulling et al. 1988; Petersen et al. 1989; Gelvin 2003; Bensaddek et al. 2008). In Ri plasmid, T_L-DNA and T_R-DNA are separated from each other by at least 15 kb of non-integrated DNA, which is represented by T-Central DNA (TC-DNA) as seen in Fig. 1.5.

The phenotype of hairy root is related with the genes whose products act as the determinants located on T_L-DNA (Tepfer 1984; Taylor et al. 1985; Jouanin et al. 1987b; Nakamura et al. 1988; Schmulling et al. 1988; Sinkar et al. 1988) whereas the genes on the T_R-DNA would only play a role in root induction (Cardarelli et al. 1985; Ryder et al. 1985; Cardarelli et al. 1987a; Smulders et al. 1991). Two fragments, defined as T_L-DNA and T_R-DNA, can be transferred and integrated independently into the plant genome during the infection process. However, the integration capacity of T_L-DNA was much higher than T_R-DNA (Chilton et al. 1982; Costantion et al. 1984; David et al. 1984; Grant et al. 1991; Phelep et al. 1991; Nilsson and Olsson 1997; Holefors et al. 1998; Sevon and Oksman-Caldentey 2002; Kumar et al. 2006; Navarrete et al. 2006; Bensaddek et al. 2008). Furthermore, the present findings imply that a higher number of Ri-T-DNA copies integrated into the plant genome increase the phenotypic effect in the Ri-line (Christensen et al 2008).

T_R-DNA

It was found that the right T-DNA (T_R-DNA) contains genes homologous to T-DNA of *A. tumefaciens* Ti plasmid (Huffman et al. 1984; Jouanin 1984; Vilaine and Casse-Delbart 1987; Hansen et al. 1991; Chandra 2012). Among them, the most important genes are those homologous to the *tms*1 and *tms*2 of the Ti-plasmid. *tms*1 and *tms*2 genes play important roles in auxin biosynthesis in *A. tumefaciens* (Inze et al. 1984; Schröder et al. 1984; Thomashow et al. 1984, 1986; Vilaine and Casse-Delbart 1987). Homology, mutagenesis and complementation experiments show that the two

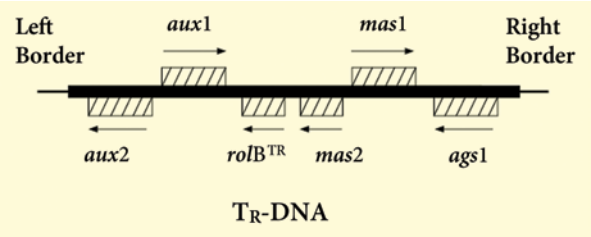

Fig. 1.6 Schematic representation of gene locations on T_R-DNA

morphogenic loci located on the T_R-DNA are counterpart of the tms loci located on the Ti plasmids and involve in hairy root tumorigenesis (White et al. 1985). In *A. rhizogenes* infected *Nicotiana glauca* tissue, the transcripts of the tms loci of Ri plasmids are found to be similar in size to those transcripts found in the tms region of Ti-plasmids (Willmitzer et al. 1983; Taylor et al. 1985; Vilaine and Casse-Delbart 1987). Similar transcripts were also found in carrot plants regenerated from tissues infected with *A. rhizogenes* (De Paolis et al. 1985; Vilaine and Casse-Delbart 1987). The root induction is probably due to auxin biosynthesis carried out by the aux loci located on T_R-DNA. The aux loci are found to be homologous to the tms loci of *A. tumefaciens* T-DNA (Vilaine and Casse-Delbart 1987).

*aux*1, *aux*2, *rol*B^{TR}, *mas*1, *mas*2, and *ags* genes located on the T_R-DNA are responsible for the biosynthesis of agropine and auxin, which cause differences in hairy root growth and morphology when compared to non-transformed roots (Fig. 1.6). It was also reported that the presence of these genes on transformed plant cells caused increase auxin sensitivity (Grant et al. 1991; Lambert and Tepfer 1992; van der Salm et al. 1997; Hansen et al. 1997; Meyer et al. 2000; Alpizar et al. 2006; Nemoto et al. 2009).

Sequence analysis revealed two open reading frames corresponding to proteins of 749 amino acids as *aux*1 gene protein and 466 amino acids *aux*2 gene protein (De Paolis et al. 1985; Camilleri and Jouanin 1991; Gaudin and Jouanin 1995; Christensen et al. 2008; Chandra 2012). Auxin biosynthetic pathway comprises two steps. The *t2m* (tryptophan 2- monooxygenase) gene product encoded by the *aux*1 catalyzes the conversion of tryptophan to indole-3-acetamide (IAM) (Comai and Kosuge 1982; Van Onckelen et al. 1986; Camilleri and Jouanin 1991). Then, IAM is converted to indole-3-acetic acid (IAA) by IAM hydrolase, the product of the *aux*2 (Jouanin 1984; Schröder et al. 1984; Thomashow et al. 1984). The T-DNA of mannopine, cucumopine and mikimopine type strains in Ri plasmids do not carry *aux* genes. Since these strains are still capable to induce a "hairy-root" phenotype, it can be said that the presence of the *aux* genes on T_R-DNA is not necessary to generate hairy root phenotype. It has been demonstrated that the *aux* genes are required to support the "hairy root" phenotype and to extend the host range of the bacterium (White et al. 1985; Cardarelli et al. 1987b; Hansen et al. 1991; Sevon and Oksman-Caldentey 2002).

Hybridization experiments also revealed that the genes encoding agropine biosynthesis (*ags*) are also located on the T_R-DNA region (Willmitzer et al. 1982; Huffman et al. 1984; Lahners et al. 1984; Vilaine and Casse-Delbart 1987; Giri and

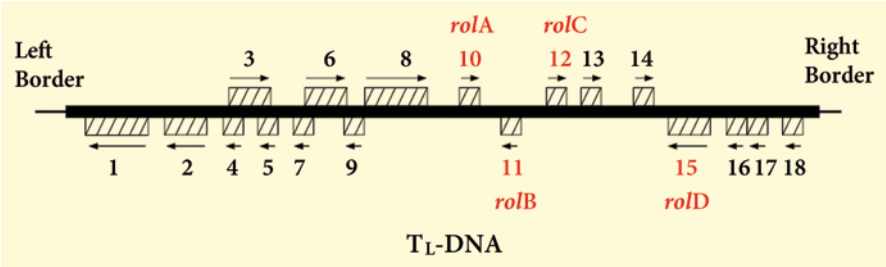

Fig. 1.7 Schematic representation of gene locations on T_L-DNA

Narasu 2000; Christey 2001). Deletion of the right border of nopaline-type or octopine-type T-DNA in Ri plasmids appears to affect virulence. Also, mutations created within this region have the same effect as removing the tms loci of Ti plasmid resulted with being avirulent on plants. The deletion of T_L-DNA in Ri plasmids is being less susceptible to oncogenic transformation than the T_R-DNA deletion (Vilaine and Casse-Delbart 1987). Expression of the T_R-DNA alone can induce root formation in some plants, but the resulting phenotype is not as strong as when both T_L- and T_R-DNA are introduced together (Vilaine and Casse-Delbart 1987).

T_L-DNA

The size of T_L-DNA of agropine type Ri-plasmid is about 19–20 kb in length but, unlike the T_R-DNA, it does not appear to be closely related to any other characterized loci of Ti-plasmids (Huffman et al. 1984; Vilaine and Casse-Delbart 1987; Aoki and Syono 1999; Chandra 2012). In many species, T_L-DNA size seems almost constant, except in *Nicotiana tabacum* consisting shorter T_L-DNA (Jouanin et al. 1987b). The mannopine/cucumopine type T-DNAs and the agropine type T_L-DNA contain two strongly conserved regions which flank an only partially homologous central region (Filetici et al. 1987; Brevet and Tempe 1988; Aoki and Syono 1999; Chandra 2012). A substance carrying out stimulation of hairy root differentiation under the influence of endogenous auxin is synthesized by genes of T_L-DNA (Ooms et al. 1986; Shen et al. 1988; Giri and Narasu 2000; Mishra and Ranjan 2008).

As a result of mutagenesis in T_L-DNA of Ri plasmid, the loss or attenuation of virulence is shown (White et al. 1985). The T_L-DNA of Ri plasmids carrying several loci is identified to be essential for hairy root induction (so-called *rol* genes for root oncogenic loci) (Fig. 1.7). Transposon mutagenesis in the T_L-DNA has identified at least four genes (*rol*A, *rol*B, *rol*C and *rol*D) involved in tumorigenesis as affecting some plants (White et al. 1985; Estramareix et al. 1986; Slightom et al. 1986; Vilaine and Casse-Delbart 1987; Meyer et al. 2000; Christensen et al. 2008). All *rol* genes have been shown to carry out formation of hairy root phenotype (White et al. 1985; Cardarelli 1987a; Jouanin 1987a; Vilaine et al. 1987a; Schmulling et al. 1988;

Petersen et al. 1989; Lee et al. 2001; Bensaddek et al. 2008). It has been reported that the T_L-DNA of the agropine-type Ri plasmid consists of at least 18 open reading frames (ORF). ORF 10, 11, 12 and 15 coincided with *rol*A, *rol*B, *rol*C and *rol*D, respectively (Slightom et al. 1986; Scorza et al. 1994).

Rol Genes

The T-DNAs have many other genes other than those opine and hormone synthesis genes. Although their functions are not well characterized, they are known to have very strong effects on growth. At least four genetic loci (rolA, B, C and D) were identified in the T-DNA regions of pRiA4 by a series of deletions and transposon insertions studies and shown to play important roles of root-inducing properties of *A. rhizogenes* on the T_L-DNA (Table 1.1) (White et al. 1985). The *rol* genes located on the T_L-DNA of Ri plasmid modify auxin and cytokinin biosynthesis and/or endogenous hormone levels and their expressions stimulate the formation of roots in transformed tissues (Nilsson et al. 1993a; Maurel et al. 1994; Moritz and Schmülling 1998; Shen et al. 1990; Bonhomme et al. 2000; Ishizaki et al. 2002; Hong et al. 2006; Bensaddek et al. 2008). Studies have focused on characterizing the three *rol* genes named as *rol*A, *rol*B, and *rol*C because they are considered essential for the hairy root initiation based on transposon "loss-of-function" analysis (White et al. 1985). Induced adventitious root formation by *rol*A, *rol*B and *rol*C genes is shown on tobacco, kalanchoe and tomato leaves (Cardarelli et al. 1987a; Spena et al. 1987; Vilaine et al. 1987; Spano et al. 1988; van Altvorst et al. 1992; Kiyokawa et al. 1994) and plants carrying these genes are morphologically equivalent to those carrying the whole T_L-DNA (Spano et al. 1988). Inactivation or overexpression of various *rol* genes in stable transgenic lines or hairy-root cultures exhibits different variations in plant phenotypes and root morphology (Schmulling et al. 1988; Martin-Tanguy et al. 1996; Casanova et al. 2004).

*rol*A

The *rol*A gene is found on all Ri plasmids and encodes a small protein with a molecular mass of approximately 11 kDa (Nilsson and Olsson 1997). The *rol*A gene sequence length differs in various *A. rhizogenes* strains ranges from 279 to 423 bp (Meyer et al. 2000). Analysis of amino acid sequences showed that *rol*A encodes a protein with basic isoelectric point (PI 11.2). It also contains a frequent sequence motif common in DNA-binding proteins (Suzuki 1989) and proposed to function as a regulatory transcription factor (Levesque et al. 1988; Veena and Taylor 2007).

A dramatic reduction in several classes of hormones, including auxin, cytokinin, gibberellic acid (GA) and abscisic acid triggered by the expression of *rol*A gene is

Table 1.1 Oncogenes of *A. rhizogenes*, their encoded proteins, functions and phenotypic changes in host plants

Gene	Protein	Function	Phenotype
*rol*A	Sequence motif common in DNA-binding proteins Regulatory transcription factor	Inhibits cell elongation via diffusible factor Decreases hormone concentrations Increase sensitivity to auxin Modulating hormone physiology of GA Interfere polyamine metabolism Correlate with plasma membrane H^+ ATPase activity	Stunted growth, dark green wrinkled leaves with an altered length to width ratio, condensed inflorescences, retarded onset of flowering, compact reduced number of flowers
*rol*B	Localizes to plasma membrane	Alterations in the reception/transduction of the auxin signal Stimulates new meristem formation Induce secondary metabolism	Fast growth, root meristem neoformation, high branching and plagiotropism
*rol*C	Phloem-specific expression in the root, low expression in the leaf, and no expression in the shoot tip	Reduces cell size Reduces abscisic acid (ABA), polyamine, and ethylene levels Formation of shoot meristems Regulate sugar metabolism and transport Stimulate the production of high levels of secondary metabolites	Increased branching, dwarfed plants with short internodes, reduced epidermal cell size in internodes, lanceolate leaves, early flowering, reduced flower size and reduced pollen production
*rol*D	Only expresses in Agropine type strains Cytosolic protein Exhibits poor tissue- or organ-specific expression	Incapable of inducing root formation on its own Provide defense response as a result of environmental stress	Increased flowering, reduced rooting, elongating and expanding tissues of each organ but not on apical meristem, callus growth giving rise to initiation of tumor resemble formation
rolB^{TR}	CX5R motif is absent N-terminal part contain 14 amino acids	*rol*B homolog on TR-DNA in the agropine type Ri plasmid	Wrinkled leaves bent strongly downward, formed shoots at the base of the stem and retarded growth
ORF3n	Modification of phenolic enzymes and involve secondary metabolism and/or the transport of hormones	Negative regulator to the dedifferentiation of tissues	Retarded flowering, less dense inflorescences, altered internode elongation and leaf morphology and necrotic tips of upper leaves, sepals and bracts no sign of necrosis on the basal leaves

Table 1.1 (continued)

Gene	Protein	Function	Phenotype
ORF8	Fusion protein consisting of N-terminal domain (NORF8) and C-terminal part (CORF8) Tryptophan monooxygenase activity	Modifies sucrose transport N-terminal domain causes sugar/starch accumulation C-terminal domain reduces sugar/starch accumulation	Growth retardation, chlorotic and necrotic leaves and accumulation of high levels of sugars (glucose, fructose and sucrose) and starch
ORF13	Contains a conservative retinoblastoma (RB)-binding motif LxCxE	Hormone homeostasis and regulation of the cell cycle Increases number of mitoses in shoot apical meristem Induces dedifferentiation (prerequisite to competence) Graft transmissible	Induce cell proliferation such as dense green and rapidly proliferating callus, including irregular formation of leaves, severe leaf nervure, shortened and variable internode length, abnormal and asymmetric flowers, agravitropic root growth and a reduced cell number and cell size in the root
ORF13a	Tissue specific manner in plants, primarily in leaf vascular tissues May interact directly with DNA SPXX repeat motif	Necessary for root induction Regulatory function of itself	Not yield a visible phenotype
ORF14	Auxin like effect	Act together with ORF13 to induce root induction	No morphological change

observed in *N. tabacum*. The reduction ratio depends on tissue type and growth stage of the plant (Dehio et al. 1993). It was demonstrated that despite low level of auxin concentration, auxin sensitivity is enhanced in transgenic plants (Maurel et al. 1991; Vansuyt et al. 1992). Additionally, the effects of *rol*A can be attenuated, probably through methylation (Martin-Tanguy et al. 1996; Lee et al. 2001). Inactivation of *rol*A leads to the formation of long, straight roots giving a less compact appearance on *Kalanchoe daigremontiana* leaves (Vilaine and Casse-Delbart 1987). Transgenic *N. tabacum* plants are also show stunted growth, dark green wrinkled leaves with an altered length to width ratio, condensed inflorescences, retarded onset of flowering, a reduced number of flowers and compact styles (Dehio et al. 1993).

A. rhizogenes infected plant tissues are 100 times more sensitive to auxin than normal phenotype exhibiting plant tissues. This suggests that the increased sensitivity of transformed plants should not be due to a particular insertion position of the *rol*A gene in the transgenic plant genome, but rather reflects the effect(s) of the *rol*A gene product (Vansuyt et al. 1992). It was found that *N. tabacum* leaves of *rol*A

transgenic clones show 40–60 % reduction of GA content compared to wild-type leaves. The reduction of GA content is indirectly cause stem elongation and planar leaf blade growth (Dehio et al. 1993). When the wild-types of *N. tabacum* treated by gibberellin biosynthesis inhibitors, *rol*A expressing plants and wild types show similar phenotypes. On the other hand, when *rol*A transgenic plants treated with GA, the phenotype of transgenic plant not completely restored (Dehio and Schell 1993; Dehio et al. 1993). All these shows that the *rol*A gene has been considered in playing an important role in modulating hormone physiology of GA and polyamine metabolism (Sun et al. 1991; Dehio and Schell 1993; Dehio et al. 1993; Prinsen et al. 1994; Martin-Tanguy et al. 1996; Veena and Taylor 2007). It was thought that the sensitivity of auxin response might correlate with plasma membrane H^+ ATPase activity observed in *rol*A expressing transgenic plants (Maurel et al. 1991; Vansuyt et al. 1992).

There is data suggesting that there is an antagonism between *rol*A and *rol*B genes in general. An observation of additional transcripts ranging from 2.1 to 2.8 kb in size explains this antagonism (Durand-Tardif et al. 1985). Size of transcription of *rol*A would be more than 2 kb. This would span the whole *rol*B sequence, leading to the generation of an antisense message for *rol*B. Its occurrence could be the major cause of antagonism between *rol*A and *rol*B in the transformed plant cells. Probably, existence of a mechanism prevents co-expression of *rol*A and *rol*B (Capone et al. 1989; van Altvorst et al. 1992; Veena and Taylor 2007).

*rol*B

The *rol*B gene size ranging 765 (strain 8196) to 840 bp (strain 2659) length depending on the strain and encodes 254–279 amino acid protein which has molecular weight of 30 kDa localized in the plasma membrane (Filippini et al. 1996; Meyer et al. 2000; Veena and Taylor 2007). *rol*B gene is present in all Ri plasmids with approximately 60 % identity between strains (Meyer et al. 2000). RolB proteins encoded by pRi1724 and pRi2659 have a 17 amino acid longer N-terminal stretch than the RolB proteins encoded by pRi1855 (pRiA4) (Meyer et al. 2000). The physical presence of the *rol*B gene in T_L-DNA segment of Ri plasmid of the infecting *Agrobacterium* in leaf tissues of plants regenerated from selected rhizoclones was demonstrated by a positive PCR amplification (Pal et al. 2012).

The reports have shown that the RolB may have a critical role in early steps of hairy-root induction (Bellincampi et al. 1996). The root induction is totally alleviated when *rol*B gene is inactivated in the pRiA4 on kalanchoe leaves (White et al. 1985). *rol*B also has capacity nearly as much as the wild type *A. rhizogenes* T-DNA for enhancing rooting and hairy root formation on wounded *N. tabacum* stems (Cardarelli et al. 1987b; Bellincampi et al. 1996; Altamura and Tomassi 1998; Binns and Costantino 1998) and leaves (Spena et al. 1987).

Phenotypical abnormalities such as root meristem neoformation on leaf discs and fast growth of *rol*B-transgenic plants and growth pattern of *rol*B-induced roots are characterized by fast growth, high branching, and plagiotropism. As a result of these

observations firstly suggested that there is a similarity between the auxin-mediated effects and morphogenic effects of *rol*B. However, further studies demonstrated that an auxin-induced hyperpolarization at the plasma membrane is exhibited by *rol*B-transformed plants. The morphogenic effects of *rol*B involve changes in either the responsiveness to auxin or in auxin content (Cardarelli et al. 1987b; Shen et al. 1988; Capone et al. 1989; Maurel et al. 1991). Activation of auxin-induced hyperpolarization through H^+ ATPase protein pump at the plasma membrane appears to be related to the proton excretion (Ephritikhine et al. 1987; Keller and Van Volkenburgh 1998). *rol*B gene causes transformed plant cells to bind more auxin than wild type and the additional auxin-binding activity is completely abolished by using anti-RolB antibodies (Filippini et al. 1994; Shoja 2010).

Estruch et al. (1991) reported that RolB protein exhibits a β-glucosidase activity able to hydrolyze biologically active indole-3-glucosidese. It can be explained by the increased auxin perception and sensitivity with releasing the hormone from β-glucoside conjugates. As a result of increase concentration of auxin cause the phenotypic alterations observed in *rol*B transgenic tissues (Shen et al. 1988, 1990; Maurel et al. 1991, 1994; Meyer et al. 2000). However, later studies showed that neither the intracellular concentration nor the metabolism of auxin was changed by *rol*B expression in plant cells. Rather, the increased auxin sensitivity of *rol*B-transformed cells results from alterations in the reception/transduction of the auxin signal (Nilsson et al. 1993b; Schmülling et al. 1993; Delbarre et al. 1994; Bellincampi et al. 1996; Veena and Taylor 2007).

Overexpressing *rol*B gene under a constitutive promoter in transgenic plants suppresses adventitious root induction (Spena et al. 1987) and necrosis in callus tissues and leaves of young plants (Schmulling et al. 1988). Both callus and root formations at wound sites are cancelled if mutations occur in *rol*B gene (Vilaine and Casse-Delbart 1987). Normal growth of these organs depends upon the expression level of *rol*B gene necessary for active growth of hairy roots. A high or low level of expression correlates with impaired growth of these organs (Tanaka et al. 2001; Veena and Taylor 2007).

A. rhizogenes rol genes enhance the biosynthesis of certain groups of secondary metabolites in transformed plant cells. It was shown that *rol*B is apparently the most powerful inducer of secondary metabolism and at the same time, the most important inhibitor of callus growth (Palazon et al. 1998; Bonhomme et al. 2000; Bulgakov et al. 2002a; Shkryl et al. 2008; Shoja 2010). *rol*B gene mediated stimulatory effect on resveratrol and anthraquinone production suppresses with the tyrosine phosphatase inhibitors proven that RolB also has tyrosine phosphatase activity (Filippini et al. 1996; Kiselev et al. 2007).

*rol*C

The *rol*C gene sequences vary in different strains but their sizes are similar and ranging between 537 bp (strain 8196) to 543 bp (strain 2659, 1724 and A4). *rol*C

gene encodes 178–180 amino acid protein (approximately 20 kDa) that share more than 65 % identity with each other (Meyer et al. 2000).

*rol*C transformed plants exhibited reduced apical dominance leading to increased branching, dwarfed plants with short internodes, lanceolate leaves, early flowering, reduced flower size and reduced pollen production (Schmulling et al. 1988). Dwarfing was caused by reduced epidermal cell size in internodes (Oono et al. 1990). Regulation of expression of *rol*C is complex, and varies depending upon the existence of the complete T-DNA sequences. In addition, root production was increased compared to untransformed plants, but decreased compared to plants transformed with the complete set of *rol* genes (Palazòn et al. 1998). Expressing *rol*C shows phloem-specific expression in the root, low expression in the leaf, and no expression in the shoot tip (Schmulling et al. 1988; Estruch et al. 1991). However, *rol*C is highly expressed in leaves when the whole T-DNA is present (Durand-Tardif et al. 1985; Leach and Aoyagi 1991). More recently, *rol*C gene has been shown to play a role in formation of shoot meristems, hence suggesting its important role in the formation of pluripotent stem cells (Gorpenchenko et al. 2006).

The *rol*C promoter is utilized extensively for phloem-specific gene expression making it a useful tool in some biotechnological programs on pathogen resistance. Replication of many plant viruses, including luteoviruses, reoviruses and most geminiviruses transmitted by hemipteran vectors occur exclusively in phloem-associated tissues. Therefore, by introducing an insecticidal gene that is toxic to hemipteran vectors under the control of phloem-specific *rol*C is a promising way for the control of such viruses through its expression in transgenic plants (Graham et al. 1997). Similarly, a plant lectin with insecticidal activity is encoded by *ASAL* (*Allium sativum* leaf agglutinin) gene and under control of the *rol*C promoter, it confers resistance against various hemipteran pests in transgenic rice, tobacco and chickpea plants (Saha et al. 2007).

*rol*C is known to stimulate rooting by an auxin-like effect of the gene (Schmulling et al. 1988; Zuker et al. 2001; Casanova et al. 2003). An increase in auxin sensitivity may lead to occurrence of the auxin-like effect. In fact, in comparison between *rol*C transgenic *N. tabacum* protoplasts and their wild-type counterparts showed that more sensitivity was recorded in transgenic *N. tabacum* in the measurement of transmembrane hyperpolarization in response to auxin (Maurel et al. 1991; Shoja 2010).

Also, abscisic acid (ABA), polyamine, and ethylene levels are extensively reduced due to *rol*C expression. The promoter of *rol*C activated by sucrose was found to be very high (Yokoyama et al. 1994; Faiss et al. 1996), implying that *rol*C may be influencing the source-sink relationship of a plant by regulating sugar metabolism and transport (Nilsson et al. 1996a, b; Martin-Tanguy 2001).

Alike *rol*B, the *rol*C gene is able to stimulate the production of high levels of secondary metabolites such as tropane alkaloids (Bonhomme et al. 2000), pyridine alkaloids, indole alkaloids (Palazon et al. 1998), ginsenosides (Bulgakov et al. 1998) and anthraquinone phytoalexins (Bulgakov et al. 2002b; Shkryl et al. 2008; Shoja 2010) in transgenic plants.

*rol*D

The *rol*D gene is found only in T_L-DNA of agropine type Ri plasmids. It is also the only *rol* gene that is incapable of inducing root formation on its own (Mauro et al. 1996). The *rol*D gene size 1,032 bp and encodes a protein of 344 amino acids (Meyer et al. 2000; Christey 2001). This is a cytosolic protein with a sequence similar to ornithine cyclodeaminase (OCD) that catalyzes the conversion of ornithine to proline. Proline is an osmoprotectant and its accumulation is considered to be a defense response as a result of environmental stress in many plant species (Mauro et al. 1996; Trovato et al. 2001; Bettini et al. 2003). High levels of proline accumulation are in flowers suggesting a role in flowering (Trovato et al. 2001). The pleiotropic effects induced by expression of *rol*D gene in transgenic plants are increased flowering and reduced rooting (Mauro et al. 1996; Trovato et al. 2001). Although flower yield is accelerated, the flowers show heteromorphic incompability, which prevents self-fertilization. Production of viable seeds is achieved through manually-selfed plants (Mauro et al. 1996). However, it should be noted that these experiments were conducted using the *rol*D sequence from pRi1855. It has been reported that the induction of flowering is not performed by *rol*D from pRiHRI (Lemcke and Schmulling 1998). *rol*D exhibits poor tissue- or organ-specific expression in comparison with other *rol* genes but is shown to have a predominantly developmental expression pattern (Vilaine and Casse-Delbart 1987). Activity is seen in the elongating and expanding tissues of each organ in adult plants, but never in apical meristems. As the plants age, expression decreases and ceases at senescence. The mutations in *rol*D appear to accentuate callus growth giving rise to initiation of tumor formation resembling the Ti-plasmid infection (Trovato et al. 1997).

*rol*B^{TR} (*rol*B Homologue in T_R-DNA)

A *rol*B homolog on T_R-DNA in the agropine type Ri plasmid was discovered and named as *rol*B^{TR}. Excluding the 5′ or 3′ flanking sequences, there is a 53 % nucleotide similarity between *rol*B^{TR} and *rol*B in their sequences (Bouchez and Camilleri 1990). The expression of *rol*B^{TR} in *N. tabacum* is shown to cause phenotypical alterations such as wrinkled leaves bent strongly downward, formed shoots at the base of the stem and retarded growth is observed which are different than *rol*B phenotype. Two big differences were noted by the alignment of protein sequences of *rol*B and *rol*B^{TR}. First, a CX5R motif is absent in *rol*B^{TR} and second, N-terminal part of RolB^{TR} contains 14 amino acids and mutations in the corresponding sequence in *rol*B^{TR} gene cause abolishment of the altered phenotype (Lemcke and Schmulling 1998).

ORF Genes

Besides *rol* (root locus) genes, there are several ORFs (Open Reading Frames) located on the T_L-DNA (Slightom et al. 1986). Many of 18 open reading frames (ORFs) nucleotide sequences identified on T_L-DNA region contain 5′ and 3′ regulatory elements similar to those found in eukaryotic genes. They have at least 255 nucleotides and start with the initiation codon ATG (Slightom et al. 1986; Holefors et al. 1998). In many cases, CCAAT and TATA elements were situated upstream of putative transcriptional initiation codons and poly(A) addition (AATAAA) elements were present in presumed 3′-noncoding regions (Slightom et al. 1986). The sequence length of coding regions of ORFs differ in ranging from 255 bp (ORF 9) up to 2280 bp (ORF8) and encode protein products ranging in size from 9,600 to 85,000 daltons, respectively. The results from analysis of insertion mutants within the T-DNA region (White et al. 1985) and transformation experiments with individual or combinations of the ORFs have showed that the open reading frames ORF10, 11 and 12, corresponding to the genes *rol*A, *rol*B and *rol*C, were able to promote the formation of hairy root syndrome (Table 1.1) (Jouanin et al. 1987b; Vilaine et al. 1987; Spena et al. 1987; Spano et al. 1988; Schmulling et al. 1988). Besides this, it has been showed that ORF3n, ORF8 and ORF13 DNA sequences are highly conserved among all known Ri plasmids, indicating that they alter plant morphogenesis or response of transgenic tissues to plant hormones (Lemcke and Schmulling 1998; Veena and Taylor 2007). The sensitivity to auxin and cytokinin in combination or auxin alone can be lowered by expressions of both ORF3n and ORF8 (Lemcke and Schmulling 1998).

ORF3n

Expression of ORF3n in transgenic *N. tabacum* caused retarded flowering, less dense inflorescences, altered internode elongation and leaf morphology and necrotic tips of upper leaves, sepals and bracts (Lemcke and Schmulling 1998). Appearance of localized necrosis was noticed on the tips of apical narrow leaves whereas there was no sign of necrosis on the basal leaves. Additionally, senescence was not altered in these leaves, and bracts became necrotic as a whole. On sepals, the necrosis emerged on the tips just when the corolla was visible through the calyx (Koltunow et al. 2001; Lemcke and Schmulling 1998). The ORF3n protein (48.7 kDa) resembles phenolic-modifying enzymes and may be involved in secondary metabolism and/or the transport of hormones (Binns et al. 1987; Jacobs and Rubery 1988; Lemcke and Schmulling 1998). A cessation was observed in the shoot formation from ORF3n callus in response to auxin and cytokinin. Also, plantlets transferred to the medium containing auxin and cytokinin showed decreased sensitivity leading to small and fewer calli than controls. Thus, it has been proposed that ORF3n may act to negative regulator to the dedifferentiation of tissues as a reaction to auxin and cytokinin, which may favor the formation of *rol* gene-induced roots from such cells during pathogenesis (Britton et al. 2008; Dodueva 2007).

ORF8

The ORF8 gene has the longest sequence of T_L-DNA and coding for a protein containing 780 amino acids (Slightom et al. 1986). The ORF8 protein has one of the most conserved amino acid sequences (81 % similarity) between different strains like pRiA4 and pRi2659 (Ouartsi et al. 2004).

The protein encoded by the ORF8 gene is a natural fusion protein consisting of N-terminal domain (NORF8) of 213 amino acids homologous to RolB protein of the *A. rhizogenes* strain A4 T-DNA and the C-terminal part (CORF8) of approximately 506–524 amino acids shows homology to the IaaM proteins of various other bacteria (Yamada et al. 1985; Slightom et al. 1986; Levesque et al. 1988; Dodueva 2007; Shoja 2010). *iaa*M genes that homologues to the coding sequence of CORF8 codes for a tryptophan monooxygenase which catalyzes the formation of indole-3-acetamide (IAM) from tryptophan (Lemcke et al. 2000).

Furthermore, ORF8 possesses a 200 amino acid stretch at its N-terminus that shows homology with the *rol*B gene product (33.5 % amino acid identity) (Levesque et al. 1988). The N-terminal part (NORF8) of this protein functions in carbohydrate metabolism such that when only NORF8 was expressed, transformed plant showed growth retardation, chlorotic and necrotic leaves and accumulation of high levels of sugars (glucose, fructose and sucrose) and starch (Otten and Helfer 2001).

However, some studies show that the auxin content can be elevated by the genes found in the T_L-DNA region on the T-DNA in some hosts, independent of the presence of the T_R-DNA (Lemcke et al. 2000). Presumably this occurs because of conversion of IAM to IAA in cells expressing only t2m protein (Klee et al. 1987; Prinsen et al. 1990). Besides this, as a characteristic functional motif of the t2m proteins that catalyzes decarboxylation of tryptophan to indole-3-acetamide exhibits 23-aminoacid- long a flavin adenine dinucleotide (FAD) binding site was identified by Levesque et al. (1988). The experimental data obtained from plants and bacteria suggest that the gene product of ORF8 of *A. rhizogenes* T_L-DNA has t2m activity responsible for the increased IAM content in transgenic tissues (Lemcke et al. 2000). Moreover, there is a physical connection between N- and C-regions of ORF8 protein required for the emergence of a specific phenotype in transgenic plants consisting ORF8 gene. This suggests a distinct specific function for the whole protein (Umber et al. 2005; Dodueva 2007).

ORF13 and ORF14

The ORF13 and ORF14 genes are found to be highly conserved among *A. rhizogenes* strains (Stieger et al. 2004). It has been demonstrated that alone A4-*rol*ABC genes carried by an *Agrobacterium* strain are showed to be incapable of inducing rooting on carrot disc and *aux* genes located on the T_R-DNA or ORF13 and ORF14 located on T_L-DNA are also required for rooting (Cardarelli et al. 1987b; Capone

et al. 1989). In *N. tabacum* leaf discs harboring *rol*B and ORF13 genes had capacity to induce rooting almost as well as the full length of T_L-DNA (Aoki and Syono 1999). The results obtained via co-inoculation of leaf discs achieved using the *rol*A, *rol*B and *rol*C with either ORF13 or ORF14 showed a limited root induction on carrot disks (Capone et al. 1989). A comparison from the studies showed that there is no homology between ORF13/ORF14 and auxin biosynthetic genes. Furthermore, unlike the genes controlling biosynthesis of auxin (Camilleri and Jouanin 1991), ORF13 and ORF14 have no activity for the induction of roots on *N. tabacum* leaf discs (Cardarelli et al. 1987b). A highly divergent gene family known as plast gene family is constituted by *rol*B, *rol*C, ORF13 and ORF14. They have similar functions and are thought to be evolutionary related (Levesque et al. 1988).

The ORF13 gene is approximately 600 bp in size, encoding a 197–200 amino acid protein, whose expression leads to higher levels in leaves and roots (Durand-Tardif et al. 1985; Veena and Taylor 2007). ORF13 gene leads to the formation of induce cell proliferation such as dense green and rapidly proliferating callus on transformed carrot root and tobacco leaf discs (Capone et al. 1989; Frundt et al. 1998; Dodueva 2007). Wound-inducible and organ-specific expression of ORF13 in transgenic plants lead to a variety of characteristic modifications including irregular formation of leaves, severe leaf nervure, shortened and variable internode length, abnormal and asymmetric flowers, agravitropic root growth and a reduced cell number and cell size in the root (Hansen et al. 1993, 1997; Lemcke and Schmulling 1998; Veena and Taylor 2007). Accelerated expression level in ORF13 gene triggered a more severe reduction of growth in stem and roots through TC-dependent overproduction of the ORF13 gene product, affecting both cell number and cell size in the root. Interestingly, growth and gravitropism was normal in the ORF13 high expressers (Lemcke and Schmulling 1998).

Expression of ORF13 provokes specific phenotype similar to cytokinin-treated plants however free or bound cytokinin content of the transformed tissues shows no difference from wild-type (Medford et al. 1989; Hansen et al. 1993; Lemcke and Schmulling 1998). Furthermore, the shoot part of the ORF13 transformed plant does not resemble cytokinin-overproducing plants, indeed the growth reduction results from the inhibition of cell division in the apical meristems and development of leaves (Lemcke and Schmülling 1998). Some of the phenotypic alterations in transgenic plants are thought to arise from interaction of ORF13 with hormone signaling pathways. ORF13 may play roles in hormone homeostasis and regulation of the cell cycle in infected cells (Veena and Taylor 2007). The observations and grafting of transgenic shoots onto wild type plants revealed that ORF13 may cause the production of a diffusible factor with cytokinin-like activity (Hansen et al. 1993; Dodueva 2007).

Since the only T-DNA gene that induces cell proliferation is ORF13, when inoculated with both carrot discs and tobacco leaf discs produce green callus (Hansen et al. 1993; Frundt et al. 1998). Application of exogenous cytokinin increases the number of roots produced from ORF13 tobacco leaf discs, but does not change root induction on untransformed, even though there was no difference in endogenous cytokinin levels (Specq et al. 1994; Lemcke and Schmulling 1998, Britton et al. 2008).

Furthermore, endoreduplication was reduced in ORF13 plants (Meyer et al. 2000), indicating an interaction of ORF13 with cell cycle control. Stieger et al. (2004) claimed that a proliferative effect of ORF13 expression in the shoot apical meristem (SAM) caused increased number of mitoses and showed no influence on meristem structure. In consequence, the reductions of cell and meristem sizes and the retardation in the formation of leaf primordia were observed. Smaller leaf sizes can be explained by an earlier cessation of leaf growth, but not explained with a reduced size of leaf cells, since the number of epidermal leaf cells per square millimeter was remain unaltered. Enhanced number of cell divisions in the shoot apical meristems and accelerated production of leaf primordia were seen in plant expressing ORF13. ORF13 is involved in the inference of the cell cycle regulation leading to an earlier stop in organ growth in the developing leaves. Furthermore, earlier flowering of plants expressing ORF13 may arrest leaf initiation and leaf expansion, explaining the fewer leaves formed in ORF13 plants (Stieger et al. 2004).

It has been also revealed that ORF13 protein contains a conservative retinoblastoma (RB)-binding motif LxCxE (Meyer et al. 2000). This motif was found in all members of the ORF13 family, including agropine-, mannopine-, cucumopine-, and mikimopine-type Ri plasmids (Stieger et al. 2004). When mutations are introduced into the Rb motif, normal leaf size is restored, but plants still show stunting and reduced apical dominance. It was also observed that ORF13 expression leads to the formation of spur between minor veins on leaves and petals *N. tabacum* (Meyer et al. 2000). Similar structures are formed on leaves, when *KNOX* (KNOTTED1-like homeobox) genes are overexpressed (Sinha et al. 1993; Chuck et al. 1996; Sentoku et al. 2000; Stieger et al. 2004). It was explained that cytokinin-like phenotype such as the formation of spikes, stunted growth, loss of apical dominance, fusion of organs, and stem fasciations observed as consequences of ectopic expression of *KNOX* genes which are induced by ORF1 and cell cycle regulations (Stieger et al. 2004).

Among the additional ORFs in the T_L-DNA, there are two genes, which may also contribute to the hairy root phenotype, ORF13a and ORF14. ORF13a is located between ORF13 and ORF14 on the opposite strand. Expression of this gene is taken place in a tissue specific manner in plants, primarily in leaf vascular tissues (Hansen et al. 1994b). ORF13a is necessary for root induction (Capone et al. 1989). ORF13a containing motifs common to phorphorylated gene regulatory proteins codes for a protein that may interact directly with DNA (Hansen et al. 1994b). Despite a higher expression rate of ORF13a was found in roots compared to leaves, its expression did not yield a visible phenotype (Lemcke and Schmulling 1998; Veena and Taylor 2007). The putative protein encoded by ORF13a has a SPXX repeat motif and is considered to have a regulatory function for this gene (Hansen et al. 1994b). ORF14 is in the same gene family as *rol*B, *rol*C, ORF8 and ORF13 (Levesque et al. 1988). Although overexpression of ORF14 in transgenic carrot and tobacco produced no morphological changes (Lemcke and Schmulling 1998), it has been shown that the *rol* genes and ORF13 act together to induce root induction (Capone et al. 1989; Aoki and Syono 1999) (Table 1.1).

A. rhizogenes and Crop Biotechnology

Genes can be transferred between species and in conjunction with this fact; plant improvements for many decades have been relied heavily upon gene transfer. Either by natural selection or through the efforts of plant breeders, development of plants has always depended upon creating, evaluating and selecting of right combination of alleles. Transgenic plants possessing useful features such as resistance to diseases, insects and pests have been developed by transferring such traits to crop varieties from different species.

Since 1970, rapid progress being made in developing tools for recombinant DNA technology has led to the creation of genetically modified plants. Genetically modified crops have been developed for improving various agricultural, nutritional and food processing traits and used commercially all over the world (Miflin 2000; Kuiper et al. 2001; James 2006; Olempska-Beer et al. 2006). Establishment of plant tissue culture techniques are the most important and preliminary steps for many direct (electroporation, biolistic, microinjection, etc.) and indirect (virus- or bacteria-mediated) gene transfer methods in biotechnology and these methods are used successfully by a lot of laboratories around the world (Ozyigit 2012). The particle bombardment and electroporation transformation methods were favored DNA delivery systems because they do not show any plant host range problems and very effective with high DNA delivery rate (Hauptmann et al. 1987; Birch 1997; Taylor and Fauquet 2002; Turgut-Kara and Ari 2010). However with these methods, gene silencing/co-suppression can be occurred as a result of high copy number of DNA inserted in host cells (Block 1993; Yasuda et al. 2005). On the other hand, *Agrobacterium*-based plant transformation is very effective method of creating plants at low cost, simple to use and with low copy number inserted. Limited number of host range is the only disadvantage (Lessard et al. 2002; Chandra 2012). For achieving transformation of plants, *Agrobacterium* based technology has been used since the mid-1990s increasingly (Hiei et al. 1994). *Agrobacterium*-mediated transformation in generating transgenic plants has been employed as a major DNA delivery system for novel transgenic technologies starting with the transformations of dicotyledonous (Zambryski et al. 1983) and monocotyledonous (Hiei et al. 1994) species in the 1980–1990s. Increasing understanding of *Agrobacterium*-plant relationship (Gelvin 2003) and the mechanisms of transgene integration and genetic recombination in plants (Vain 2007) will lead to achieve further advances in these areas. Conducting efficient and controlled research on targeted gene replacement/alteration, overexpression and mis-expression could provide valuable resource to define gene regulation/function and traits in further in crops. Achievements on *Agrobacterium*-based transformation technologies enable large-scale transgenic studies in a range of important plant and crop species (such as indica rice, wheat, barley, etc.) (Vain 2007) and also bring opportunity to define and select plant cultivars, which could not be obtained by conventional breeding methods (Christou 1997).

For many crops, aim of breeding program is altering plant forms. Establishment of plants with reduced size is favorable in many crops ranging from fruit trees to

annual bedding plants (Mayo 1987). Breeding strategies empowered by genetic engineering will lead to the development of more useful and productive crops for plant breeders. While transferring genes to plants for being resistant against diseases and insects, they might have been affected in other ways having altered properties (Oono et al. 1987; Spena et al. 1987; Schmulling et al. 1988; Fladung 1990; Smigocki and Hammerschlag 1991; Scorza et al. 1994). Legumes are not only providing a main source of protein and oil for human and animal nutrition but also contributing to the biological fixation of nitrogen. Moreover, a better understanding of plant-microbe interactions such as symbiotic nitrogen fixation, mycorrhizal associations, and legume-pathogen interactions can be possible with legume studies (Chilton et al. 1982; Christey 2001). Studies on aspects of hairy roots in legumes showed that proliferous root growth and abundant lateral branching are important for improving nitrogen fixation (Cheng et al. 1992).

Most plant structures, such as the hypocotyl, leaf, stem, stalk, petiole, shoot tip, cotyledon, protoplast, storage root, and tuber, have shown capacity to be infected and genetically transformed by *A. rhizogenes* resulting in stimulation of hairy root formation (Mugnier 1988; Han et al. 1993; Bajrovic et al. 1995; Arican et al. 1998; Drewes and Staden 1995; Giri et al. 2001; Krolicka et al. 2001; Azlan et al. 2002; Veena and Taylor 2007). Applications of plant biotechnology favor hairy-root cultures because of their special properties such as fast growth, short doubling time, ease of maintenance, and ability to synthesize a range of chemical compounds and proteins. Hairy root cultures are usually able to produce the same compounds found in wild-type roots of the parent plant, without the loss of concentration (Kim et al. 2002; Veena and Taylor 2007). Above all, hairy roots have an ability to regenerate stable transgenic plants either by a process of somatic embryogenesis or adventitious bud formation, so that genetically modified generations can be achieved (Spano and Costantino 1982; Tepfer 1984; Han et al. 1993; Cho and Wildholm 2002).

It is also known that modification of the cell hormonal balances occurring in response to infection causes root formation at the infected site (Gaudin et al. 1994; Aarrouf et al. 2012). However, the response varies depending upon the strain and its interaction with the plant. One of the most important advantages is that hairy root formation can be used as a verification of transformation. The use of antibiotic resistance markers in the development of transgenic plants is given rise to substantial public attention because of their unknown effects (Christey 2001).

Hairy roots have been used for infection of bacteria, fungi and nematodes and shown to successfully complete their life cycles (Cho et al. 1998; Collier et al. 2005). The resistance genes of nematode have been studied through using hairy roots (Cai et al. 1995; Remeeus et al. 1998; Kifle et al. 1999; Hwang et al. 2000). Development of plants using hairy roots have become of interest because of great potential for building up tolerance to biotic stresses and abiotic stresses (Porter 1991). Hairy root cultures provide an advantage related with making possible the analysis of the changes in enzyme activities and their isoenzyme patterns (Messner and Boll 1993; Kärkönen et al. 2002; Talano et al. 2006).

A variety of dicotyledonous plants are susceptible to *A. rhizogenes*. As a result of stable transformation, root cultures have been established from a range of spe-

cies of plants (Tepfer 1990). In 1997, Christey reported plant species that had been genetically modified produced from hairy roots of 60 different taxa, representing 51 species from 41 genera and 23 families including Pinaceae Fabaceae, Brassicaceae and Solanaceae Araliaceae, Caricaceae and Rutaceae. In 2001, it was reported that, transgenic plants have been derived via transgenesis using in 89 different taxa, representing 79 species from 55 genera and 27 families (Christey 2001). Because lack of susceptibility, monocotyledonous plants are not a host for *A. rhizogenes* for and still there is no example for transgenic monocotyledonous plant except onion (Dommisse et al. 1990) and asparagus (Hernalsteens et al. 1993; Christey 2001). According to Web of Science, currently there are more than 500 studies conducted on *A. rhizogenes*. Table 1.2 summarizes the studies conducted, the plants and the genes transferred via *A. rhizogenes* in chronological order.

Conclusion and Future Perespective

This chapter deals with current research on *A. rhizogenes*-mediated transformation and its applications in crops. *A. rhizogenes* is responsible for the development of hairy root disease in a wide range of dicotyledonous plants and characterized by a proliferation of excessively branching roots. Containing case studies demonstrating the result of *A. rhizogenes*-mediated transformation includes biosynthesis pathways in plants created a valuable platform in the last years. Furthermore, the plants transformed with *A. rhizogenes* are become increasingly popular for offering approaches to create cost-effective options in mass-producing desired plant metabolites and expressing foreign proteins. The data from numerous proof-of-concept studies including improved the nutritional quality, agronomical characteristics, production of plant-derived products encourages for the realization of scaling up *Agrobacterium* based practices. Recently, transgenic plants produced by *Agrobacterium*-mediated transformation have also been shown to have immense potential for applications in phytoremediation. This chapter highlights recent progresses in the field of *A. rhizogenes*-mediated transformation and outlines future perspectives for the exploitation of it.

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Table 1.2 Summary of the studies conducted, the plants and the genes transferred via *A.rhizogenes* in chronological order

Daucus carota	Carrot	*rol*	David et al. 1984
Kalanchoe daigremontiana	Devil's backbone	*rol*	White et al. 1985
Arabidopsis thaliana	Mouse ear cress	*rol*	Pavingerova and Ondrej 1986
Cucumis sativus	Cucumber	NPTII	Trulson et al. 1986
Lycopersicon esculentum	Tomato	NPTII	Shahin et al. 1986
Petunia hybrida	Petunia	*rol*	Ondrej and Biskova 1986
Armoracia lapathifolia	Horseradish	*rol*	Noda et al. 1987
Lycopersicon peruvianum	–	NPTII	Morgan et al. 1987
Nicotiana debneyi	Debney's tobacco	NPTII	Davey et al. 1987
Nicotiana plumbaginifolia	–	NPTII	Davey et al. 1987
Solanum nigrum	Black nightshade	NPTII	Davey et al. 1987
Anagallis arvensis	Pimpernel	*rol*	Mugnier 1988
Convolvulus arvensis	Morning glory	*rol*	Mugnier 1988
Foeniculum vulgare	Fennel	*rol*	Mugnier 1988
Linum usitatissimum	Flax	*rol*	Zhan et al. 1988
Nicotiana glauca	Tree tobacco	*rol*	Sinkar et al. 1988
Nicotiana hesperis	–	*rol*	Walton and Belshaw 1988
Brassica oleracea var. *acephala*	Ornamental kale	*rol*	Hosoki et al. 1989
Catharanthus roseus	Periwinkle	*rol*	Brillanceau et al. 1989
Glycine argyrea	Wild soybean	NPTII	Rech et al. 1989
Glycine canescens	Wild soybean	NPTII	Rech et al. 1989
Lotus corniculatus	Bird's-Foot trefoil	GUS	Forde et al. 1989
Solanum tuberosum	Potato	NPT II, GUS	Visser et al. 1989
Stylosanthes humilis	Townsville stylo	NPT II	Manners and way 1989
Trifolium repens	White clover	*rol*	Diaz et al. 1989
Brassica napus	Rapeseed	NPTII	Boulter et al. 1990
Nicotiana rustica	Mapacho	ODS	Hamill et al. 1990
Nicotiana tabacum	Tobacco	NPTII	Hatamoto et al. 1990
Vicia faba	Fava bean	NPTII	Ramsay and Kumar 1990
Actinidia deliciosa	Kiwifruit	*rol*	Rugini et al. 1991
Allocasuarina verticillata	Drooping she-oak	*rol*	Phelep et al. 1991
Cichorium intybus	Chicory	*rol*	Sun et al. 1991
Hyoscyamus muticus	Egyptian henbane	*rol*	Oksman-Caldentey et al. 1991
Medicago arborea	Tree medick	HPT	Damiani and Aricioni 1991
Medicago sativa	Alfalfa/lucerne	*rol*	Golds et al. 1991
Olea europaea	Olive	*rol*	Rugini et al. 1996
Onobrychis viciifolia	Sainfoin	*rol*	Golds et al. 1991
Pistacia vera	Pistachio	*rol*	Rugini and Mariotti 1991
Malus domestica	Apple	*rol*B	Rugini and Mariotti 1991
Solanum dulcamara	Nightshade	NPTII, *rol*	McInnes et al. 1991
Anthyllis vulneraria	Kidney vetch	NPTII, *ipt*	Stiller et al. 1992
Atropa belladonna	Deadly nightshade	*bar*	Saito et al. 1992
Brassica campestris	Turnip	NPT II	Christey and Sinclair 1992

Table 1.2 (continued)

Brassica campestris var. *rapifera*	Turnip	GUS, NPTII, ALS	Christey and Sinclair 1992
Brassica oleracea var. *acephala*	Forage kale	GUS, NPTII, ALS	Christey and Sinclair 1992
Malus pumila	Apple	*rol*	Lambert and Tepfer 1992
Medicago truncatula	Barrel clover	NPTII	Thomas et al. 1992
Papaver somniferum	Opium poppy	*rol*	Yoshimatsu and Shimomura 1992
Coffea arabica	Coffea	*rol*	Spiral et al. 1993
Eucalyptus sp.	Eucalyptus	*rol*	MacRae and van Staden 1993
Glycine max	Soybean	GUS	Olhoft et al. 2007
Ipomoea batatas	Sweet potato	NPTII, GUS	Otani et al. 1993
Populus trichocarpa × *P. deltoides*	Cottonwood	NPTII	Han et al. 1993
Robinia pseudoacacia	Black locust	NPTII	Han et al. 1993
Vicia hirsuta	Hairy vetch	*rol*	Quandt et al. 1993
Vigna aconitifolia	Moth bean	SbPRP1	Suzuki et al. 1993; Lee et al. 1993
Diospyros kaki	Japanese persimmon	*rol*	Tao et al. 1994
Larix decidua	European larch	NPTII, *aro*A, BT	Shin et al. 1994
Pelargonium graveolens	Lemon geranium	*rol*	Pellegrineschi et al. 1994
Rosa hybrida	Hybrid tea rose	NPTII, GUS	Firoozabady et al. 1994
Rubia peregrina	Wild madder	ICS	Downs et al. 1994
Vinca minor	Lesser periwinkle	NPTII, GUS	Tanaka et al. 1994
Vitis vinifera	Grapevine	NPTII, GUS	Nakano et al. 1994
Casuarina glauca	Swamp she-oak	GUS	Diouf et al. 1995
Gentiana scabra	Japanese gentian	*rol*	Suginuma and Akihama 1995
Solanum tuberosum L.	Potato	*rol*	Bajrovic et al. 1995
Rudbeckia hirta	Black-Eyed susan	*rol*	Daimon and Mii 1995
Verticordia grandis	Scarlet featherflower	NPTII, GUS	Stummer et al. 1995
Citrus sinensis	Sweet orange	*rol*	Li et al. 1996
Ajuga reptans	Blue bugle	GUS	Uozumi et al. 1996
Begonia tuberhybrida	Begonia	*rol*	Kiyokawa et al. 1996
Brassica campestris	Turnip	GUS	Christey et al. 1997
Brassica oleracea	Wild cabbage	GUS	Christey et al. 1997
Carica papaya	Papaya	NPTII, GUS	Cabrera-Ponce et al. 1996
Eustoma grandiflorum	Lisianthus	NPTII, GUS	Handa 1992
Ipomoea trichocarpa	Blue morning glory	NPTII, GUS	Otani et al. 1993
Juglans regia	Walnut	*rol*B	Caboni et al. 1996
Lotus angustissimus	Slender bird's-foot trefoil	NPTII, GUS	Nenz et al. 1996
Pelargonium fragrans	Nutmeg geranium	*rol*	Pellegrineschi and Davolio-Mariani 1996

Table 1.2 (continued)

Pelargonium odoratissimum	Apple geranium	*rol*	Pellegrineschi and Davolio-Mariani 1996
Pelargonium quercifolium	Oak-Leaved geranium	*rol*	Pellegrineschi and Davolio-Mariani 1996
Pinus contorta	Lodgepole pine	*rol*	Yibrah et al. 1996
Pinus halepensis	Aleppo pine	*rol*	Tzfira et al. 1996
Pinus nigra	Austrian pine	*rol*	Mihaljevic et al. 1996
Populus tremula	Aspen	NPTII, GUS	Tzfira et al. 1996
Rosa sp.	Rose	*rol*	Van der Salm et al. 1997
Scoparia dulcis	Licorice weed	*rol*	Yamazaki et al. 1996
Aconitum heterophyllum	Indian atees	*rol*	Giri et al. 1997
Artemisia annua	Sweet wormwood	*rol*	Banerjee et al. 1997
Brassica napus	Oilseed rape	GUS, NPTII, ALS	Christey et al. 1997
Brassica oleracea	Wild cabbage	GUS, NPTII	Christey et al. 1997
Datura arborea	Angel's trumpets	*rol*	Giovannini et al. 1997
Datura sanguinea	Red Angel's trumpets	*rol*	Giovannini et al. 1997
Digitalis lanata	Grecian foxglove	*rol*	Pradel et al. 1997
Gentiana cruciata	Gentian	GUS	Momčilović et al. 1997
Gentiana purpurea	Purple gentian	*rol*	Momčilović et al. 1997
Gentiana triflora × *G. scabra*	–	*rol*	Hosokawa et al. 1997
Lotus japonicus	Lotus japonicus	*rol*	Stiller et al. 1997
Nierembergia scoparia	Tall cupflower	*rol*	Godo et al. 1997
Peganum harmala	Harmal	TDS	Berlin et al. 1993
Antirrhinum majus	Snapdragon	bar, NPTII	Hoshino and Mii 1998
Arachis hypogaea L.	Groundnut	*rol*	Akasaka et al. 1998
Astragalus sinicus	Chinese milk vetch	GUS	Cho et al. 1998
Citrus aurantifolia	Mexican lime	NPTII, GUS	Pérez-Molphe-Balch and Ochoa-Alejo 1998
Nicotiana spp.	–	*rol*	Palazon et al. 1998
Panax ginseng	Ginseng	*rol*	Yang and Choi 2000
Prunus avium	Sweet cherry	*rol*	Gutierrez-Pesce et al. 1998
Brassica campestris var. *pekinensis*	Chinese cabbage	NPTII, EAS	Christey et al. 1999
Brassica oleracea L. var. *italica*	Broccoli	*rol*	Henzi et al. 1999
Brassica oleracea var. *botrytis*	Cauliflower	NPTII, GUS	Christey et al. 1999
Brassica oleracea var. *capitata*	Cabbage	NPTII, GUS	Christey et al. 1999
Brassica oleracea var. *gemmifera*	Brussels sprouts	NPTII	Christey et al. 1999
Brassica oleracea var. *italica*	Broccoli	NPTII, EAS	Christey et al. 1999
Gentiana punctata	Spotted gentian	GUS	Vinterhalter et al. 1999
Pimpinella anisum	Anise	*rol*	Andarwulan and Shetty 1999
Pyrus communis	Pear	*rol*C	Bell et al. 1999

Table 1.2 (continued)

Rubia tinctorum	Common madder	*rol*	Ercan et al. 1999
Ulmus spp.	Elm	*rol*	Rinallo et al. 1999
Ziziphus jujuba	Jujube	*rol*	Hatta et al. 1996
Crotalaria juncea	Sunn hemp	*rol*	Ohara et al. 2000
Trifolium pratense	Red clover	*rol*	Díaz et al. 2000
Brassica napus var. *rapifera*	Swede (Rutabaga)	bar	Christey and Braun 2001
Oryza sativa var. *japonica*	Japanese Rice	*rol*A, NPTII	Lee et al. 2001
Spinacia oleracea	Spinach	*rol*	Ishizaki et al. 2002
Citrus aurantium	Bergamot orange	*rol*	Chavez-Vela et al. 2003
Ginkgo biloba	Ginkgo	*rol*	Ayadi and Tremouillaux-Guiller 2003
Rauvolfia micrantha	–	*rol*	Sudha et al. 2003
Sesbania rostrata	Pea	*rol*	Van de Velde et al. 2003
Aesculus hippocastanum	Horse-chestnut	GUS	Zdravkovic-Korac et al. 2004
Alstroemeria sp.	Peruvian lily	NPTII, GUS, *rol*	Akutsu et al. 2004
Camptotheca acuminata	Happy tree	*rol*	Lorence et al. 2004
Genista tinctoria	Greenweed	*rol*	Luczkiewicz and Kokotkiewicz 2005
Typha latifolia	Common bulrush	*rol*	Nandakumar et al. 2005
Brassica oleracea var. *sabauda*	Savoy cabbage	GUS	Sretenovıc-Rajicic et al. 2006
Brassica oleracea var. *sabauda*	Savoy cabbage	*rol*	Sretenovic-Rajicic et al. 2006
Eustoma grandiflorum	Lisianthus	*rol*	Popa et al. 2006
Echinacea purpurea	Purple coneflower	*rol*B	Wang et al. 2006
Phaseolus vulgaris	Common bean	GFP, GUS	Estrada-Navarrete et al. 2006
Tylophora indica	Indian ipecac	*rol*	Chaudhuri et al. 2006
Asimina triloba	Pawpaw	*rol*B, C	Ayala-Silva et al. 2007
Pueraria candollei	–	*rol*B	Medina-Bolivar et al. 2007
Beta vulgaris	Red beet	NPTII	Thimmaraju et al. 2008
Glycyrrhiza glabra	Licorice	*rol*	Mehrotra et al. 2008
Musa sp.	Banana	*rol*	Matsumoto et al. 2009
Plumbago rosea	Plumbago	*rol*	Satheeshkumar et al. 2009
Podophyllum hexandrum	Himalayan mayapple	*rol*	Lin et al. 2003
Psoralea corylifolia	Babchi	*rol*	Shinde et al. 2009
Drosera burmannii	Tropical sundew	*rol*	Putalun et al. 2010
Echium rauwolfii	Echium rauwolfii	*rol*	Abd El-Mawla 2010
Fagopyrum esculentum	Buckwheat	GUS	Kim et al. 2010
Mangifera indica	Mango	*rol*	Chavarri et al. 2010
Przewalskia tangutica	–	*rol*	Lan and Quan 2010
*Corchorus capsularis*t	Jute	GUS	Chattopadhyay et al. 2011
Nasturtium officinale	Watercresses	*rol*	Park et al. 2011
Prunus sp.	–	Egfp, NPTII	Bosselut et al. 2011
Amaranthus spinosus	Spiny amaranth	*rol*B	Pal et al. 2012
Capsicum annuum	Pepper	GFP	Aarrouf et al. 2012
Clitoria ternatea	Butterfly pea	*rol*	Swain et al. 2012

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