Khalid Rehman Hakeem
Parvaiz Ahmad
Munir Ozturk *Editors*

Crop Improvement

New Approaches and Modern Techniques

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Foreword

Crop improvement is now one of the most significant subject matters in agriculture, which includes the genetic alteration of plants to satisfy ever increasing human need. A large number of genetic techniques were developed and refined in the twentieth-century. It has been suggested that many of the limitations of conventional breeding can be overcome with advances in molecular biology. The aim in crop development is to support innovative and excellent research to underpin the development of improved crop varieties that deliver increased productivity and consistent, high quality end products. The limitations of the new breeding methods include technical problems, such as the difficulty of transformation, problems of gene expression, or the lack of knowledge concerning suitable genes to transfer.

Biotechnology is generally accepted as the use of living systems and organisms to develop or make useful products. Increases in crop yield is one of the most obvious applications of modern biotechnology in agriculture, it is also recognized the most difficult one. Many of the genetic characteristics associated with yield (e.g., enhanced growth) are controlled by a large number of genes, each of which has a minimal effect on the overall yield. Most of the current commercial applications of modern biotechnology in agriculture are related to reduce the dependence of farmers on agrochemicals. There is a need much scientific work to be done in this area.

The book contains state-of-the-art new research results in crop improvement and related disciplines in crop development. It provides up-to-date information for researchers, educators, graduate students and industry. It consists of 17 Chapters. The *first Chapter* provides the reader with extensive information on *A. rhizogene, s* which is responsible for the development of hairy root disease in a wide range of dicotyledonous plants and its T-DNA system components. *Second Chapter* talks about recent advances in bioinformatic tools, together with advance molecular technology under clear biological categories. *Chapter 3* deals with tissue culture, which is employed for large-scale propagation of disease free clones and gene pool conservation. It covers *in vitro* propagation and role of biotechnology in crop improvement. *Chapter 4* describes about mutagenesis, which is a crucial step in crop improvement program.

Chapter 5 explains the importance of biofertilizers in sustainable ecosystem. Biofertilizers are now gaining ground as they are used to maintain the soil health,

curtail the environmental pollution and cut down on the use of chemicals in agriculture. *Chapter 6* indicates the importance of Arbuscular mycorrhizal fungi (AMF) for soil quality and tolerance of plants to biotic and abiotic stresses. Biotic stress is the subject matter of *Chap. 7* is the subject matter of how wheat genetic variability is obtained. New and useful genetic variations exist in the wild wheat progenitor species that can be utilized for the enhancement of the existing wheat breeding pools and improve yield stability. This was followed by *Chapter 8* dealing specifically with Variability in *Fusarium* Species causing wilt disease in crops. Abiotic stresses including salinity are a major threat to agricultural productivity and hence global food security are described in *Chapter 9*. Crop plants have adopted specialized strategies to reduce the impact of stress.

Chapter 10 is devoted to wheat grain quality advances in the genomics of grain quality are considered crucial for defining genes and their networks underpinning functional flour qualities. *Chapter 11* talks about N use efficiency (NUE) in agriculture and future development. The use of N in agriculture and its significance in the sustaining human society is addressed, especially in the developing countries. *Chapters 12 and 13* covers the issue of heavy metals toxicity in soils, uptake by plants. They throw light on the arsenic toxicity in plants and their tolerance mechanism in plants.

Chapter 14 describes the in vitro production of secondary metabolites using elicitor *in Catharanthus roseus.* Elicitation has been carried out in a large number of medicinal plants, this article deals with the *Catharanthus roseus*, as it is an important source of anticancer compounds Vinblastine (VLB) and Vincristine (VCR). Handling soybeans under stress is the topic of *Chapter 15.* Soybean is among the most important leguminous plants with the ability to establish symbiotic association with the N-fixing bacteria, *Bradyrhizobium japonicum*. One of the most important processes, affecting the performance of soybean under stress is the inhibited exchange of the signal molecules, specifically genistein, between the host legume and *B. japonicum* during the initiation of symbiosis. *Chapter 16* is a review on the *genus Atriplex*. This review is a contribution to the knowledge on the ecological and socioeconomical potential of some plant genus *Atriplex*. The last *Chapter 17* deals with' the role of polyamines in stress responses'. Genetic manipulation of crop plants for altered regulation of PA biosynthesis/catabolism may lead to improved stress tolerance potential.

This book will be a new contribution on crop improvement and be useful for scientists and graduate students in the area.

Prof. Dr. Ahmet Ruhi Mermut
President, Federation of the European Soil Scientists, Turkey & Prof. of Soil Sciences, University of Saskatchewan, Canada.

Preface

The improvement of crop species has been a basic pursuit since cultivation began thousands of years ago. To feed an ever increasing world population will require a great increase in food production. Wheat, corn, rice, potato and few others are expected to lead as the most important crops in the world. Enormous efforts are made all over the world to document as well as use these resources. Everybody knows that the introgression of genes in wheat provided the foundation for the "Green Revolution". Later also demonstrated the great impact that genetic resources have on production. Several factors are contributing to high plant performance under different environmental conditions, therefore an effective and complementary use of all available technological tools and resources is needed to meet the challenge.

The developments in biotechnology, genomic research, and molecular marker applications has brought to the forefront an interdisciplinary science that is revolutionizing 21st century crop improvement. Many new genomics technologies like next generation sequencing, omics technologies have emerged as powerful tools for understanding genome variation in crop species at different molecular levels.

The era of genomics seems to be upon us and new techniques will probably enable us to access the genetic basis of metabolomics associated traits much more rapidly. The information and developments related to the metabolomics, transcriptomics analysis and extensive phenotyping of genetically diverse populations together with bioinformatics is going to prove of great help in the field of crop biotechnology. These technologies will unveil the metabolic pathways for under-resourced crop species.

In this book attempt has been made to bring together chapters from different authors and highlight the current status of crop productivity in the light of developments in crop biotechnology, and at the same time provide information on some recent genomic tools and novel genetic and breeding approaches with a final aim of crop improvement. Emphasis has been laid on the topics related to advances in crop biotechnology, the key principles influencing the current practice in crop improvement programs and elucidate the nature of new approaches as well as modern techniques in crop improvement and how molecular plant breeding opens new avenues for research and is contributing to discoveries in this field.

We hope that a new generation of researchers will benefit much from this book and share the respect for the crop plants we all live by and concern for the maintenance of diversity.

The final objective of this book is to refresh and emphasize the fact that we are compelled to save our biodiversity, otherwise plant breeding possibilities will decrease to the extent that it will cost us much.

Dr. Khalid Rehman Hakeem
Dr. Parvaiz Ahmad
Prof. Munir Ozturk

Contents

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Dr. Khalid Rehman Hakeem, PhD is Post Doctorate Research Fellow at Universiti Putra Malaysia (UPM), Serdang, Selangor, Malaysia. He has completed his Post Graduation (Env. Botany) as well as Ph.D. (Botany) from Jamia Hamdard, New Delhi, India in 2006 and 2011 respectively. Dr. Hakeem has more than 6 years of teaching/research experience in Plant Eco-Physiology, Biotechnology, as well as Environmental sciences. Recipient of several fellowships at both national and international levels, Dr. Hakeem has so far edited/authored more than seven books with International publishers. He has also to his credit more than 20 research publications in peer reviewed journals and 10 book chapters. Dr. Hakeem is currently engaged in studying the plant processes at ecophysiological as well as proteomic levels.

Dr. Parvaiz Ahmad, PhD is Assistant professor in Botany at A.S. College, Srinagar, Jammu and Kashmir, India. He has completed his post-graduation in Botany in 2000 from Jamia Hamdard New Delhi India. After receiving Doctorate degree from Indian Institute of Technology (IIT) Delhi, India he joined International Centre for Genetic Engineering and Biotechnology, New Delhi in 2007. His main research area is stress physiology and molecular biology. He has published more than 30 research papers in peer reviewed journals and 16 book chapters. He is also editor of 6 volumes (5 with Springer and 1 with Studium Press-New Delhi, India). He is recipient of Junior Research Fellowship and Senior Research Fellowship by CSIR, India, and has been awarded Young Scientist Award under Fast Track scheme in 2007, by the Department of Science and Technology, Govt. of India. Dr. Parvaiz is actively engaged in studying the molecular and physio-biochemical responses of different agricultural and horticultural plants under environmental stress.

Prof. Munir Ozturk, PhD is a Consultant Fellow at Faculty of Forestry, Universiti Putra Malaysia. He has completed his Post-Graduation in Botany in 1964 Jammu & Kashmir University, India, Ph.D. and D.Sc. in 1970, 1975 in the field of Eco-Physiology from Ege University, Izmir, Turkey. Dr. Ozturk was appointed as full Professor in 1980 at the Ege University and served as Chairman of Botany from 1983–1986, and served as Director of Centre for Environmental Studies between

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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

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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

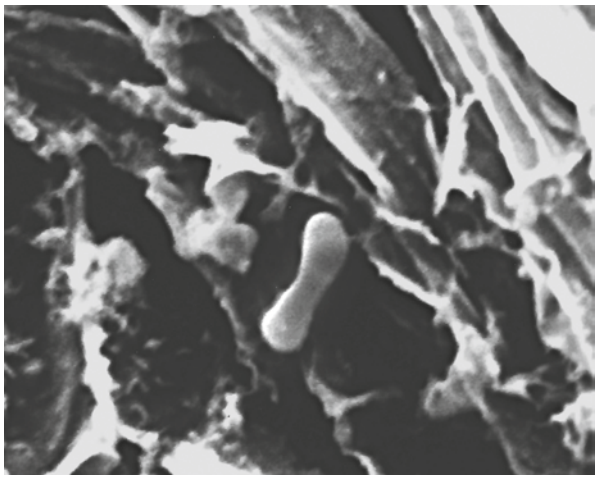

Fig. 1.1 Scanning electron micrograph of attachment of *Agrobacterium rhizogenes* strain R1000 to sunflower (*Helianthus annuus* L.) cotyledonary node cell

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).

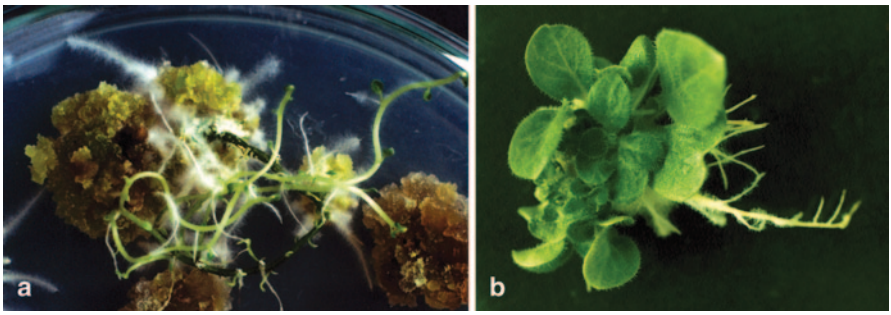

Fig. 1.2 Hairy root formation induced by *A. rhizogenes* strain 8196 in potato (*Solanum tuberosum* L.) callus cultures (**a**), regenerated tobacco (*Nicotiana tabacum* L.) plantlets (**b**). (From Arican)

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*

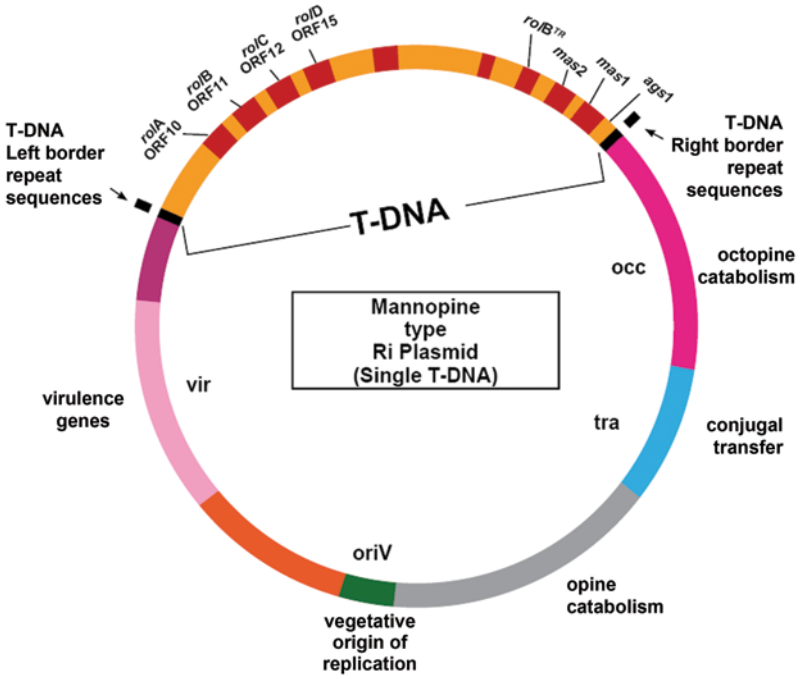

Fig. 1.4 Schematic representation of Mannopine type Ri plasmid of *A. rhizogenes*

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).

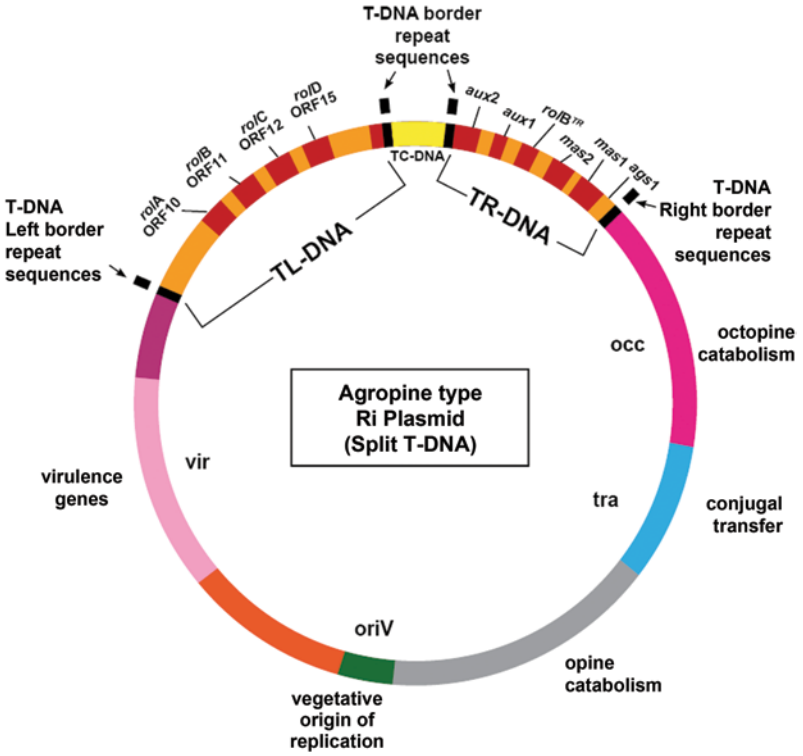

Fig. 1.5 Schematic representation of Agropine type Ri plasmid of *A. rhizogenes*

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

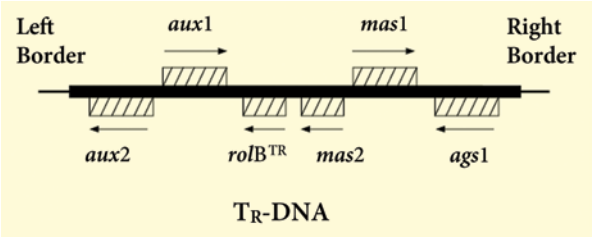

Fig. 1.6 Schematic representation of gene locations on T_R-DNA

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

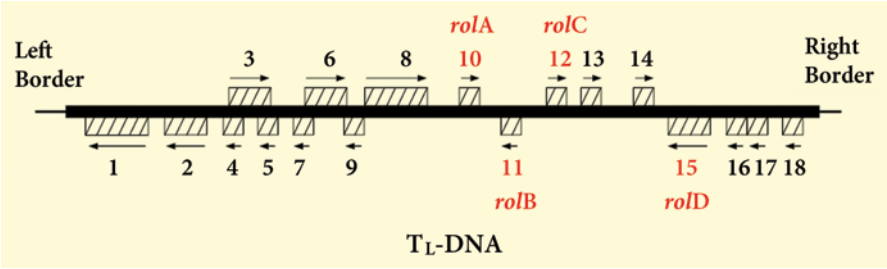

Fig. 1.7 Schematic representation of gene locations on T_L-DNA

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