Cloning of *myc* tagged *rolA* gene in *Agrobacterium*-mediated plant transformation vector

By

Muhammad Arslan Khan

Department of Biochemistry

Faculty of Biological Sciences

Quaid-i-Azam University

Islamabad, Pakistan

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"In the name of ALLAH, The Beneficent and The Merciful"

The Guardian of faith, The Majestic,

The Bestower and The Forgiver.

Whose help and guidance, I

always implore at every step.

CERTIFICATE

This thesis, submitted by **Mr. Muhammad Arslan Khan** to the Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan, is accepted in its present form as satisfying the thesis requirement for the Degree of Master of Philosophy in Biochemistry/Molecular Biology.

Supervisor:

Dr. Bushra Mirza
Professor

External Examinar:

Dr. M. Sheeraz Ahmad
Assistant Professor
(PMAS) University of Arid
Agriculture, Rawalpindi.

Chairperson:

Professor

Dated: September 04, 2014

DEDICATION

I dedicate this dissertation to,

MY COMPASSIONATE PARENTS
ENDEARING SISTERS
AMIABLE BROTHERS
RESPECTABLE TEACHERS
AND
AFFABLE COLLEAGUES

CONTENTS

Acknowledgements		x
List of Abbreviations		xiii
List of Figures		x
List of Tables		xi
Abstract		xii
Chapter 1: Introduction and Review of Literature		
1.1	Importance of medicinal plants and secondary metabolites	1
1.2	Chemical synthesis and pharmaceutical industry perspective	2
1.3	Techniques for secondary metabolites production	2
1.4	Strategies for enhancing secondary metabolites production	3
1.4.1	Selection of highly productive cell lines	3
1.4.2	Optimizing plant culture media	4
1.4.3	Genetic transformation and synthetic biology	4
1.5	The science of metabolic engineering and need of functional genomics	5
1.6	Artemisinin a classic example	6
1.7	Methods of genetic transformation	7
1.8	*Agrobacterium*-mediated transformation	7
1.8.1	Taxonomy and diseases caused by *Agrobacterium*	8
1.8.2	Host range	9
1.8.3	What is T-DNA	9
1.8.4	Molecular mechanisms of *Agrobacteirum*-mediated transformation	10
1.8.5	The oncogenes of *Agrobacteriumtumefaciens*	11
1.8.6	The oncogenes of *Agrobacteriumrhizogenes*	12

1.8.6.1	The *rol*genes and their importance in secondary metabolite production	16
1.8.7	Types of *Agrobacterium* vector systems	18
1.8.7.4	Features of binary vectors	19
1.8.7.5	Drawbacks of binary vector systems	21
1.8.7.6	Disarmed Agrobacterium strains	21
1.8.7.7	*E.Coli (DH5α)* as holding strain	22
1.8.7.8	Feature of pEarleyGate binary vectors series	22
1.8.7.9	Choice of expression vector for plants	23
1.8.8.10	Production of transformed plants with Ti plasmid	23
1.9	Molecular Cloningand Gateway cloning	24
1.10	c-Myc oncogene to *myc* tag	25
1.11	Epitope(*Myc*) tagging for functional proteomics	25
1.12	PCR encoded epitope tagging (PET)	26
1.13	The bioethics and biosafety of gene transfer	27
1.14	Aims and Objectives	27

Chapter 2: Materials and Methods

2.1	Laboratory plasticware	28
2.2	Laboratory glassware	28
2.3	Chemicals, enzymes, kits, and DNA ladders	28
2.4	Bacterial strains	28
2.4.1	*Agrobacterium tumefacienes* strain *GV3101*	28
2.4.2	*Agrobacterium rhizogenes* strain *ARqual*	29
2.4.3	*E.Coli (DH5α)* as holding strain	29
2.4.4	*E.Coli (DB 3.1)*	29

2.5	Culturing media, conditions and selection for bacteria	30
2.6	Plasmid constructs	30
2.6.1	Expression vector or destination vector	30
2.6.2	Transformation vector pMBQAU1001	30
2.6.3	pRiA4 of *Agrobacterium rhizogenes* ARqua1	30
2.7	Cloning of recombinant plasmid pMBQAU1001	31
2..7.1	Extraction of plasmid DNA (pEarleyGate203 and pRiA4)	31
2.7.2	Agarose gel electrphoresis	35
2.7.3	Designing of primers and PET tagging	35
2.7.4	Restriction of vector and insert	36
2.7.5	Eluting through gel	36
2.7.6	Ligation of vector and insert to make pMBQAU1001	37
2.8	Transformation of *E.Coli DH5α* with pMBQAU1001	37
2.8.1	Competent cells Preparation of *E. Coli* and *Agrobacterium tumefacienes* strain GV3101	37
2.8.2	Electroporation of pMBQAU1001 in competent *E. coli* cells and *Agrobacterium tumefacienes* strain GV3101	38
2.9	Molecular screening of positive clones	38
2.9.1	PCR confirmation	38
2.9.2	Restriction mapping	39
2.9.3	Sequencing	39
2.10	Preparation of recombinant bacterial glycerol stock	40

Chapter 3: Results

3.1	Cloning of pMBQAU1001 and transformation in *E.Coli (DH5α)*	41
3.1.1	Plasmid isolation	41

3.1.2	PET tagging of *rolA* gene	42
3.1.3	Purification and restriction	43
3.1.4	Ligationand transformation	45
3.1.5	Molecular analysis of clones by PCR	45
3.1.6	Restriction map analysis	47
3.2	Moving pMBQAU1001 in *Agrobacterium tumefaciencs GV3101*	49
3.2.1	Molecular analysis of clones by PCR	49
3.3	Sequencing of pMBQAU1001	51
Chapter 4: Discussion		53
Chapter 5: References		58

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Muhammad Arslan Khan

List of Abbreviations

ACTs	Artemisinin-based combination therapies
AcV5	*Autographacalifornica Multiple nucleopolyhedrosis virus*
ags	Agropine synthase gene
AMP	Adenosine monophosphate
AQ	Anthraquinone
AS	Acetosyringone
Avg.	Average
BAC	bacterial artificial chromosome
Basta^R	Bialaphos resistance or phosphinothricin acetyltransferase gene
BIBAC	Binary bacterial artificial chromosome
BiFC	Bimolecular fluorescence complementation
BRET	Bioluminescence resonance energy transfer
CAMV35S	Cauliflower tobacoo mosaic virus 35Spromotor
cMyc	Myelocytomatosis cellular oncogene
con	Conjugation
CTAB	Cetyltrimethyl ammonium bromide
dNTPs	Deoxyribonucleotide triphosphates
Dof	DNA binding with one finger
DSBR	Double strand breaks repair
DTCS	Dye Terminator Cycle Sequencing
EDTA	Ethylene Diamine Tetra Acetic Acid
FDA	Food and Drug Administration
FLAG	FLAG tag for affinity purification
FRET	fluorescence resonance energy transfer
GA	Gibberellic acid
GFP	Green fluorescent protein
GMO	Genetically modified organism
GS	Glutamate synthetase
GST	Glutathione S-transferase

GUS	β-glucuronidase
HA	Human influenza hemagglutinin
HDR	Homology directed repair
HTS	High-throughput screening
IAA	Indole-3-acetic acid
ILA	Indole-3-lactic acid
IncP	incompatibility group P of plasmids
IR	Inverted Repeat
LA	Luria Agar
Lb	Luria-Bertani broth
LB	Left border
LMW	Low molecular weight
LSC	Large Single-Copy
mM	Mili molar
NADPH	Nicotinamide adenine dinucleotide phosphate
nBlast	Nucleotide BLAST
NCBI	National Center for Biotechnology Information
NLSs	Nuclear target signals
NPTII	*Neomycin phosphotransferase II*
NtBBF1	*Nicotianatabacumrol*B domain B factor 1
OCD	Ornithine cyclodeaminase
OD	Optical density
ORFs	Open-reading frames
ori	Origin of replication
PA	Polyamine
PET	PCR encoded epitope tagging
pMBQAU1001	Recombinant plasmid having myc-rolA fragment in pEarleyGate203
pmol	Pico mole
pRiA4	Ri plasmid of *A. rhizogene A4* strain
RB	Right border

RBF	Rol Binding Factor 1
Ri	Root inducing
Rol	Root Oncogenic Loci
rps	Ribosomal Proteins
SDS	Sodium Dodecyl Sulphate
SLS	Sample loading solution
SNP	Single Nucleotide Polymorphism
SSC	Small Single Copy
SSR	Simple Sequence Repeat
T4SS	type IV secretory system
TAE	Tris Acetate Ethylene Diamine Tetra Acetic acid
TBE	Tris- borate- EDTA
TCLs	Tobacco thin cell layers
T-DNA	Transfer DNA
TE	Tris-EDTA buffer
Ti plasmid	Tumor inducing plasmid of *A.tumifaciens*
TL-DNA	Left Transfer DNA of pRiA4
TR-DNA	Right Transfer DNA of pRiA4
UTR	Untranslated regions
vir	Virulence

List of Figures

Sr. #	Title	Page #
1.1	Typical components of T-DNA binary vector.	20
1.2	Types of *Agrobacterium* T-DNA vector.	20
2.1	Genetic elemental map of pHarleyGate203.	39
2.2	Genetic map of pMBQAU1001 with myc tagged *rolA* gene.	40
2.3	PCR encoded epitope tagging of *rolA* gene and cloning in pHarleyGate203 to make recombinant plasmid pMBQAU1001.	41
3.1	Colonies of *Agrobacterium rhizogenes strain A4*.	42
3.2	Midiprep of *A. rhizogenes* strain *ARqua1*.	42
3.3	Midiprep of pHarleyGate203 from *E.Coli DB3.1*.	42
3.4	PCR amplicon after 3rd round of *rolA* gene (352bp) PHT tagging.	43
3.5	myc tagged *rolA* gene (352bp) sequence after 3rd round of PCR with different restriction enzymes sites and breathing site.	43
3.6	myc tagged *rolA* gene purification on 0.8% agarose gel by Qiaprep.	44
3.7	Final checking of insert and vector before restriction.	44
3.8	Transformed colonies of recombinant *E.Coli DH5α*.	45
3.9	PCR amplification of 352 bp fragment of the myc-*rolA* gene from pMBQAU1001 in recombinant *E.Coli DH5α* colonies.	46
3.10	PCR screening of recombinantspMBQAU1001 for *NPTII* gene.	46
3.11	Repeating PCR amplification of 791bp fragment of the *NPTII* gene.	47
3.12	Plasmid pMBQAU1001 qauntification before restriction.	48
3.13	Restriction digestion of plasmid pMBQAU1001 for confirmation.	48
3.14	Colonies of recombinant *Agrobacterium tumifaciens LBA4404* on kanamycin 50mg/l and gentamycin 10mg/l selection.	49
3.15	PCR amplification of 303bp fragment of the *rolA* gene.	50
3.16	PCR amplification of 791bp fragment of the *NPTII* gene from pMBQAU1001 recombinant in *A. tumifaciens LBA4404*.	50
3.17	*myc-rolA* gene PCR amplicon with pfu DNA polymerase for sequencing.	50
3.18	First purification for sequencing of *myc-rolA* gene.	51

List of Tables

Sr. #	Title	Page #
1.1	Commonly used disarmed *Agrobacterium* strains	22
2.1	Conditions for gene amplification using PCR	33

Abstract

It is a proven fact that secondary metabolites like alkaloids, steroids etc. are crucial for lead identification, optimization, drug discovery and development. Because most of the small molecule drugs are complex organic molecular scaffolds that are difficult to synthesise in vitro, currently plants are considered the best source for their commercial supplies. However, small amount of these metabolites in cells is bottleneck in their large scale extraction, due to the high cost in terms of time and money. So understanding the molecular biology of plants as well as various genes that boost these metabolic fluxes is a good strategy in this regard. The *rol* genes derived from *Agrobacterium rhizogene* are widely used in this regard but the exact mechanism of how *rol* genes enhance secondary metabolites is not known. Hence *rol* genes functional proteomic detailed understanding is essential in their rational manipulation. This study was designed to shed light on this aspect by constructing epitope tagged *rolA* gene by adaptor PCR (N-terminal *myc* tagging by using overlapping set of primers) at 5' end of *rolA* gene followed by its forced cloning into pEarlyGate203 expression vector for *Agrobacterium tumifaciens* mediated transformation. Thus resulting recombinant plasmid pMBQAU1001 harbours *CaMV 35S* promoter, *Kan* bacterial selectable marker gene and plants selectable marker gene *BASTAr*. Initially competent cells of *E.Coli DH5α* were transformed with this construct by electroporation and colonies were successfully screened by PCR as well as by restriction mapping. Later, pMBQAU1001 was electroporated into competent cells of *disarmed Agrobacterium tumifaciens GV3101* followed by screening and validation of cloning by using gene specific PCR primers for both *NPTII* (Kan) and *rolA* gene as well as final confirmation by sequencing of myc-*rolA* gene fragment. Prospectively, this transformed *Agrobacterium tumifacien GV3101*strain can be used in future for functional proteomics studies of *rolA* gene including subcellular immunolocalization and affinity purification.

Chapter 1

Introduction and Literature Review

Introduction

1.1 Importance of medicinal plants and secondary metabolites

Plants are crucial for life on earth and an indispensable resource for human welfare. Essentially all we eat is their product in direct or indirect way. Moreover, plants are blessed with an amazing array of biochemical pathways producing molecules that help to elicit an effective defense response to biotic and abiotic stress situations. In addition these sophisticated molecules supply us with a variety of natural chemicals such as drugs, nutraceuticals, agrochemicals, food additives, fuels and biomass (Eloff, 1998; Orhan, 2012). Exceptional medicinal properties of plant secondary metabolites such as terpenes, phenolics, coumarins, anthocyanins, iridoids, and cardiac glycosides, mark their importance as drug candidates (Vormfelde and Poser, 2000). Natural products significance in drug discovery is proven by the fact that more than 50 percent drugs approved by FDA are either natural products or their derivatives including big names like aspirin, eribulin, tetracycline, artemisinin, camptothecin, lovastatin, paclitaxel, penicillin, reserpine and morphine (Kingston, 2011; Bickerton *et al.*, 2012; Lachance *et al.*, 2012; Newman and Cragg, 2012).

These secondary metabolites have complex but flexible structures because their synthesis involves a series of different enzymes. Their diversified binding groups and configurations allow them to modulate a variety of cellular effector proteins (Ji *et al.*, 2009). During early 1990s, research focus shifted on combinatorial chemistry and high-throughput screening (HTS) for lead identification. However, this strategy was unsuccessful as statistics indicate a decrease of new drug entities approved by Food and Drug Administration (FDA) from 53 in 1996 to just 17 in 2007. That is why drug discovery experts at pharmaceutical industries are therefore returning to nature's toolbox and harnessing the potential of various '-omics' technologies along with the HTS in targeted drug designing for many devastating diseases including cancer, diabetes, infectious diseases and dementia (Baker *et al.*, 2007; Beghyn *et al.*, 2008; Hunter 2008; Ji *et al.*, 2009). In addition chemi-informatics tools are used synergistically for screening and selection of appropriate scaffolds or drug-like chemical space (Bickertoni *et al.*, 2012; Lachance *et al.*, 2012). Therefore secondary metabolites are of great scientific, economic and therapeutic interest.

1.2 Chemical synthesis and pharmaceutical industry perspective

Over the past 30 years, only two novel classes of antibiotics have been launched on the market: the oxazolidinone (linezolid) and the cyclic lipopeptide (daptomycin). So, there is growing need of novel drugs especially antibiotics because of emergence of resistance strains of microorganisms.

Large scale and cost effective total chemical synthesis of many low molecular weight (LMWs) drugs particularly with multiple N-heterocyclic rings from petrochemicals is still a challenge. That is why essentially natural products are the special compounds for which biosynthesis is competing with chemical synthesis. However, the main barrier in the usage of plants as a source for these metabolites is their inability to ensure the sustained and efficient supply of these compounds because the yield can be affected depending on the genetic background, the geographic location and climatic conditions of cultivation. In addition there are potential difficulties in harvest and transport methods.

1.3 Techniques for secondary metabolites production

The in vitro culturing can be started from plant parts like stem, leaf, seed, root, etc. But most frequently actively dividing parts of plant, such as meristematic regions are used. For cell culture, differentiated explant must be used for initiating callogenesis, having growth regulators to facilitate cell division and dedifferentiation. The process can be split up into three stages: induction involving active metabolism of cell prior to mitosis, cell division involving the cells division in explant, followed by differentiation of cells along with enhancement of certain pathways of metabolism involved in secondary metabolites formation (Dodds, 1982). For secondary metabolites generation through in vitro techniques, cell suspension and tissue culture are primarily used. In tissue culture, small explants of tissue are inoculated in semisolid media, having growth regulators that induce callus. While in the case of suspension culture, the calli are kept with continuous stirring in a liquid medium. This technique is attractive for the synthesis of secondary metabolites because of easy handling, uniformity, simplicity, limited number of cell types and rapid growth (Rhodes, 1987). The form of culture bears on product formation, cell growth, refinement and the kind of bioreactors used. At commercial level, mainly cell suspension cultures (Zhao *et al.*, 2005) and transformed root cultures have been used

(Guillon *et al.*, 2006). Organ culture is used because production of some secondary metabolite is critically affected by the level of differentiation of cell, making the use of tissue culture imperative. Likewise, hairy root cultures are important source for production of commercially important secondary metabolites on global scale (Flores and Curtis, 1992; Wilson, 1997). Hairy roots formed from genetic transformation of roots by *Agrobacterium rhizogenes* often grow faster than plant cell cultures (Flores *et al.*, 2007) plus do not need hormones in the medium. In addition these are genetically stable with greater biosynthetic capacity (Banerjee *et al.*, 1998).

1.4 Strategies for enhancing secondary metabolites production

Multiple strategies are being employed to synthesize sufficient quality and quantity of these secondary metabolites at industrial scale. Current advancements in the field of plant biotechnology and molecular biology techniques demonstrated the potential of exploiting plant cell and tissue cultures for the large-scale synthesis of valuable medicinal metabolites rather using whole plants which require extensive land exploitation (Orhan, 2012).

Recently, cell culture techniques are being focused for boosting the synthesis as well as accumulation of industrially important secondary metabolites (Stafford, 1991). An alternative approach for enhancing the synthesis potential of plant for medicinal compounds is by using biotechnological procedures including cell, tissues and organ cultures. In addition following approaches improve production and reduce the time to isolate the active ingredients of plants.

1. Selection of highly productive cell lines
2. Culture media optimization as well as physicochemical parameters likes cell permeability, eliciting systems, cell immobilization and biotransformation
3. Genetic engineering

All these procedures are a viable option to boost the synthesis of secondary metabolites:

1.4.1 Selection of highly productive cell lines

The selecting type of explant is one of the critical factor in achieving a highly productive cell line of a desired metabolite (Fedoreyev *et al.*, 2000. Oncina *et al.*, 2000). The selection is based on performance, growth rate, stability and maintenance

of cell culture line having desired properties (Trejo *et al.*, 2008). This selection is done by challenging them to toxic inhibitors, some abiotic stress, or extreme conditions.

1.4.2 Optimize culture media and other physicochemical parameters

During cell growth and differentiation the synthesis of secondary metabolites depends on the various parameters (Raval *et al.*, 2003) like carbon, nitrogen, phosphate source, growth regulators and light. These are important factors for increasing biomass, growth, nutrient consumption and yield of secondary metabolites (Akalezi *et al.*, 1999; Sato *et al.*, 1996; Ramachandra and Ravishankar, 2002; Dörnemburg,1995; Yeoman and Yeoman, 1996; Zhang *et al.*, 2002). Temperature is also an important factor as the heat stress stimulates the production of metabolites (Georgiev *et al.*, 2004). Cell Permeability affects release of intracellular secondary metabolites in the culture medium so this facilitates the recovery and purification of the product by electropermeation or ultrasonication (Dörnenburg and Knorr, 1992; Brodelius and Pedersen, 1993; Dornemburg, 1995). In the plant cell cultures elicitors enhance the synthesis of secondary metabolites which are classified into two types first biotic including polysaccharides, glycoproteins. Secondly abiotic including osmotic shock, heavy metal ions and UV rays (Furzes *et al.*, 1991; Dornemburg, 1995). Cell immobilization is the confinement of cell on or inside solid matrix just to facilitate the reuse of biocatalyst as well as release of the product (metabolite). By employing different types of gels like agarose, calcium alginate or gelatin this technique leads to clustering of cells(Osuna *et al.*, 2008).

1.4.3 Genetic transformation and synthetic biology

Recombinant DNA technology is fast, free of sexual barriers and independent of variability. Through transformation we can also enhance the production of secondary metabolite by inserting our desired genes. Recombinant DNA technology involves:

1. Identification and isolation of gene of interest
2. Modifying the gene to express in the target plant system
3. Cloning in a suitable expression vector
4. Transformation

In synthetic biology living entities are metabolically engineered with a few or more transgenes for commercial scale production of costly secondary metabolites. Engineering of the metabolic fluxes is done by cloning a specific critical enzyme or even complete metabolic pathway of a genetically complex or intractable organism to those simple organisms that are readily engineered (Malpartida and Hopwood, 1984). In this way new heterologous production systems are on the rise. Transgenic moss, lemna, fungal or yeast, animals and plants systems have the potential to become economically and industrially applicable.

1.5 The science of metabolic engineering and need of functional genomics

Before manipulation of genes from synthetic biology perspective first mapping of the metabolic pathways of secondary metabolite is done by functional genomics approaches. Although the recombinant DNA technology is now almost two and half decade old, but there is still lack of high-throughput methods to speed up the screening and designing of new molecules by recombination of pathways in an automated fashion. Gene knockout experiments, complementation of blocked mutants and heterologous expression reveals the details of biosynthetic gene clusters, ultimately leading to the isolation and characterization of entire biosynthetic pathways (Hutchinson, 1997). Furthermore, it is necessary to continuously enhance the variety of heterologous hosts. For example studies on polyketide antibiotics are benchmark as these established the new solutions for the engineering of major metabolic pathways employing transposons and episomes, overcoming one of the main methodological bottlenecks for the heterologous expression of these pathways. Sequencing by high throughput technologies followed by structural prediction of enzymes by computational algorithms have expanded opportunities to explore the metabolic potential of organisms across the spectrum of life (Gomes *et al.*, 2013) along with ability to measure reaction fluxes using carbon-13 isotopic labeling and Gas chromatography-mass spectrometry.

There is a paradigm change since the adaptation of holistic approach for pathways exploration based on genomics, high-throughput biology, bioinformatics, and functional genomic, in plant secondary metabolism studies (Liu *et al.*, 2002). In a milestone study, UniGen collected data on global scale and built a library of about 40,000 secondary metabolites from medicinal plants by 2005. As in silico models

predicts that changes in genes of metabolic enzymes involved in carbon fluxes can help in reengineering or redirecting metabolic refluxes optimally. Hopefully efficient cellular factories for bio-production can be built on this rational design based strategy (Becker *et al.*, 2011). Multivariate modular metabolic engineering is new approaches that assesses and eliminate regulatory pathways choke points by stipulating the network of metabolic pathways as an assembly of separate and self-contained components. Prospectively the slumping costs of *de novo* gene synthesis along with ever enhancing sensitivity, and automation of investigational and bioinformatics tools will result in the ontogeny of metabolic engineering science (Yadav *et al.*, 2012).

Moreover, supplementation of synthetic chemistry with synthetic biology can substantially advance our ability to synthesize cost effectively, complex drugs on commercial scale. A rational combination of advanced techniques with the de-replication strategies for eliminating known compounds will prove to be a gateway for not only identification of novel lead compounds in drug discovery but also lead optimization (Rocha *et al.*, 2014).

1.6 Artemisinin a classic example

Artemisinin is a drug of choice in malaria recommended by World Health Organization but it is in short supply and too expensive for malaria affected third world population. Although the total organic synthesis is established, but chemical synthesis of artemisinin is not commercially feasible because of its complex chemical space and low yield. To date leaves, roots and flowers of Artemisia species are the only source of Artemisinin that is present in very low quantities. Beside their other limitations, selection and other non-conventional approaches are being employed for obtaining high artemisinin yielding clones. In addition, in vitro culture system of *A. annua* has been exploited in this regard (Delabays *et al.*, 1993). Prospectively, a rational mix of synthetic chemistry and synthetic biology has the potential for the large-scale, high-output semi-synthesis of artemisinin that is extracted from the plant *Artemisia annua* (Enserink, 2005). *A. annua* genes *YP71AV1, CPR1, CYB5; ADH, ALDH1* that are involved in oxidation step of amorphadiene to artemisinic alcohol, artemisinic alcohol to artemisinic aldehyde; artemisinic aldehyde to artemisinic acid, respectively (Paddon *et al.*, 2013) are isolated, combined and supplemented with genes of other organisms into a single strain of the *Saccharomyces*

cerevisiae growing by fermentation process by using simple sugars (Ro *et al.*, 2006) to make high-yielding artemisinic acid, a precursor of artemisinin and latter converting it to artemisinin by just a few chemical synthesis steps (Zeng *et al.*, 2008). This technique holds the promise to be an industrially viable way for the semi-synthesis of artemisinin to ensure its steady supply for incorporation into artemisinin-based combination therapies (ACTs) (Paddon *et al.*, 2013). The process is cost effective, environmentally friendly and reliable, that needs to be further improved for large scale production of the drug. Similar approaches are being perused for other complex drugs like paclitaxel holding the promise of breakthroughs in future.

1.7 Methods of genetic transformation

Recombinant DNA technology and plant tissue culture are the landmark techniques that allow sophisticated genetic transformation of medicinal plants. Introducing foreign DNA to the plant cell may enhance the production of chemical structures as phenylpropanoids, alkaloids, terpenoids, quinones and steroids. There are broadly two main categories of genetic transformation tools.

(1) Physical methods

The physical methods of genetic transformation include microinjection, gene gun or biolitic, Poly ethylene glycol mediated.

(2) Biological Agents

While biological methods include *Agrobacterium*-mediated, Viruses mediated.

1.8 *Agrobacterium*-mediated Transformation

The soil bacterium *Agrobacterium tumefaciens* has historically a significant character in the field of transgenic technologies of plants. Vision of exploiting it as vector to generate transgenic plants is almost three decade old. Because it harbors the Ti plasmid, causing crown galls on plants (Brown *et al.*, 2001). To date more than 50% of transformed plants are produced by *Agrobacterium*-mediated transformation. Transformation involves the integration of T-DNA segment of Ti-plasmid in the host plant genome (Chilton *et al.*, 2003). Which bears many oncogenes facilitating the bacterial growth (Garfinkel *et al.*, 2006; Joss *et al.*, 1989) as well as for synthesizing and catabolizing the opines (Murai and Kemp, 1988) that are source of nitrogen for

Agrobacterium. Recombinant *Agrobacterium* strains who's natural T-DNA is replaced with metabolically important genes. is the most important tool for generating transgenic plants (Tzfira *et al*., 2006). It is estimated that in developed countries already a major area is cultivated by economically significant transgenic crops including tomatoes, canola, potato, corn, soybeans and cotton (Gelvin *et al*., 2003).

1.8.1 Taxonomy and diseases caused by *Agrobacterium*

Kingdom	Bacteria
Phylum	Proteobacteria
Class	Alpha Proteobacteria
Order	*Rizobiales*
Family	*Rhizobiaceae*
Genus	*Agrobacterium*
Species	*Agrobacterium tumefaciens, A. rhizogenes, A. albertimagni, A. Larrymoorei, A.radiobacter, A. rubi, A.vitis.*

The genus *Agrobacterium* has variety of species depending on the symptoms of neoplastic disease and host range. Thus, *Agrobacterium radiobacter* is an "avirulent" specie, *A. tumefaciens* causative agent of crown gall, *Agrobacterium rhizogenes* possess an agropine like root inducing (Ri) plasmid and is responsible for hairy root disease means excessive root formation (Gelvin *et al*., 2003; Offringa *et al*., 1986). *Agrobacterium rubi* is implicated in cane gall disease and *Agrobacterium vitis* is involved in pathologies of grape gall disease (Otten *et al*., 1984). Although this nomenclature is contemplated by bergeys manual but its is confusing and complex. Perhaps a more important system of classify the genus Agrobacterium into "biovars" on the basis of metabolic and growth characteristics (Keane *et al*., 1970). According to which most *Agrobacterium tumefaciens* and *Agrobacterium rubi* (Tighe *et al*., 2000) strains classified in biovar I, *Agrobacterium rhizogenes* strains represent biovar II and *Agrobacterium vitis* strains fall in biovar III. In addition *Agrobacterium* is categorized according to the synthesis and catabolism of opine into Agropine type (strain EHA105) that carry genes for agropine synthesis and catabolism. Octopine type strain LBA4404 that carry genes to synthesize octopine in the plant and catabolism in the bacteria. Nopaline type strain GV3101::pMP90 carrying gene for

synthesizing nopaline in the plant and for utilization in the bacteria (Hellens *et al.*, 2000).

1.8.2 Host range

Initially, it was envisaged that gymnosperm, dicots and a few species of monocot can only be transformed using this technique. Currently this narrative is totally changed and it is shown that many "recalcitrant" species like fungi as well as monocots can also be transformed (Chan *et al.*, 2004; Bundock *et al.*, 2005). In addition, the transformed cells mostly carry one copy or a few copies of heterologous gene integrated stably in genome with little rearrangement, and recently relatively large segments of DNA can be transformed into the plants (Hamilton *et al.*, 1996; Liu *et al.*, 1999).

1.8.3 What is T-DNA

During infection the transfer of T-DNA to plant genome is the reason for genetic manipulations by *Agrobacterium* (Rao *et al.*, 2009). A cluster of neoplastic genes along with opine metabolism genes are the weapons of *Agrobacterium*. The expression of these genes in plants causes oncogenic phenotype of transgenic plant. The opines are basically the derivatives of amino acids so a food for *Agrobacterium* (Tzfira, *et al.*, 2006). The Ti plasmids found in nature are of 200 to 800 kb size. The T-region on Ti plasmid inside bacteria is called T-DNA which is of 10 to 30 kb about 10% of complete Ti plasmids (Gelvin *et al.*, 2003). Its characterization is based on the T-DNA borders which are highly homologous sequences about 25 bp long direct repeats. The T-strand which is a single stranded molecule is only transferred to the plants (Gelvin *et al.*, 2003). In the plant cell cytoplasm it is termed as mature T-complex. The variety of cellular mechanisms are employed by *Agrobacterium* to genetically transform the host cell are discussed later (Tzfira *et al.*, 2006). The tumor inducing plasmids of *Agrobacterium* also contain some other vital *virulence* regions of about 40kb size in addition to having *chv* genes which are chromosomally encoded (Tzfira *et al.*, 2006). Vir genes are vital and located on the Ti plasmid in a fragment of 40 kb known as virulence region or on the chromosome (chv genes) (Paul *et al.*, 1992). The VIR proteins by suppressing the host innate immune response facilitate the this genetic transformation (Gelvin *et al.*, 2003).

1.8.4 Molecular mechanisms of *Agrobacteirum*-mediated transformation

Ti or tumor inducing plasmids have T-DNA that is transferred to plants in addition it has other essential regions like origin of replication (*ori*), virulence (*vir*) and conjugation (*con*). For successful tumor formation in plant through a natural infection of *Agrobacterium* both vir genes and T-DNA are simultaneously required. Following are exact sequence of events.

1.8.4.1 Attachment

The infection begins at the wounded sites with the physical attachment of bacteria with the cell surface of plant. The bacterium recognizes the molecules of cell wall of the plant cell including vitreonectin protein that serve as a receptor for bacterium. Several genes play role in modification, exopolysaccharide synthesis, secretion (*pscA/exoC, chvA, pscA, cel* and *chvB*), bacterial attachment to plant cells and enforcement of contact by cellulose filaments, and regulation of vir gene stimulation (*chvD*) are constitutively expressed. These chromosomal genes along with *VirC* and/or *VirF* recognize the host plant cells (matthysse *et al.*, 2000). ChvA/B and cyclic β-1,2-glucans helps in stabilizing this attachment (Weising and Kahl., 1996; Winans, 1991).

1.8.4.2 Induction of virulence genes

Plant cells respond to injury by releasing some wound signals such as the phenolic acetosyringone (AS), sugars, pH which helps in recruiting *agrobacterium* by activating ChvE and virA that is a membrane histidine kinase through autophosphorylation. The *virA* subsequently phosphorylate asparate residue of *Vir*G protein that is a transcription factor leading to its activation (Peng *et al.*, 1998). Upon activation virG upregulates the other *vir* gene's expression by specifically binding an upstream region called *vir* box (Stachel *et al.*, 1986).

1.8.4.3 Transfer of T-DNA

The virD1 (a helicase) and virD2 (an endonuclease) generates the T-strand by specifically reading and cutting lower strand right borders as well as on left border and it is interesting to know that right border is the start site (Jayaswal *et al.*, 1987; Wang *et al.*, 1987). The transport channel type IV secretory system (T4SS) for T-DNA and accompanying virE2 and virF transfer is made up of virD4 and 11 virB

protein subunits. The virD4 which functions as a "linker" assists the docking of T-DNA/virD2 complexes with the T4SS. While a plethora of other proteins like virB2, virB5 and most probably virB7, make up the T-pilus that act as a conduit for both T-DNA as well as vir proteins channeling or probably act as a simple "hook" to ensure the contact with plant cell (Christie *et al.*, 2005). During transfection the virD2 and virE2 proteins are crucial players as they bind the T-DNA. The T-DNA journey is made possible by virE2 protein that make pore in the plant's plasma membrane (Gelvin *et al.*, 2003) (Ward and Barnes, 1988; Howard *et al.*, 1992).

1.8.4.4 Integration

The *vir*D2 cuts the 5' end of T-strand and gets covalently linked with it in the form of a complex. Then it is targeted to the nucleus due to the presence of nuclear localization signals (NLSs) on associated *vir*D2 and *vir*E2. The integration of T-DNA is random and copy number is variable (Howard *et al.*, 1992; Zupan *et al.*, 1996). Recently it is revealed that virD2 is actually a DNA ligase (Tzfira *et al.*, 2006). The virD2 mutant show decline in 40% transformation efficiency because only T-DNA is transported but not integrated that efficiently. Role of virE2 is also to guard T-strands from nucleases mediated breakdown in cytoplasm and nucleus of plant cells (Yusibov *et al.*, 1994; Rossi *et al.*, 1996). VirF probably unload the trans protein from the DNA before integration (Tzfira *et al.*, 2004). There are two models for Single or double-stranded T-DNA integration into genome, first is based on microhomology of T-strand homology directed repair (HDR) secondly double strand T-DNA integration occur via double strand breaks repairs (DSBR).

1.8.5 The oncogenes of *Agrobacterium tumefaciens*

1.8.5.1 Auxin and cytokinin synthesis gene

The auxin synthesis genes *iaaH* and *iaaM* of Ti plasmid encode two enzymes indole-3-acetamide hydrolase and tryptophan monooxygenase respectively. These enzymes catalyzes the modification of tryptophan into auxin indole-3-acetic acid (IAA) resulting in the higher amount of IAA inside crown galls. Like many viruses the *Agrobacterium tumefaciens* can also compromise plant defense through RNA silencing, allowing unchecked expressions of oncogenes like *iaaM* and agropine synthase (ags) gene. These genes further suppresses the plant regulatory pathways.

Similarly the cytokinin synthesis in plant is induced by ipt gene of Ti plasmid by encoding an enzyme isopentenyl transferase which catalyze a rate limiting condensation reaction of adenosine monophosphate (AMP) with an isoprenoid precusrsor (Astot *et al.*, 2000).

1.8.5.2 Gene 6b

Tml (tumor morphology locus) is a 1.25 kb region on T-DNA embracing a couple of genes: gene 6a and gene 6b involved in crown gall tumors (Garfink el *et al.*, 1981; Hooykaas *et al.*, 1988). Gene 6a encodes an enzyme opine permease without any direct involvement in tumor formation (Messens *et al.*, 1985). While gene 6b is a oncogene having intrinsic ability to induce small tumors on a limited spectrum of plants hosts its own (Hooykaas *et al.*, 1988).

1.8.5.3 Gene 5

The gene5 transcript is abundant in teratomas (Joos *et al.*, 1983) and encode an enzyme its transformed plants displays a major enhancement of ILA (Indol lactic acid) more than 1000 fold, a slight decline in IAA but higher permissiveness to even lethal amounts of externally provided IAA (Körber *et al.*, 1991). The gene5 inactivation leads to a subtle phenotype that matches to only *iaaH* and *iaaM* mutants (Leemans *et al.*, 1982).

1.8.6 The oncogenes of *Agrobacterium rhizogenes*

T-DNA of Ri plasmid contain multiple open reading frames (ORFs) *A. rhizogenes* harbours an agropine Ri plasmid which is responsible for hairy root disease means excessive root formation due to oncogenes on its TL-DNA and TR-DNA and such plants are also able to grow on hormone free medium (Gelvin *et al.*, 2003; Offringa *et al.*, 1986). The overall organization of TL-DNA is comparable to T-DNA of Ti plasmid like sequences of left and right border as well as the eukaryotic 5' and 3' regulatory elements. The TL-DNA of two agropine Ri plasmids was sequenced and it has 18 open reading frames (ORFs). The *ORF 10, 11, 12* and *15* are regarded as root loci and named as *rol A, B, C* and *D* respectively (White *et al.*, 1985; Slightom *et al.*, 1986)). Besides *rolA,B,C and D*, the *ORFs 8,13* and *14* are also regarded as oncogenes. It is interesting to note that mannopine, mikimopine and cucumopine

strains of *Agrobacterium rhizogenes* have one T-DNA region instead of two which shares similarity with agropine TL-DNA but missing *rolD*.

1.8.6.1 *rolA*

The *rolA* gene is present among all *Agrobacterium rhizogenes* Ri plamsids having highly conserved N-terminal of the protein. In different *Agrobacterium rhizogenes* strains *rolA* gene length varies between 279 to 423 bp (Meyer *et al.*, 2000). Its protein is highly basic with isoelectric point (PI) of about 10. Its presence increases sensitivity to auxin (Maurel *et al* . 1991) that relates to H^+ ATPase activity in plasma membrane (Vansuyt *et al* . 1992). It tilts the auxin/cytokinin ratio in favour of cytokinins. The tobacco protoplast transformed with *rolA* gene show enhanced sensitivity to auxin as studied by trans-membrane differences (Maurel *et al.*, 1991). The *rolA* transformed tobacoo plants show a typical phenotype of dwarfism, long internodes, delayed senescence, bushy having dark green wrinkled leaves, abnormally small flowers with male and female reduced fertility (van Altvorst et al . 1992; Schmülling et al . 1993; Schmulling *et al.*, 1988; Carneiro and Vilaine, 1993). The *rolA* expression is stronger in phloem cells of stem than that of leaves and root (Sinkar *et al.*, 1988; Carneiro and Vilaine, 1993). The suppression of elongation of parenchyma cells in leaf which are adjacent to vascular bundles leads to wrinkled leaf phenotype. Initially it was hypothesized that *rolA* encodes a diffusible protein and later proved by an experiment involving grafting *rolA* rootstock and scion to an previously untransformed plant changed its phenotype (Guivarch *et al.*, 1996a). The *rolA* expression impressively decreases the several phytohormone level like auxin, cytokinin, gibbrellins, polyamines. This decrease is highly correlating with the tissue type and developmental stage (Dehio *et al.*, 1993; Moritz and Schmulling, 1998). Regardless of the less concentration of auxin in *rolA* transgenic plants, the sensitivity to auxin is drastically increased especially in protoplast and young seedling (Vansuyt *et al.*, 1992; Maurel *et al.*, 1991). In 1994, an intron was discovered in 5' UTR of the *rolA* and it was shown that targeted mutation in the splice site eliminated the phenotype of *rolA* but it transcript level was unchanged (Magrelli *et al.*, 1994). This intron has transcription initiation site and actually it was a bacterial promotor (Pandolfini *et al.*, 2000), hence broadening the expression host profile including *A. rhizogenes* and eukaryotic plants where interestingly that intronic promoter is spliced out. The

fundamental biochemical role of rolA protein is unknown yet but its promotor hass similarities with auxin related genes (Carneiro and Vilaine, 1993). In spite of the fact that *RolA*:GUS fusion localizes to plasma membrane but it does not possess the hydrophobic transmembrane domain so hypothesis is rolA protein is non-integral but a membrane peripheral protein (Vilaine *et al.*, 1998). Most interestingly it stabilizes the GUS activity when expressed as fusion therefore probably it interferes with the protein degradation pathways (Barros *et al.*, 2003).

1.8.6.2 *rolB*

It is known that *rolB* is the important most Ri oncogene as transformation with only *rolB* can lead to the phenotype of hairy root. The resulting roots are fast growing, highly branched and show no geotropic behavior (Altamura, 2004). The *rolB* gene highly conserved among all Ri plasmids, with about 60% homology between strains (Meyer *et al.*, 2000). The tobacoo thin cell layers (TCLs) experiments indicated that *rolB* simultaneously enhance the adventitious flowering and roots by influemcing at the developmental stage of meristemoids formation (Altamura *et al.*, 1994). In addition, the research shows a strong correlation between auxin and *rolB*. In tobacco, the NtBBF1 (*Nicotiana tabacum rolB* domain B factor 1) transcription factor binds to a cis regulatory element by its Dof domain (DNA binding with one finger) for specifically inducing auxin expression in meristem (De Paolis *et al.*, 1996). Another transcription factor, RBF1 (Rol Binding Factor 1), binds to its promotrt domain leads to its expression in non-meristematic cells of root. The RBF1 expression remains indifferent between *rolB* transgenic and non-transgenic tobacco (Filetici *et al.*, 1997). It demonstrates localization to cell membrane as well as due to presence of conserved CX5R motif a tyrosine phosphatase activity. It perturbs the auxins signaling pathways (Filippini *et al.*, 1996; Lemcke and Schmulling, 1998b). Currently it is hypothesized that the *RolB* changes the perception of auxins (Maurel *et al.*, 1994). *RolB* may do this by either enhancing the expression of auxin binding proteins and/or stimulating their activity (Venis *et al.*, 1992). This leads to induction of roots which is the frequent most adventitious organ formed.

1.8.6.3 *rolB*$_{TR}$ (*rolB* homolog in T_R-DNA)

This gene is 53% homologous to *rolB* but lacks a CX5R motif of native *rolB* (Bouchez and Camilleri, 1990). It induced bent down wrinkled leaves, shoots at the base of the stem and retarded growth (Lemcke and Schmulling, 1998b).

1.8.6.4 *rolC*

The *rolC* transformants are important in floricultural applications because under its native promoter plants are small, having decreased apical dominance, having lanceolate leaves and small flowers with early inflorescence as well as reduced pollen formation (Schmulling *et al.*, 1988). The rolC transformed plant under the 35S promoter show pronounced phenotype and are male sterile having leaves with pale green color (Schmülling *et al.*, 1988).The *rolC* protein have β-glycolytic activity hence releasing free cytokinins from their inactive glucosides (Estruch *et al.*, 1991a). Its seedlings have increased sensitivity to cytokinin plus increased tolerance to auxins, gibberelins and abscisic acid (Schmülling *et al.*, 1993). It causes a decrease in ethylene synthesis in flowers while the enhances the collection of conjugates that are water insoluble (Martin-Tanguy *et al.*, 1993). The *rolC* gene has only phloem companion cells expression profile especially of roots (Sugaya *et al.*, 1989; Oono *et al.*, 1990; Leach and Aoyagi, 1991). Since *rolC* is induced by sucrose in the phloem parenchyma, companion and ray cells. In this way *rolC* might create the sink required to enhance the synthesis of sugar (Nilsson and Olsson, 1997).

1.8.6.5 *rolD*

The rolD is the only *rol* gene present in the TL-DNA agropine Ri plasmids and unable to induce the hairy root phenotype individually (Mauro *et al.*, 1996). It causes early and enhanced flowering but decreased rooting in a hormone independent fashion (Mauro *et al.*, 1996; Altamura, 2004). Expression of *rolD* is ontogenetically modulated. It is switched on in the every expanding and elongating tissue in fully grown plants except meristematic part (Trovato *et al.*, 1997). It is a gene that is induced by auxin in later stages. It has a Dof-binding element in its promotor that is likely to be involved in auxin induction (Mauro *et al.*, 2002). Probably *rolD* is implicated in later stages of formation of meristem and also may be involved in the fate determination of meristem during stress conditions (Altamura *et al.*, 2004). The

rolD has a sequence homology to ornithine cyclodeaminase (OCD) an enzyme that is involved in proline production which is an osmoprotectant in this way it can also affect flowering as flowers have high proline levels and it is postulated that proline in this way may stimulate the synthesis of hydroxylproline-rich glycoprotein, a cell wall component that is considered to be involved in cell division and extension regulation (Trovato *et al.*, 2001). It is proposed that this gene may be implicated in changing the hormonal balance of plants decreasing levels of auxin (Mauro *et al.*, 1996).

1.8.6.6 Other *ORF's*

In addition to *rol* genes TL-DNA of Ri plasmid has several ORF including ORF3n, ORF8, ORF13, (Slightom *et al.*, 1986; Ouartsi *et al.*, 2004). These prefer the generation of *rol* gene mediated root formation and decrease sensitization to cytokinins as well as auxins (Lemcke and Schmülling 1998a; Cardarelli *et al.*, 1987a; Capone *et al.*, 1989). In addition these are involved in changes in intermodal lenght, leaf morphology, growth, and delayed starting of flowerings with less dense inflorescences. ORF13 is the special gene that enhance the growth of cell by encoding diffusible substance (Hansen *et al.*, 1993; Fründt *et al.*, 1998).

1.8.6.7 Importance of *rol* genes in secondary metabolite synthesis

Many precious secondary metabolites are formed in roots in vivo, and importantly their synthesis is coupled with degree of root differentiation (Robins *et al.*, 2003; Flores *et al.*, 2007). The neoplastic roots have typically high growth rate, characterized by lateral branching, high bulk of neoplastic roots of a particular phenotype as well as high yield of secondary metabolites (Flores and Filner, 2004). The three *rol* genes *rolA*, *rolB*, and *rolC* genes of T-DNA individually or in combination are regarded as the most important inducers of secondary metabolites because they have crucial role in drug's synthetic pathways upregulation (Shkryl *et al.*, 2008). The amount of secondary metabolites produced varies with the type of secondary metabolite and plant species. It may upregulated from 2-300 times. The *rolA* gene has egressed as enhancer of growth and secondary metabolism (Bulgakov VP 2008). Although it is acknowledged that the *rol* genes function by transcriptional up-regulation of defense genes but exact molecular events are not clear. Data indicate that uncommon signal transduction pathways are regulated by *rol* genes in plants. Probably, they have role in calcium-dependent NADPH oxidase

activity and phytoalexin biosynthesis. Due to the importance of the genetic interactions altering the metabolite content in the hairy root, most of the current research concentrated on role of individual genes that are being expressed under the constitutive and inducible promoters mostly the Cauliflower Mosaic Virus 35S promoter to study changes in the pattern of hormones and phenotype. *T-DNA* promoters driving the expression of *GUS* reporter gene are used for elucidating the facio temporal expression profiles of desired genes.

From plant biotechnological secondary metabolite production aspect the most important genes of choice are *rolB* and *rolC* genes but *rolA* does have synergistic effect (Spena *et al.*, 1987). Researchers are convinced that *rol* genes unique individual role in plant metabolism (Bulgakov *et al.*, 2003). *A. rhizogene's* genes find their application in floriculture, for plant development (Christensen *et al.*, 2009).

Moreover, these roots has been producing higher amounts of secondary metabolites in comparison to undifferentiated plant cell suspensions, such as indole alkaloid along with ginsenoside synthesis in the cultures of *Catharanthus roseus* (Palazon *et al.*, 2003), ginsenoside synthesis in the cultures of *Panax ginseng* (Bulgakov *et al.*, 2002), anthraquinone synthesis in callus cultures of *Rubia cordifolia* (Bulgakov *et al.*, 2002), tropane alkaloids production in hairy roots of *Atropa belladonna* and *Datura stramonium* (Kamada *et al.*, 2008; Saito *et al.*, 2001; Aoki *et al.*, 2008; Spena *et al.*, 2002; Palazon *et al.*, 1997). Every characteristic phenotype of hairy root in transgenic plants is expressed in the presence of all three *rolA,B,C* loci. Hence each of *rolA,B,C* genes independently modulate the host functions involved in the differentiation of roots (Spena *et al.*, 2002). Several reports suggest that *rol A* and *rol C* change Polyamine (PA) metabolism hence plant phenotype (Michael *et al.*, 1998; Sun *et al.*, 1999; Martin *et al.*, 2006). It appears that the degree of expression of *rol A* and/or *rol C* phenotype depends on the penetration level, tissue type, strength of promoter and foreign gene integration site in the host genome. Putrescine, spermidine and traces of spermine were present in all samples, both in free and bound forms, while *rol A* roots causes high levels of free as well as bound polyamines (Altabella *et al.*, 2007). The *rol A* transgenic roots have higher polyamine and nicotine contents along with higher ornithine and arginine decarboxylase activities (Altabella *et al.*, 2007). The studies with the protein phosphatases 1 and 2A inhobitors have indicated that various

phosphatases are implicated in AQ biosynthesis both transgenic and no transgenic *R. cordifolia* cultures (Bulgakov *et al.*, 2003).

1.8.7 Types of Agrobacterium vector systems

There are various strategies to enhance effectiveness and ease of manipulation of *Agrobacterium*-mediated transformation for plant by designing novel vectors with changing combinations of genetic elements of Ti-plasmids (Figure 1.2).

1.8.7.1 The co-integration vectors

This technique exploit the natural homology directed repair mechanism of bacteria by inserting a foreign plasmid (pBR322) that has transgene inserted into its unique site flanked by the homologues sequences of *Agrobacterium tumefaciens* Ti-plasmid T-DNA inside borders. In this way the new gene get integrated into T-DNA after recombination. Later infecting the plant with this construct leads to integration of transgene into plant genome (Brown, 2001).

1.8.7.2 The binary vectors

A binary vector consists of T-DNA and the vector backbone. This system is based on the crucial observation that although *vir* genes are essential for transfer of T-DNA but their physical attachment to T-DNA of Ti plasmids in cis, is not necessary. Rather these can be equally effective if present on helper plasmid that is devoid of T-DNA. This two-plasmid system was a breakthrough design facilitating the high throughput construction of Ti plasmids because in this way their size is reduced hence easy to handle and manipulate using standard techniques decreasing the cost and time for cumbersome genetic manipulations of cointegrate vectors (Garfinkel *et al.*, 1981; Zambryski *et al.*, 1983; Fraley *et al.*, 1985). In fact similar binary vector system was found in some *Agrobacterium tumifaciens*. The small T-DNA plasmids have unique restriction sites for ease of cloning (Hoekema *et al.*, 1983; de Framond *et al.*, 1983). T-DNA is the region bounded by the right (RB) and the left border (LB) sequences and in addition to other genes may have multiple cloning sites, a selectable marker gene and a repotor gene (Hellens *et al.*, 2000). Previously used wild-type Ti/Ri-plasmids were of very large size, low copy number in *Agrobacterium*, in vitro manipulation and isolation was cumbersome and without any *Escherichia coli* origin

of replication which is favorably used for genetic manipulation in addition there were superfluous genes like opine synthesis and other oncogenes of T-DNA.

1.8.7.3 Super binary vector

A super-binary vector an modified version of a binary vectors that exhibits very high frequency of transformation, as it carries additional virulence genes from a pTiBo542 plasmid namely the *virB*, *virC* and *virG* genes that are creditworthy for the super virulence character of an *Agrobacterium tumefaciens* strain A281 (Jin *et al.*, 1987; Komari, 1990). So, it is valuable for recalcitrant monocotyledonous plants such as rice and maize (Komari, Takakura *et al.*, 2006; Hiei *et al.*, 1994; Ishida *et al.*, 1996).

1.8.7.4 Features of binary vectors

The genetic elements of binary vectors include the left, right borders of T-DNA, multiple cloning sites, origins of replication both for *Escherichia coli* and *A. tumefaciens*, marker genes for plant and bacteria, reporter genes for transient assays, promotor, 3' and 5' untranslated regions (UTRs), terminators and other accessory elements that can enhance the efficiency and capability of the system (Figure 1.1).

(1) Firstly the T-DNA 25 bp left and right border repeat sequences are conserved in all Ri and Ti plasmids define and delimit T-DNA (Waters *et al.*, 1991). However nucleotide 4 to 25 of LB remains within the T-DNA as VirD1/VirD2 endonucleases nick between 3 and 4 nucleotides, whereas on the RB nucleotides 1 to 3 remain intact (Wang *et al.*, 1987). Initially there was a problem that placing the gene of interest near LB leads to its partial of complete deletion due to the polarity of T-DNA transfer. For troubleshooting this issue now plant selectable marker genes are placed near LB and gene of interest near RB along with some overdrive sequences to increase the T-DNA transmission (Rossi *et al.*, 1996; Bevan, 1984; Peralta *et al.*, 1986; Wang *et al.*, 1984).

(2) Secondly binary vectors have plant selectable marker gene that usually encode resistance to antibiotic such as kanamycin, hygromycin or herbicide like phosphinothricin formulations such as Basta (Hoechst). In addition there are metabolic marker genes like phospho-mannose isomerase (Todd and Tague, 2001).

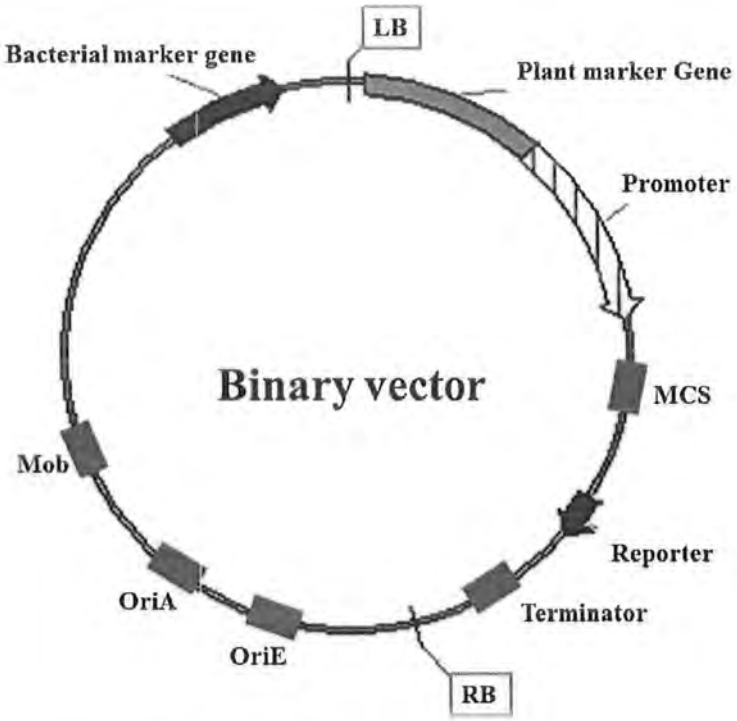


Figure 1.1: Typical components of T-DNA binary vector.

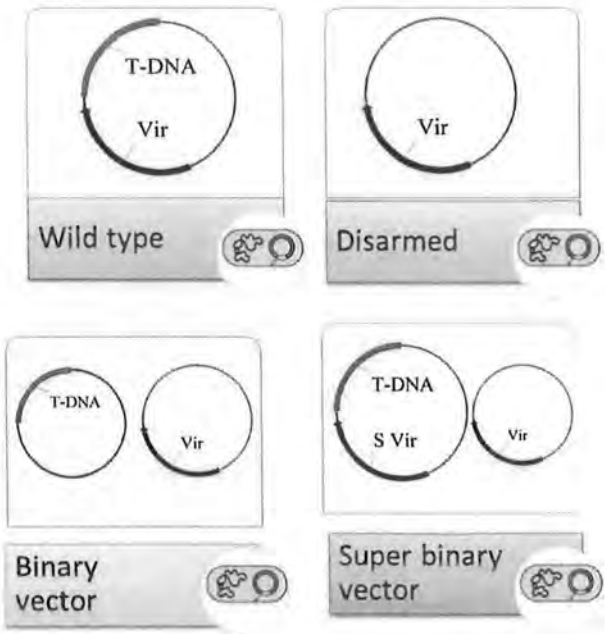


Figure 1.2: Schematic expression of strains of *Agrobacterium tumefaciens* and vector. S vir, is a fragment containing virB and virG genes from pTiBo542.

(3) Thirdly rare cutting restriction endonucleases or homing endonucleases sites flanked by promoters, UTRs and polyA addition signals within T-DNA to facilitate the insertion and expression of gene of interest (Chung *et al.*, 2005; Tzfira *et al.*, 2005). Some vectors fuse various tags for protein-protein interaction or affinity purification or subcellular localization studies like 6xHis, GFP, myc (Tzfira *et al.*, 2005; Citovsky *et al.*, 2006). Most importantly for fast and efficient jumping the gene of interest between different vectors lambda phage recombination sites are used to facilitate this as well as it allows modular design of genes by using gateway cassettes. Moreover for large gene inserts bacterial artificial chromosomes (BAC) are developed for cloning genes of more than 100kb size (Hamilton, 1997; Liu *et al.*, 1999).

(4) Origin(s) of replication are of enormous importance both in terms of broadening the host range and determining the copy number that are crucial factors moreover functions of different incompatibility groups that are essential to co-exist two plasmids in a bacterium are related to origin of replication. Some of these like pCGN are host specific while others are broad host range pVS1 that are compatible with both *E.Coli* and *A. tumifaciens*. ColE1 origin is added to Ti plasmids for enabling replication in *E.Coli*. The pVS1 origin encodes 7 to 10 copies per *Agrobacterium* cell.

(5) Chromosomal antibiotic resistance genes beside selectable marker genes in binary vectors are necessary for differentiating between empty strains of *Agrobacterium* or *E.Coli* for example *Agrobacterium tumifaciens strain GV3101* have chromosomally encoded rifampicin resistance gene.

1.8.7.5 Drawbacks of binary vector systems

Preference for multi copy binary vector plasmid causes two untoward effects first multiple copies of transgene are integrated into plant genome secondly vector backbone integration in plants. But this can be mitigated by using binary vectors showing low copy number or launching the T-DNA from chromosome of *Agrobacterium* (Ye *et al.*, 2007).

1.8.7.6 Disarmed Agrobacterium strains

The commonly used Agrobacteria strains are having C58 chromosomal background with the disarmed Ti-plasmid pTiC58 (Wood *et al* . 2001) and its derivatives GV3101::pMP90 (Koncz and Schell, 1986). These strains confer gentamycin

resistance due to pTiC58 and GV3101::pMP90 carries an additional resistance to Rifampicin in chromosome

Table 1.1: commonly used Disarmed *Agrobacterium* strains.

Name of strain	Chromosomal background	Ti-Plasmid derivation	Antibiotic resistance	Ref.
AGL-1	C58	pTiBo542	rif, carb	Lazo et al. (1991)
EHA101	C58	pTiBo542	rif, kan	Hood et al. (1986)
GV3101 :: pMP90	C58	pTiC58	rif, gent	Koncz and Schell (1986)
LBA4404	Ach5	pTiAch5	rif	Ooms et al. (1982)

1.8.7.7 *E.Coli* (*DH5α*) as holding strain:

It is gram negative bacterium having a genome size of 4.6Mb and can harbours one or more extra chromosomal DNA called plasmid of varing size from 1kb to more than 200kb. The strain used in this perticular study is empty strain and *recA, EndA* knockout strain so transgene of recombinent vector is stably holded in this strain. Moreover, these cells can be easily transformed and give good quality plasmid DNA after transformation.

1.8.7.8 Feature of pEarleyGate binary vectors series

These are a large number of Gateway compatible destination vectors for plants for AcV5, HA, FLAG, and cMyc epitope tagging of proteins of interest to enable their immunoblot detection. The importance of these tags and vectors is increased as antibodies detecting these epitopes have little cross-reaction or affinity to endogenous proteins of a variety of dicotyledonous and monocotyledonous plants (Earley *et al.*, 2006).

Broadly there are three subseries of **pEarleyGate series**, first ranging from **pEarleyGate 100–105** having vectors that translationally fuse fluorescent tags like YFP, GFP, CFP for localization or 6X-His for affinity purification. Secondly **pEarleyGate 201–205** series have epitope-tagging vector that can be used for localization as well as affinity purification for example $P^{EARLYGATE203}$ binary vector

with myc tag that is transnationally fused to protein of interest at N-terminus (Earley *et al.*, 2006). Thirdly **pEarleyGate 301–304** series contain promotorless epitope-tagging vector. But all these are constructed by taking the genetic elements (35S, BAR, OCS 3', LB, RB, KanR etc.) from pCAMBIA1302, pCAM-35SEYFP-C1 and pFGC5941 (Fritze and Anderson, 2000).

1.8.7.9 Choice of expression vector for plants

The basics of binary vector systems was explicated in 1980s, and panoptic betterments have unremittingly been made resulting in a wide range of binary and super binary vectors now a day (Hellens *et al.*, 2000; Komari *et al.*, 2006). Although a diverse variety of vectors is available but there is not all in one vector. Most of these vectors can be used in a range of experiments means they are flexible. Selection depends on the characteristics of the gene of interest, the *Agrobacterium* strain to be used, plant species under investigation and most importantly the goals of the study. For larger gene than 15 kb, BIBAC, BAC and IncP vectors are appropriate. Generally rang of vectors have been designed that are convenient to use due to high copy number plasmids, have diverse multiple cloning sites, wide pole of selectable marker genes, high frequency of transformation and GATEWAY recombination sites for modular design. There are various series of vectors specifically constructed for a particular task e.g. pSAT vector series for RNA interference studies. Still classic vectors find a lot of applications; but newly customized vectors have features that are user-friendly. Most importantly there are vectors that are designed to address regulatory problems, like elimination of marker genes and decrease in vector backbone transfer. As new frontiers of science are emerging so the vector designing will evolve accordingly.

1.8.8.10 Production of transformed plants with Ti plasmid

Introduction of transgene uniformly into every cell in the plant is desired. For this reason plant cells cultures and protoplasts are infected in liquid medium. A mature plant obtained in this way will not be a chimera thus stably inheriting the transgene in offspring. Moreover regeneration of a healthy transformed plant without any cancerous phenotype is possible only if the Ti vector has been "disarmed" by removing the oncogenes between the LB and RB sequences. For example GV3101::pMP90 is a disarmed strain of *A. tumefaciens* so can be transformed by Ti

binary vector like pEarleyGate203. But the initial cloning steps are done in *E. coli*, later the correct and validated recombinant molecule is transferred to *A. tumefaciens* and selection on agar medium containing appropriate antibiotic followed by plant transformation (Brown, 2001; Vergauwe *et al.*, 1998).

1.9 Molecular Cloning and Gateway cloning

Without cloning of individual or multiple genes in simple systems and revealing the underlying mechanisms the fruits of genomics cannot be reaped. In this regard, the significance of recombinational cloning methods like Gateway systems is increased manifold because of ease and speed of assembling DNA fragments in a desired order, orientation, reading frame and in modular fashion.

Full potential of genomics technologies cannot be exploited without exploring the functional aspect of it, and understanding the bigger picture of system biology. Hence, utility of automated systems and approaches for cloning and expressions of plant genes transiently or stably are highly relevant. Transient expression is preferred over stable because of its less time and cost (Mansour *et al.*, 2007).

The currently only about 55% of Arabidopsis genes are annotated for a putative role. However, only fewer than 8% of them are having direct experimental evidence (Mark and Ueli, 2003).. To better understand the biochemical role of genes there is need for generation of chimeric transgenes for inducible or constitutive ectopic expression, gene complementation studies, promoter characterization, subcellular localization studies, gene knockdown by RNA interference, antisense expression, analyzing protein/protein interactions through bimolecular fluorescence complementation (BiFC), bioluminescence resonance energy transfer (BRET), or fluorescence resonance energy transfer (FRET). But the bottleneck in these large scale studies is laborious conventional cloning methods of restriction digestion and ligation.

For the provision of rapid, efficient functional analysis, cloning/subcloning, high throughput gene expression analysis tools, the freely available Gateway Cloning Technology by Invitrogen's is deemed to be the breakthrough. Gateway Cloning Technology is is basically derived from sequence specific recombination mechanisms of bacteriophage lambda (*att*L x *att*R _ *att*B x *att*P) that is well characterized. Simply it is a 2-step cloning process; in the first step a sequence of interest is cloned into an

Entry Clone followed by its transfers into a variety of *att*B-containing Expression Destination vectors (binary) that may bears the suitable genetic elements derived from pCambia T-DNA cloning vectors, for propagation and expression in a versatile range of host cells for desired outcomes. For protein expression a range of destination vectors for *E. coli*, yeast, mammalian and insect cells marketed by Invitrogen (Carlsbad, CA, USA).

The pEarleyGate plasmid vectors are widely used for generation of transgenic plants for study the functional proteomics details of gene of intrest by fusing it with fluorescent and non-fluorescent tags (Mark and Ueli, 2003).

1.10 c-Myc oncogene to myc tag

The c-myc is an decapeptide epitope with (EQKLISEEDL1) sequence. It is basically derived from residue 410-419 of human c-myc protein which is member of a family of nuclear proteins that are implicated in carcinogenesis. Importantly it can be recognized by mouse monoclonal anti-c-myc antibody (clone 9E10) that was developed for studying *MYC* oncoprotein (Evan *et al.*, 1985).

1.11 Epitope (Myc) tagging for functional proteomics

The protein tagging is the most important tool for purifying and subcellular localization of novel protein that do not have antibodies against them. Interestingly many proteins retain their biochemical function when they are C-terminally tagged or N- terminally tagged if otherwise then multiple tagging also stabilizes the protein function.

Epitope tagged recombinant proteins can be conveniently isolated from cellular mixture by using immobilized anti epitope tag affinity matrices followed by their detection by applying fluorescent anti-epitope tag antibodies through fluorescence microscopy. Such strategies can help in characterizing proteins function in terms of protein-protein interactions and its localization in cell.

1.11.1 Affinity purification

The epitope tagging pEarleyGate vectors series can be used for production of recombinant proteins because latter it can be affinity purified due to epitope tags like *myc*. Anti-HA, FLAG, or cMyc antibodies are conjugated onto the agarose beads which can be employed to capture and enrich the target protein in bead-associated

fractions. Anti c-myc conjugated agarose beads can be used for immunoblot detection of a protein which is c-myc or 3x-myc tagged due to its affinity (Kagale, Uzuhashi *et al* . 2012). This is highly pertinent for profiling the newly discovered protein and proteins having low immunogenicity (Brizzard 2008). Remarkably, eluting the protein through some matrixes by purging excess epitope polypeptides is more difficult in certain antibody–epitope combinations. The influenza virus hemagglutinin (HA) and the c-myc epitopes are widely used in this regard (Field *et al.*, 1988; Tyers *et al.*, 1993; Evan *et al.*, 1985; Munro and Pelham, 1987). The *Schistosoma japonicum* glutathione S-transferase (GST) (Smith *et al* .1986) is another valuable dual use tag employed for protein's localization (Bi and Pringle, 1996) and their fast single step isolation of tagged protein along with its associated proteins by glutathione-conjugated agarose beads (Smith and Johnson, 1988; Ausubel *et al.*, 1995).

1.11.2 Immunolocalization

Epitope-tagging vectors like pEarleyGate200 series are useful for Immunolocalization studies in transgenic plants cells. Particularly protein tagging can facilitate localization studies by enabling double staining. Due to the availability of best quality monoclonal antibodies against HA and myc tags, hence HA/myc epitope tagged protein is generally localizable in complexes with a known protein having non-mouse antibody. In addition, against HA (Berkeley Antibody Company) and GST (Bi and Pringle, 1996) the commercial availability of rabbit antibodies allows double staining of a dually tagged proteins. For dynamic double labeling of protein with native GFP (S65T) and several GFP variants are used to study living cells (Prasher, 1995; Heim and Tsien 1996; Niedenthal *et al.*, 1996). FLAG-tagged protein can be eluted from column by increasing concentration of competing peptide, but cMyc and HA tagged proteins cannot be eluted by this method except using denaturing SDS–PAGE sample buffer having low pH (Lawrence *et al.*, 2004; Zhou *et al.*, 2004).

1.12 PCR encoded epitope tagging (PET)

Overlap PCR homologies of 12–15 bp to other PCR products or another set of primers are used to combine PCR products or small fragments of insert into larger molecules to make deletions, insertions or add larger domains or epitope tags to genes of interest (Horton *et al.*, 1990). PET is a simple strategy; all that needs is designing overlapping sets of primers in a back to back fashion and sequentially amplifying with them the

gene of interest so ultimately sequence of epitope that is initially in the primers, is encoded into gene of interest at desired flank. This is very useful technique as well as accurate because we can amplify the sequence by using pfu polymerase in PCR in reaction mixture.

1.13 The biosafety and bioethics of gene transfer

The development of genetically modified (GM) crops is leading to grievous controversies some of them are due to conflicts of interest. Importantly the differences are in the way of perception of technology, its risks and potential gains. From perspective of Scientists, farmers and plant breeders GM crops are good for plant breeding, high yields, profitable, fulfilling important societal needs and less vulnerability to disease (Madsen *et al.*, 2003). On the contrary it is claimed that GM-foods are dangerous as well as ethically wrong as the poses a global threat as it critically affects: food chain, human health, Beneficial insect, insect predator, coexistence, seed purity, Herbicide (glyphosate or glufosinate) resistance, Pest resistance, pathogen resistance and insect resistance. The Vertical gene flow, horizontal gene flow, pollen dispersal, transgene escape, outcrossing, cross pollination, spontaneous hybridization, gene introgression, are the ways GMO's negative effects may go global. So a broader Public discussion on GM crops from different angles is need of the time. The strategies for minimizing the potential threats and increase the confidence on new technologies include minimizing transgene escape, focusing on marker free GMO, chloroplast transformation (paternally inherited), regular studies on plant and soil microflora, updated labeling precautions, integrated pest management strategies, post marketing surveillance, databases of transgenic plants, capacity enhancement in detection and characterization of transgenic plants by the environment protection agencies across the world.

1.14 Aims and Objectives

1. Cloning of *myc* tagged *rolA* gene in pEarleyGate203 destination vector for exploring *rolA* gene's functional proteomics in plants secondary metabolism.
2. Transformation of *E.Coli* and *Agrobacterium tumefaciens* disarmed strain GV3101 with this recombinant plasmid pMBQAU1001.

Chapter 2

Materials and Methods

Materials and Methods

The present research has been carried out at the Department of Biochemistry, Faculty of Biological Siences, Quiad-I-Azam University Islamabad, Pakistan.

2.1 Laboratory plasticware

The plasticware used in this study was of polystyrene. The micropipette tips, parafilm were obtained from Axygen Scientific USA. The 1.5 ml and 2 ml microfuge tubes were purchased from eppendorf germany. While the syringes as well as their filters et al.,

2.2 Laboratory glassware

The borosilicate glassware from Pyrex® was utilized throughout the study which was washed with tap water followed by rinsing with distilled water. The bioburden of glassware was reduced by dipping in the commercial grade bleach again rinsed thrice by doubled distilled water. While the test tubes and flasks were after washing were plugged with cotton and petri plates were wrapped in brown paper followed by autoclaving for 30 mins at 121 °C temperature at a pressure of 15 psi during cylce.

2.3 Chemicals, enzymes, kits, and DNA ladders

The analytical or molecular grade reagents or chemicals utilized in this study were procured from Sigma Chemical Co., USA. Molecular biology reagents, ladders of 100bp, 1kb and enzymes like pfu polymerase, Taq polymerases were purchased from Invitrogen, Sigma and Fermentas. While various molecular biology kits like quick gel extraction kit were obtained from Invitrogen. Moreover, Agar and Luria Bertini medium for microbiological work were acquired from "DIFCO" laboratories, USA.

2.4 Bacterial strains

2.4.1 *Agrobacterium tumefacienes* strain GV3101

This is a strain with C58 chromosomal Background that is Disarmed means it did not have binary vector carrying oncogenes or foreign gene of intrest, but only have vir helper plasmid pMP90 with borders derived from pTiC58, having resistance to gentamycin on helper plasmid, additionally resistance to rifampicin is chromosomally

encoded and was kindly provided by Prof. Kim Jae Yean Gyeongsang National University , Korea. (Wood et al., 2001; Koncz and Schell, 1986; Hellens et al., 2000)

2.4.2 *Agrobacterium rhizogenes* strain ARqua1

It was kindly provided by Mireille Chabaud LIPM, INRA-CNRS, BP 52627, 31326-Castanet Tolosan Cedex, France, and Prof. Dr. Bettina Hause, Institute of Plant Biochemistry, Weinberg Halle/Saale, Germany separately, it harbour pRiA4 T -DNA region carrying *rol* A gene (Quandt *et al.*, 1993)

2.4.3 *E.Coli (DH5α)* as holding strain:

E.Coli DH5α is gram negative bacterium having a genome size of 4.6 Mb and can harbours one or more extra chromosomal DNA called plasmid of varing size from 1kb to more than 200 kb. The strain used in this perticular study is empty strain and *recA, EndA* knockout strain so transgene of recombinent vector is stably held in this strain. Moreover, these cells can be easily transformed and give good quality plasmid DNA after transformation.

2.4.4 *E.Coli (DB 3.1)*

Expression vector $p^{EARLYGATE203}$ was extracted from *E.Coli (DB 3.1)* that was received from Prof. Kim Jae Yean Gyeongsang National University , Korea.

2.5 Culturing media, conditions and selection for bacteria

2.5.1 For *E.Coli*

E.Coli wa grown in Luria Bertini (Lb) medium with or without kanamycin 50 mg/L selection. The broth was placed at 37 °C in shaker incubator with continuous 120 rpm shaking. After the growth the optical density was determined ($O.D_{600}$=1.0). followed by streaking on Lauria Agar (LA) plates followed by their incubation at 37 °C to obtain the colonies. Then a single pure colony was picked up from petri plate of bacteria with the help of a sterile bacterial loop and inoculated in a flask having 50 mL LB broth supplemented with 50 mg/L kanamycin for plasmid other puposes.

2.5.2 For *Agrobacterium*

Agrobacterium tumefaciens and *A. rhizogene* were cultured in Luria Bertini medium (Lb) with or without the supplementation of kanamycin and/or rifampicin or gentamycin at 50 mg/L, 25 mg/L and 10 mg/L respectively. The LB was placed at 28

°C in shaker incubator with at 120 rpm. The optical density (OD) of the culture was checked after the attaining the growth. These grown cultures were used to streak the agar plates having solidified Luria Agar (LA) or for the purpose of plasmid isolation. The LA petri plates were incubated at 28 °C to obtain the growth, only pure colonies were chosen from the plates with the help of sterile bacterial loop and inoculated in 50 mL of LB broth on selection.

2.6 Plasmid constructs

Following plasmids were employed in cloning of *rolA* gene in pEarleyGate203.

2.6.1 Expression vector or destination vector

Destination vector pEarleyGate203 is a contruct with N-terminal *Myc* tagged gateway cassette having pVS1 origin of replication for agrobacterium with 7-10 copies per cell and ColE1 ori for *E.Coli* (Figure 2.1). Plant and bacterial Selection Marker genes are *Bar* also called as bialaphos resistance or Phosphinothricin acetyl transferase (*PAT*) and Kan (kanamycin or neomycin phosphotransferase II gene, *NPTII*) respectively (De Block *et al*., 1989). Moreover, it has left, right border, 35S promotor drive the expression of transgene and 3' *OCS* or octopine synthase enhancer elements are also present. Mainly pEarleyGate203 vector facilitates protein tagging. This vector backbone is basically derived from pCAMBIA vectors (Earley *et al*., 2006).

2.6.2 Transrofmation vector (pMBQAU1001)

Final transformation vector with N-terminal Myc tagged rolA gene contruct pMBQAU1001 having pVS1 origin of replication with 7-10 copies per cell ColE1 ori for *E.Coli*. Plant and bacterial Selection Marker genes are *Bar* (bialaphos) and *Kan* (kanamycin) respectively. Moreover, 35S promotor drives the expression of transgene, and 3' OCS or octopine synthase enhancer elements are also present (figure 2.2).

2.6.3 pRiA4 of *Agrobacterium rhizogenes ARqua1*

The pRiA4 plasmid having *rolA* oncogene on TL-DNA was used for its native amplification.

2.7 Cloning of recombinant plasmid pMBQAU1001

RolA gene PCR amplified fragment from pRiA4 was subsequently cloned by restriction digestion cloning in the gateway destination vector pEarlyGate203 to make

the expression vector *pMBQAU1001* with an N-terminal cMyc fusion tag for immunological protein detection (Figure 2.3).

2.7.1 Extraction of plasmid DNA

Isolation of plasmids pEarleyGate203, pRiA4 and pMBQAU1001 was done by alkaline lysis method (Sambrook and Russell, 2001) from *E.Coli DB3.1, Agrobacterium rhizogenes strain A4* and both *E.Coli DH5α, Agrobacterium tumifaciens GV3101* respectively. The falcon tubes of 15 ml having 10 ml bacterial culture, grown in Lb medium overnight at 37 °C or 28 °C from a single colony of different strains, at appropriate selection, were centrifuged at 4,000 rpm for 20 minutes at 4°C to get pellet of bacterial cells. The dried pellet was suspended in 200 µl solution 1 (NaOH 0.2 M, SDS 1%) by vigorous vortexing and transfer to microfuge. After it, 400 µl of fresh lysis buffer (solution 2; tris base 10 mM, EDTA 1 mM) was added and was left on ice for 10 minutes after inverting 5 times. It was followed by the addition of ice cold 300 µl of solution 3 (sodium acetate 2.5M, PH 4.8) and disperse the mixture by inverting several times then left on ice for 3-5 mins. The lysate was centrifuged in eppendorf at 13,000 rpm for 10-15 minutes and the 600 µl of supernatant was transferred carefully in the fresh tubes. An equal volume of phenol:chloroform (1:1 ratio) was added. Then after vortexing, it was centrifuged at 13,000 rpm for 15 minutes to take supernatant in fresh tube, and this step was repeated by only using the chloroform and supernatant was transferred to fresh eppendorf. Subsequently 600 µl of isopropyl alcohol was added to precipitate plasmid DNA at room temperature, after 10 mins collect the pellet by centrifuging at max speed for 5 mins and after drying wash it with 70% ethanol followed by centrifugation at max speed to again recover the pellet. Finally, the plasmid DNA pellet was air dried and dissolved in 20 µl of TE buffer (pH 8.0) followed by analysis by agarose gel electrophoresis and storage at -20 °C.

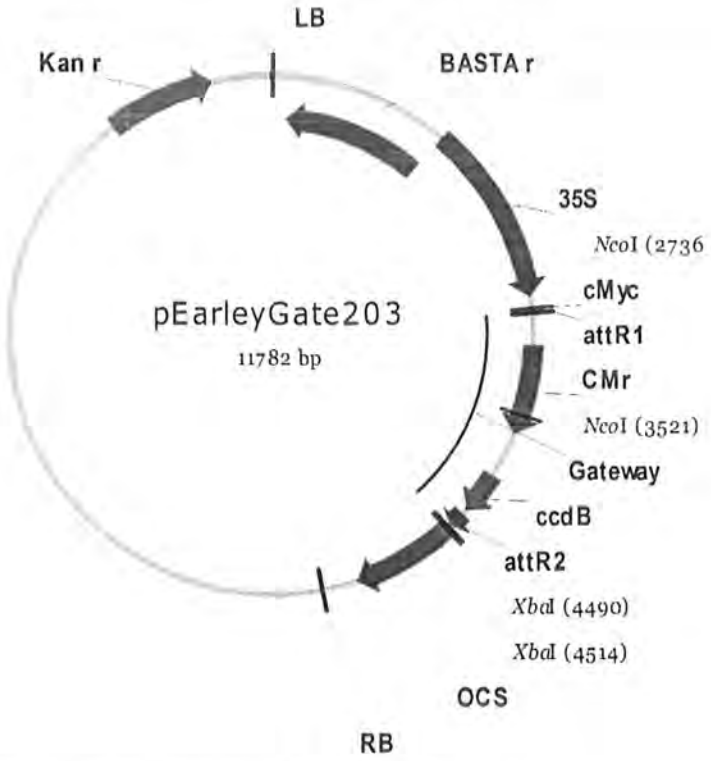


Figure 2.1: Genetic elemental map of pEarleyGate203.

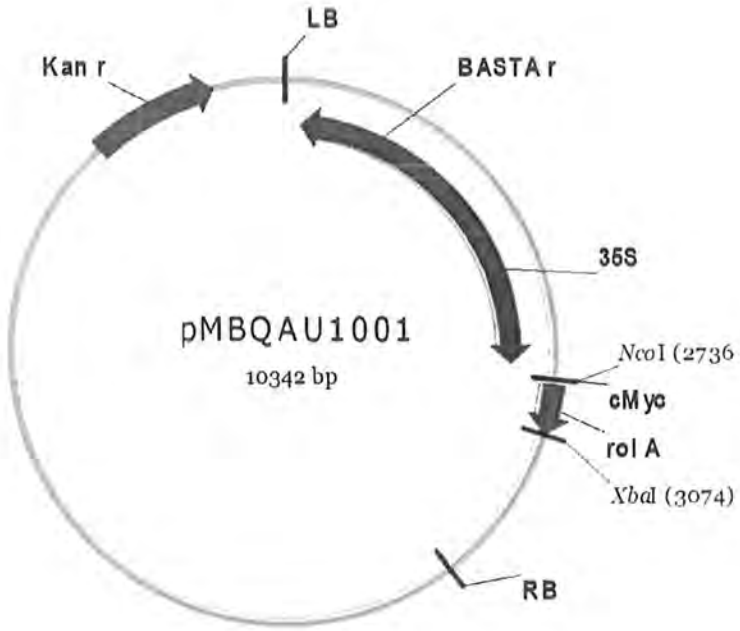
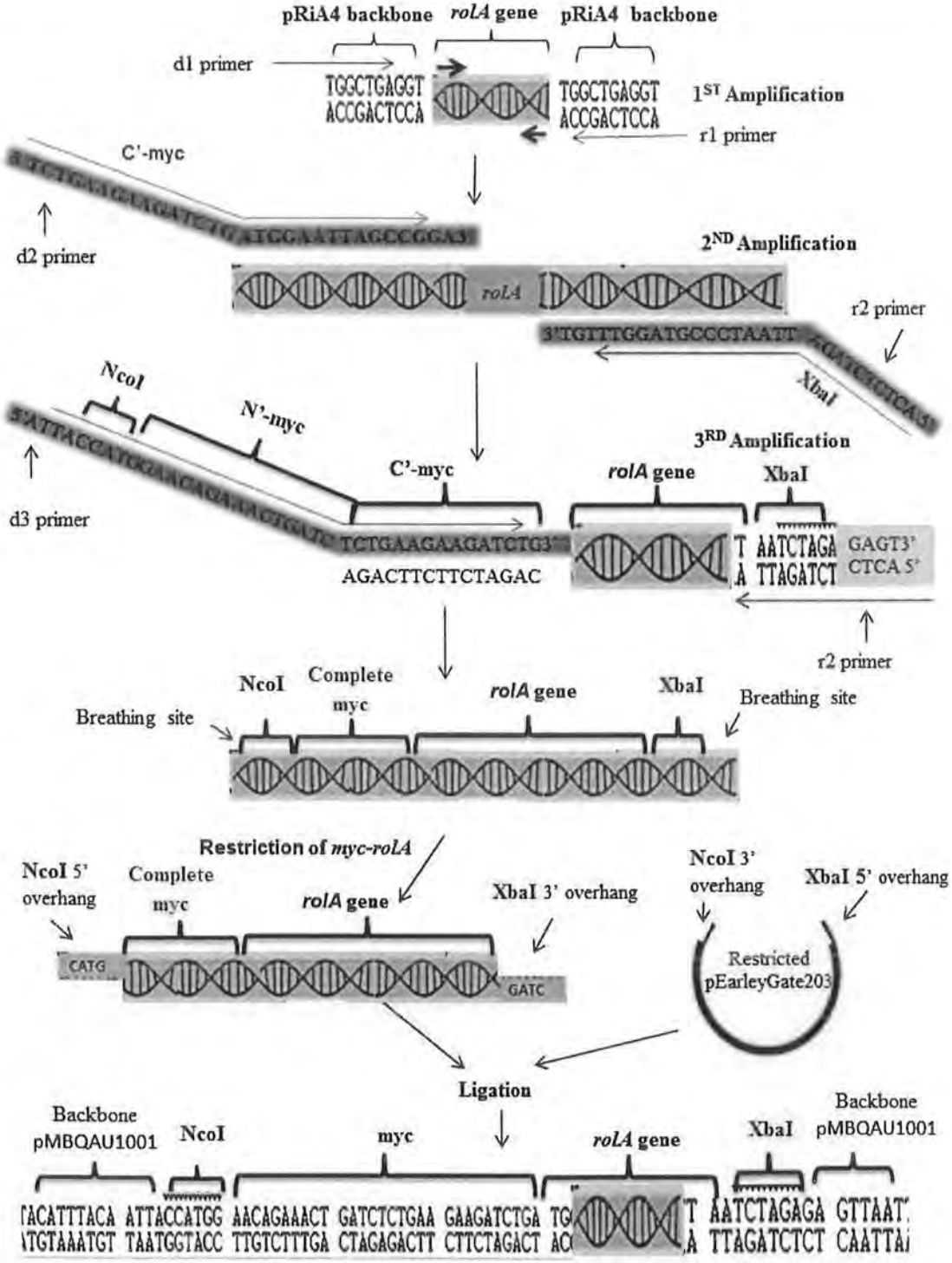


Figure 2.2: Genetic elemental map of T-DNA region of pMBQAU1001 with *myc* tagged *rolA* gene.

Table 2.1: Primers and conditions of gene amplification by PCR.

Primer	Sequence (in 5′ to 3′ Direction)	Tm	Product size (bp)	PCR Conditions		
				Temp	Time	Cycle
NcoI - *N'-Myc* **-d3**	5′ATTACCATGGAACAGAAAC TGATCTCTGAAGAAGATCTG3′	**65.3**	**352**	94 °C	5 min	1
				94 °C 53 °C 72 °C	35 sec 35 sec **90 sec**	35
				72 °C	10 min	1
C'-Myc-rolA **-d2**	5′TCTGAAGAAGATCTG-ATGGAATTAGCCGGA3′	**62.7**	**328**	94 °C	5 min	1
				94 °C 53 °C 72 °C	35 sec 35 sec **90 sec**	35
				72 °C	10 min	1
rolA-XbaI-**r2**	5′ACTCTCTAGATTA ATCCCGTAGGTTTGT3′	**59.6**	**328 /352**	94 °C	5 min	1
				94 °C 53 °C 72 °C	35 sec 35 sec **90 sec**	35
				72 °C	10 min	1
***RolA* (d1 and r1)**	5′ATGGAATTAGCCGGACTAAA3′ 5′TTAATCCCGTAGGTTTGTTT3′	**53**	**303**	94°C	5 min	1
				94 °C 53 °C 72 °C	35 sec 35 sec **90 sec**	35
				72 °C	10 min	1
NPTII	5′AAGATGGATTGCACGCAGGTC3′ 5′GAAGAACTCGTCAAGAAGGCG3′	**54**	**791**	94 °C	5 min	1
				94 °C 54 °C 72 °C	35 sec 35 sec 45 sec	35
				72 °C	10 min	1



pMBQAU1001

Figure 2.2: PCR encoded epitope tagging (PET) of *rolA* gene and cloning in pEarleyGate203 to construct recombinant plasmid pMBQAU1001.

2.7.2 Agarose gel electrphoresis

PCR amplified product as well as extracted plasmid DNA were analysed on 1.5 and 0.8 %w/v agarose gel respectively. It was prepared by melting 0.8 gm of agarose in 10 ml of 10 X TBE buffer (Tris 890 mM, Boric acid 25 mM, EDTA 0.1 mM pH 8.3) and 90ml of distilled water in microwave oven. To stain DNA 6 µl of ethidium bromide were added (10 mg/ml). The samples of DNA were mixed with loading dye having 0.25% bromophenol blue in 40% sucrose solution. It was followed by electrophoresis in 1 X TBE buffer at 90 volts (70 mA) for 1.25 hr and visualization in a UV-trans illuminator (Life Technology, USA).

2.7.3 Designing of primers and PET tagging

Sequential addition of overlaping primers with homologies of 15 bp to 2nd and 3rd sets of primers are used for synthesizing cMyc-rolA gene fragment flanked by NcoI/XbaI sites and breathing sites (Horton *et al.*, 1990; Taylor, 1991). This technique is also called as Adaptor PCR (Table 2.1).

2.7.3.1 Sequence after 1ST round of PCR amplification (303)

5'**ATG**GAATTAGCCGGACTAAACGTCGCCGGCATGGCCCAGACCTTCGGA
GTATTATCGCTCGTCTGTTCTAAGCTTGTTAGGCGTGCAAAGGCCAAGAGG
AAGGCCAAACGGGTATCCCCGGGCGAACGCGACCATCTTGCTGAGCCAGC
CAATCTGAGCACCACTCCTTTGGCCATGACTTCCCAAGCCCGACCGGGAC
GTTCAACGACCCGCGAGTTGCTGCGAAGGGACCCTTTGTCGCCGGACGTG
AAAATTCAGACCTACGGGATTAATACGCATTTCGAAACAAACCTACGGGA
T**TAA**3'

2.7.3.2 Sequence after 2nd round of PCR amplification (328)

5'TCTCTAGAAGAATTC**ATG**GAATTAGCCGGACTAAACGTCGCCGGCATG
GCCCAGACCTTCGGAGTATTATCGCTCGTCTGTTCTAAGCTTGTTAGGCGT
GCAAAGGCCAAGAGGAAGGCCAAACGGGTATCCCCGGGCGAACGCGACC
ATCTTGCTGAGCCAGCCAATCTGAGCACCACTCCTTTGGCCATGACTTCCC
AAGCCCGACCGGGACGTTCAACGACCCGCGAGTTGCTGCGAAGGGACCCT
TTGTCGCCGGACGTGAAAATTCAGACCTACGGGATTAATACGCATTTCGA
AACAAACCTACGGGAT**TAA**-TCTAGAGAGT3'

2.7.3.3 Sequence after 3rd round of PCR amplification (352)

5'ATTA-CCATGGAACAGAAACTGATCTCTGAAGAAGATCTG-**ATG**-rolA-**TAA**-TCTAGA-GAGT 3'

2.7.4 Restriction of vector and insert

Selection of restriction enzymes and their buffer for double digest was based on genetic map of vector and double digest compatability check recommendations at thermoscientific website in its five buffers system as well as in unique buffers (http://www.thermoscientificbio.com/webtools/doubledigest).

1X Tango buffer was selected because both enzymes show 100% activitiy without any star activity. As per literature of Restriction enzymes from fermentas following recepie was adopted. For a total of 20 µl reaction 16 µl of Water, 2 µl of 10x tango buffer, 500-1000ng of DNA (i.e. pEarleyGate203, *myc-rolA* gene) each separately in a volume of 1 µl, 1 µl of each NcoI and XbaI were used.

2.7.5 Eluting through gel

After completion agarose gel electrophoresis (0.8%) for *myc-rolA* gene fragment only cut the band of DNA on gel using a clean, sharp razor blade. Gel slice was Weighed on a sensitive scale after putting into a 1.5 mL microcentrifuge tube, it was dissolved by adding 3 volumes Gel Solubilization Buffer (L3) for every 1 volume of gel and then incubated into a 50 °C water bath for at least 10 minutes while Inverting the tube by hand every 3 minutes to ensure gel dissolution according to the manufacturer instructions. After the gel slice appears dissolved, incubate it for another 5 minutes. Quick Gel Extraction Column was put inside a Wash Tube and loaded at the center with dissolved gel piece containing the DNA followed by centrifugation at >12,000 × g for 1 minute. After discarding the flow-through, the Wash Tube was again put back into the Quick Gel Extraction Column and 500 µL Wash Buffer (W1), containing ethanol was Added followed by again centrifugation at >12,000 × g for 1 minute. Simillarly flow-through was discarded and the column was replaced into the Wash Tube. The column was centrifuged again at maximum speed for 1–2 minutes to remove any residual Wash Buffer and ethanol. Now discarded the Wash Tube and a

recovery Tube was placed in the Quick Gel Extraction Column. Finally 40 µL of pre-warmed Elution Buffer (E5) was added to the center of the Quick Gel Extraction Column and incubated the column for 10 minute at room temperature followed by centrifugation at >12,000 × g for 5 minute to recover the purified DNA which was stored at 4°C for immediate use or at –20 °C for long term storage.

2.7.6 Ligation of vector and insert

After Inserts was purified by gel extraction kit (Invitrogen), the ligation was done using T4 DNA ligase at 22 °C temperature for 10 mins to make pMBQAU1001. As per literature of ligase enzyme from fermentas 10 µl of *myc-rolA* insert having 300ng of DNA, 100 ng of Vector (pEarleyGate203) in 4 µl, 2 µl of 10X T4 DNA Ligase Buffer, 1 unit of T4 DNA Ligase in 1 µl and 3 µl of deionized nuclease free PCR Water to make a total of 20 µl reaction (Chen *et al.*, 2013).

2.8 Transformation of *E.Coli* DH5α with pMBQAU1001

Transformation of *E.Coli* involves following steps:

2.8.1 Competent cells Preparation of *E. Coli* and *Agrobacterium tumefacienes* strain GV3101

A single colony of DH5α a strain of *E .Coli* was picked and culured in test tube in 3ml Lb medium on selection at 37 °C and 28 °C for *E.Coli* and *Agrobacterium tumefacienes* respectively, with 120 rpm shaking for overnight. The starter culture was shifted to 50 ml Lb medium with kanamycin for *E.Coli* and rifampicin selection for *Agrobacterium*, and was again incubated at 37 °C and 28 °C with 120 rpm shaking until the OD_{500} was 0.5. Subsequently the bacterial culture was centrifuged at 3500 x g for 50 min at 4 °C and pellet was washed with 15% glycerol. The cells were resuspended in 15% glycerol for 15 mins at 4 °C. All the steps were performed on the ice. The process of washing was repeated thrice. After final washing the bacterial suspension were divided into 50-100 µl aliquots and stored at -70 °C.

2.8.2 Electroporation of pMBQAU1001 in competent *E. coli* cells and Agrobacterium tumefacienes strain GV3101

Electro-competent cells for both strains were prepared by the same above method as mentioned for DH5α except that the incubation temperature for *Agrobacterium* was 28 °C. The electroporation was done by Biorad micropulser™ using standard parameters (Sambrook *et al.*, 1989). In this experiment 2 ul of pMBQAU1001 plasmid, 50 µl of competent cells were taken in quartz cuvette and given the shock of 2.5 mA, 2.0 mA for DH5α and *Agrobacterium* respectively (Dower et al. 1988; Miller et al. 1988).

The eppendorf tubes were placed on incubator shaker at 37 °C with speed of 120 rpm for one hour at 37 °C and 28 °C for *E.Coli* and *Agrobacterium tumefacienes* respectively, with 120 rpm shaking for 1 hr. This culture was spreaded to LB agar plates with 75mg/L Kanamycin selection, and incubated at 37 °C and 28 °C for 2-3 days. After the appearance of colonies several were picked up, cultured on LB broth on selection with continuous shaking at 120 rpm for overnight.

2.9 Molecular screening of positive clones

The putative recombinant bacterial clones growing on Lb medium plus kanamycin were subjected to molecular analysis through PCR, restriction mapping and sequencing to validate transformation constructs.

2.9.1 PCR confirmation

The plasmid DNA was extracted from transformed bacteria *E. Coli DH5α* and *Agrobacterium tumefacienes* strain GV3101 with *35S::myc:ROLA* gene construct (Sambrook et al. 1989). Qualitative and quantitative estimation of extracted DNA was done through agarose gel electrophoresis by comparing band intensity with known quantity of ladder DNA.Polymerase chain reaction for the amplification, detection and PCR encoded epitope tagging of *rolA* gene was done (Taylor, 1991). PCR was done in 0.2 ml PCR tubes of 25 µl total reaction volume. Plasmid DNA 15 ng was used in a final volume of 25 µl with 10 pmole (1 µl) of reverse and forward primers each, 0.2 mM each of d ATP, d GTP, d CTP, and d TTP and 1.5 mM MgCl2 (for taq only) and 0.5 unit of Taq polymerase (or pfu plymerase) and 2.5 µl of 10X PCR

buffer (10X pfu buffer with $MgSO_4$). The reaction mixture was centrifuged for few seconds thorough mixing.

The reaction mixture was taken through thermo cycling conditions as: 5 min of 95 °C for template denaturation followed by 30 cycles of amplification each consisting of 3 steps; 30 seconds at 95 °C for DNA denaturation into single strands; one minute at 53 °C for *rol* A primers (native and cloning) to hybridize or "anneal" to their complementary sequences on either side of the target sequence; and one minute at 70°C for extension for Taq (1.5 minute for pfu) of complementary DNA strand from each primer. Final 10 minutes at 70 °C for polymerase to synthesize any unextended strands left. PCR reactions were carried out in Biometra thermocycler according to the standard protocol (Taylor. 1991). Plasmid DNA from non-transformed pEarleyGate203 was used as negative control while pRiA4 plasmid DNA of *A. rhizogene* strain ARqua1 was used as positive control in PCR reactions. PCR conditions for amplification of *NPTII. ROLA* gene along with the size of amplified product are given in table 2.1. After completion of PCR reactions, the amplified products were analyzed on agarose gel stained with Ethidium bromide using a horizontal electrophoresis apparatus.

2.9.2 Restriction mapping

Selected colonies were inoculated in Lb medium after growth on selection the plasmid was isolated for restriction analysis to confirm the presence of inserted recombinant gene. The plasmids were restricted with Nco1 and Xba1 according to the instruction given on the literature by Thermo Scientific and visualized on Ethidium Bromide stained 1.2 % agarose gel. In a 20 µl total reaction 16 µl of Water, 2 µl of 10x tango buffer, 500 ng (1 µl) of pMBQAU1001, 1 µl of Nco1 and Xba1 each, were mix gently and spin down for a few second and incubate at 37 °C for 1-16hr.

2.9.3 Sequencing

The *rolA* gene was PCR amplified with Pfu DNA polymerase before sequencing. Then the amplicon is purified through Fermentas GeneJET™ PCR purification kit. The binding buffer in a ratio of 1:1 was added to amplicon and mixture was loaded on purification column having collection tube followed by centrifugation at 13,000 rpm for 1 minute. The flow-through was discarded and 650 µl wash buffer was loaded to column again centrifuged at 13,000 rpm for min. after discarding the flow-through,

the column was re-centrifuged for 2 minutes to remove residual wash buffer. Then the column was put in a new collection tube and incubated for 10minuts with 20 µl of elution buffer at room temperature and followed by centrifugation at 13,000 rpm for 1 minute for collecting the purified product. This purified product was checked on 1.5% agarose gel before proceeding to PCR for sequencing having following recipe: Template $p^{MBQA1001}$, PCR product by pfu 4 µl, Primer F (N'-myc) 2.00 µl, DTCS 2.5 µl, H2O 1.5 µl, Total 10 µl, at following conditions: 96°C for 10 mins, 96°C for 30 sec, 53°C for 20 sec, 60°C for 4 mins(2-4 for 34cycles), 65°C for 10mins,4°C for 2 mins. Later store the product in dark after until sequencing. Second PCR product was purified by ethanol precipitation. A fresh stop solution having of 1 µl of 3M NaAc (PH 5.2), 1 µl of 100 mM Na_2EDTA (pH 8) and 0.5 µl of 20 mg/ml Glycogen was made. Sequencing PCR product was shifted to 1.5 µl microfuge then 2.5 µl of stop solution as well as 70 µl of 100% ethanol was added followed by vortexing and centrifugation at 13,000 rrpm for 20 minutes. After removing supernatant and adding 70% ethanol, the solution was again subjected to centrifugation for 15 mins at 13,000 rpm. Again the supernatant was discarded and samples were vaccum dreid at low temperature. Finally the pellet was resuspended in the SLS through vortexing and spunned for a few seconds and loaded on CEQ8800 DNA sequencer (Beckman Coulter, USA).

2.9.3.1 Mutational analysis

DNA sequences were obtained from CEQ8800 DNA sequencer (Beckman Coulter, USA), and compared with native *rolA* gene sequence of Arqua1 strain of *A. Rhizogene* to validate that there is no frame shift or any other mutation in *rolA* gene along with bioedit, sequence alignment software version 6.0.

2.10 Preparation of recombinant Bacterial glycerol stock

In a sterile conical flask having 50ml Lb medium, one validated colony from the plate was inoculated. The cells were grown to late log phase (OD_{600} = 0.8–1.0). Then in sterile eppendorfs aliquot of bacterial culture and 90% glycerol were added in 1:1 ratio. This glycerol stock was stored at –80 °C.

Chapter 3

Results

Results

The *rol* genes functional protcomicsisnecessary for their exploitation in enhancing the secondary metabolite production. The goal of this study was to construct N-terminal mycepitope tagged *rolA*gene by adaptor PCR (overlapping set of primers) followed by its forced cloning into pEarlyGate203 expression vector for *Agrobacterium tumifaciens* mediated transformation which can reveal subcellular localization and other details of *rolA* gene after plant transformation. Thus resulting recombinant plasmid designated as pMBQAU1001 harbouring*CaMV 35S* promoter, *Kan* bacterial selectable marker gene and plants selectable marker gene *BASTAr*, waselectroporated in*E.Coli DH5α* and laterin disarmed *Agrobacterium tumifaciens GV3101*and it was successfully screened and validated using gene specific PCR primers for both *rolA* and *NPTII* (Kan) as well as by restriction mapping.

Cloning of pMBQAU1001 and transformation of *E.Coli DH5α*

3.1 Plasmid isolation

Isolation of plasmids pEarleyGate203 and pRiA4 (*rolA* gene) was done by alkaline lysis method (Sambrook and Russell, 2001) from *E.ColiDB3.1*and *Agrobacterium rhizogenes strain A4* respectivelywere grown in Lb media later streaked on LB agar supplimented with kanamycin (Figure 3.1),then single colony from plate was picked and inoculated again in falcon tubes of 15ml having 10ml antibiotic supplemented LB brothand incubated overnight at 37°C or 28°C for *E.Coli*and *Agrobacterium rhizogenes* respectively. Purified plasmids wereof right size and good quantitywithout shearingwhen analysed on 1 % agarose gel(Figure 3.2 and 3.3).

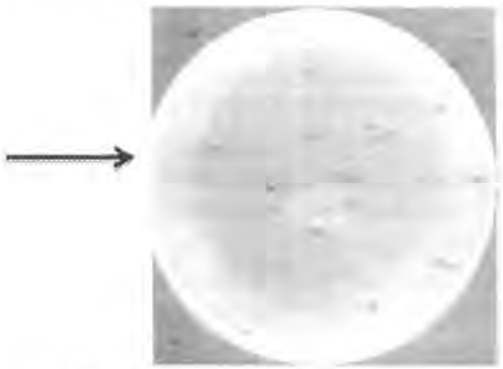


Figure 3.1: Colonies of*Agrobacterium rhizogenes strain A4* on Lb agar plates.

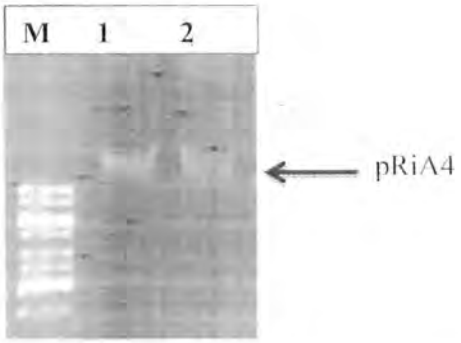


Figure 3.2 Midiprep of *A. rhizogenes* strain *ARqua1*.Lane 1: Fermentas 1Kb DNA ladder, Lane 2,3,4 pRiA4 plasmid

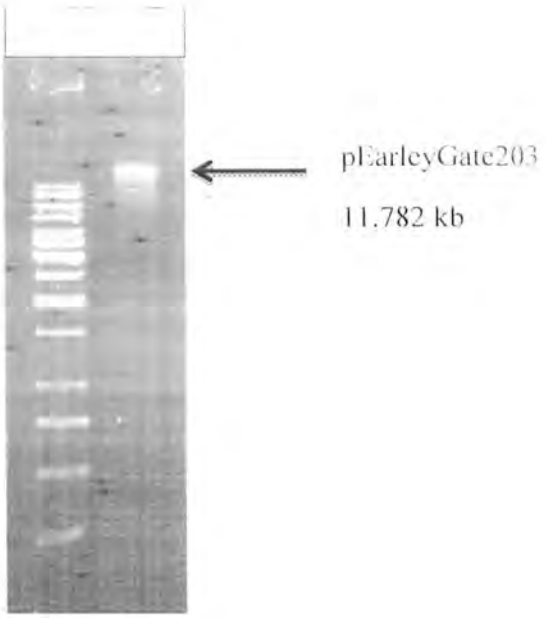


Figure 3.3 Midiprep of pEarleyGate203 from *E.Coli DB3.1*. Lane 1:Fermentas 1Kb DNA ladder, Lane 2: pEarleyGate203.

3.1.2 PET tagging of *rolA* gene for hard copies

For adaptor PCR mediated epitope tagging, sequential amplification was carried out with overlaping multiple sets ofprimer (having homologies of 15 bp) used for synthesizing m*yc-rolA* genefragment flanked by NcoI/XbaI sites and breathing sites (Horton *et al.*, 1990; Taylor, 1991). Amplicon of 1st round was used as template for next round of amplification. After third round of amplification 33bp of *myc* and restriction endonuclease sites and breathing sites were successfully added as shown by a good glowing band of 352bp as predicted (Figure 3.4 and 3.5).

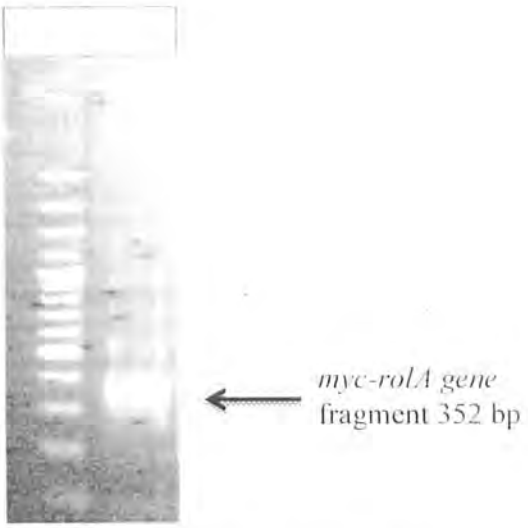


Figure 3.4PCR amplicon after 3rd round of *rolA* gene (352bp) PEI tagging (myc tagging)Lane 1: Fermentas 100 bp DNA ladder, Lane 2:*rolA*.

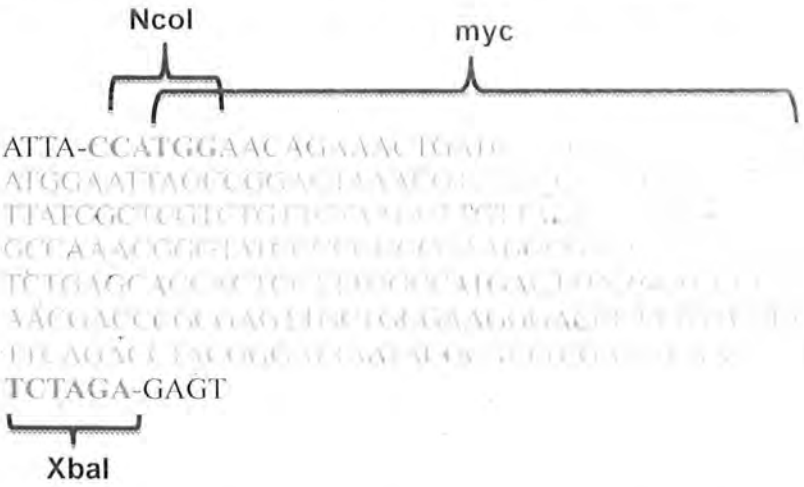


Figure 3.5 myc tagged rolA gene (352bp) complete sequence after 3rd round of PCR also showing two different restriction enzymes sites for sticky ends production plus breathing site for their proper binding.

3.1.3 Purificationand restriction

Beforedouble digest of vector and insert with NcoI and XbaIthe insert DNA was purified by eluting through 0.8% gel by gel extraction kit for this bigger comb was used in gel making to hold large quantity of amplicon and the resulting band was of exact size and good quality before (Figure 3.6) and after elution (Figure 3.7).

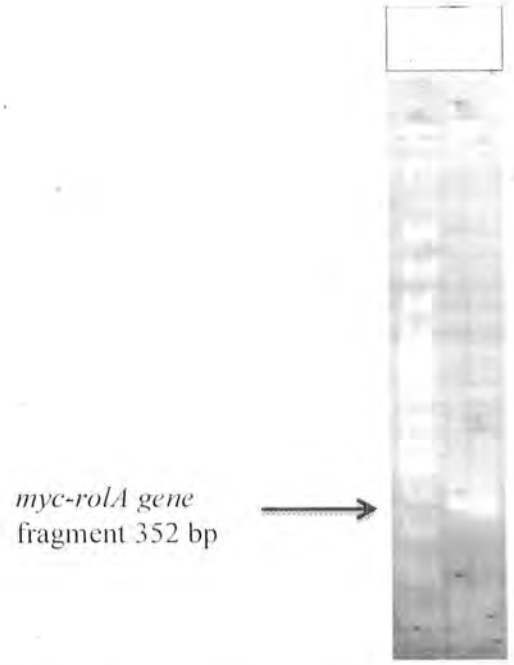


Figure 3.6*myc-rolA* genepurificationby eluting through0.8% agarosegel.Lane 1: Fermentas 100 bp DNA ladder, Lane 2 = *myc-rolA* gene.

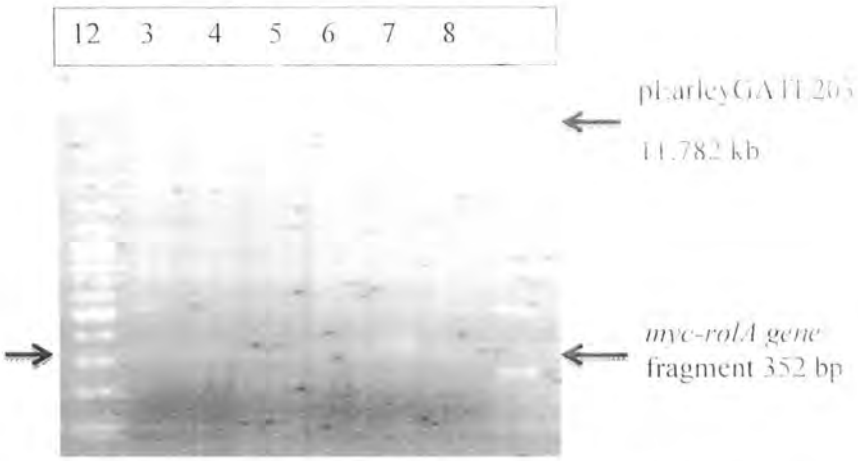


Figure 3.7 Checking elution success as well as insert and vector integrity before restriction. Lane 1 = molecular weight marker (100 bp ladder); lane 2 = sheared *rolA* PCR product; lane 3 = sheared *rolA* PCR product; lane 4 = sheared *rolA* PCR product; lane 5 = sheared *rolA* PCR product 2; lane 6= myc-rolA PCR amplicon; lane 7= pEarleyGate203 (expression vector); lane 8 = 1kb DNA Marker.

3.1.4 Vector/insert Ligation and Transformation

After Inserts was purified by gel extraction kit (Invitrogen), the ligation was done using T4 DNA ligase fermentas to make pMBQAU1001. The electroporation was done after preparing Electro-competent *E.Coli DH5α*cellsby Biorad micropulserTM using standard parameters (Sambrook *et al.*, 1989). The recombinant *E.Coli DH5α* was incubated in shaker for 1 hr followed by spreading on LB agar at kanamycin selection 100mg/L (Figure 3.8).About 50 colonies appeared on one petri plate after 2 days of incubation. A few of them were selected and screened for presence of *rolA* and *NPTII* gene by PCR.

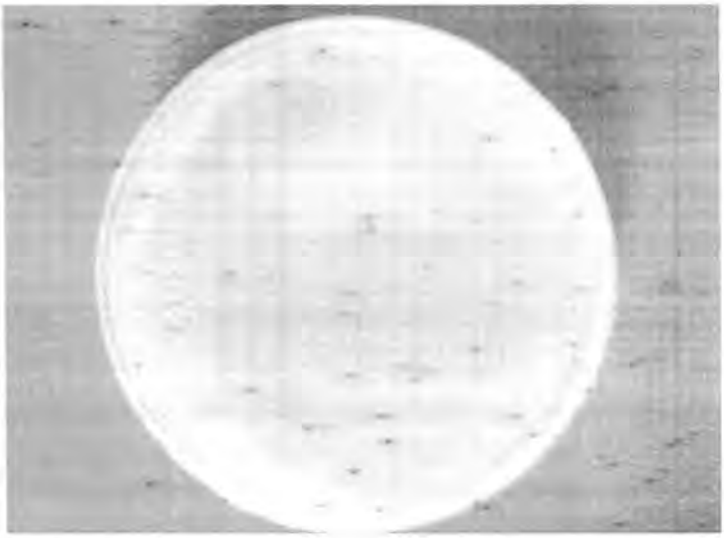


Figure3.8 Transformed colonies of *E.Coli DH5α* on kanamycin on LB agar plates.

3.1.5 Molecular analysis of clones by PCR

The confirmation of transgenic status of the bacteria was done by the PCR from plasmid DNA isolated after growing several colonies in Lb medium using forward and reverse cloning primers of *rolA* genes as well as simple primers of*NPTII* gene (Figure 3.9,3.10 and 3.11). *A. rhizogenes* ARqua1 plasmid served as the positive control and pEarleyGate203 was used as the negative control for *rolA* gene while pEarleyGate203 served as positive control for *NPTII* gene. Presence of the amplified products of the expected size in the positive control and colonies, confirmed the transgenic status of the colonies and 6th colony was stored as glycerol culture.

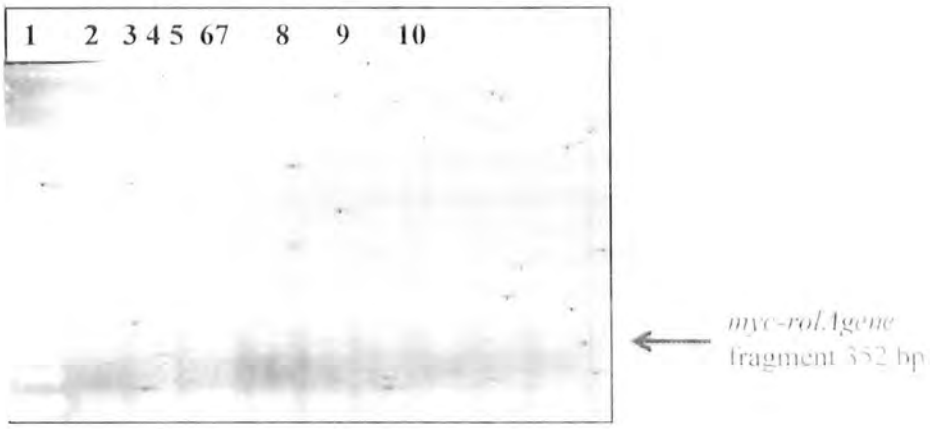


Figure 3.9 PCR amplification of 352 bp fragment of the myc-*rolA* gene from pMBQAU1001 in recombinant *E.Coli DH5α* colonies. Lane 1 = molecular weight marker (100 bp ladder); lane 2 = *A. rhizogenes*plasmid pRiA4 (positive control); lane 3 = pEarleyGate203 negative control for *rolA* gene;lane 4-10 = colony 1-7.

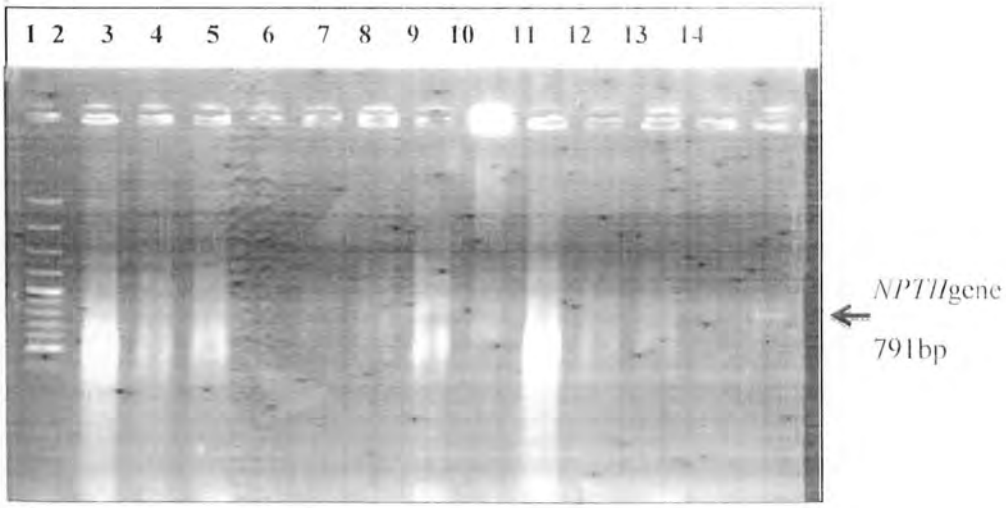


Figure 3.10 PCR screening of recombinantsfor *NPTII* gene. Lane 1 = molecular weight marker (100 bp ladder); lane 2-8 = colony 1-7 (*rolA*); lane 9-14 = colony 1-6(6^{th} colony +ve)

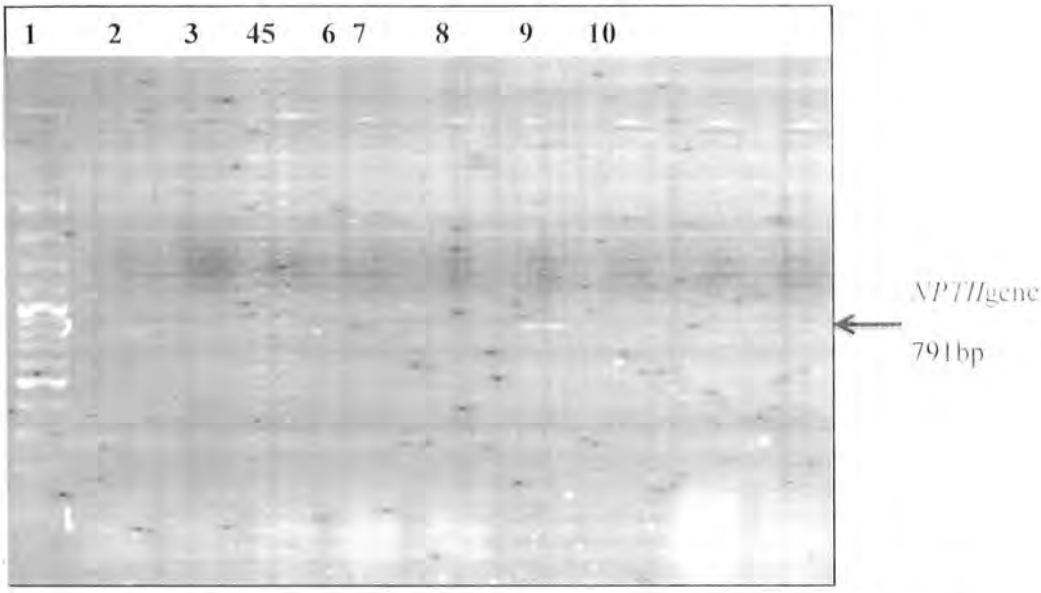


Figure 3.11 Repeating PCR amplification of 791bp fragment of the *NPTII* gene. Lane 1 = molecular weight marker (100 bp ladder); lane 2 = pHarleyGate203 (positive control); lane 3-6 = colony 1-4 ; lane 7-10 = colony 6-9 respectively notably 6th colony is positive again.

3.1.6 Restriction map analysis

Selected 6thcolony was inoculated in Lb medium after growth on selection. The plasmid was isolated for restriction analysis to confirm the presence of inserted recombinant gene. Firstly plasmid was quantitified by loading 1,2,3,4ul of plamid on 0.8% gel, it was found of good quality, without shearing and comparing band intesity with known qauntity of 100bp ladder (Figure 3.12). After concentrating about 10ul were having 500ng of Plasmid DNA (figure 3.12) then it was restricted with Nco1 and Xba1 in a 20ul total reaction volume and were run on 1.2% agarose gel followed by visualization with ethidium bromide (figure 3.13). The restriction analysis of pMBQAU1001 showed the correctbands of 10kb and 342 bp fragments after double digestion with Nco1 and Xba1 in a recommended 1X Tango buffer system because after restriction 8 bp breathing site and 2bp of recognition sites for restriction endonucleases are removed (Figure 3.13).

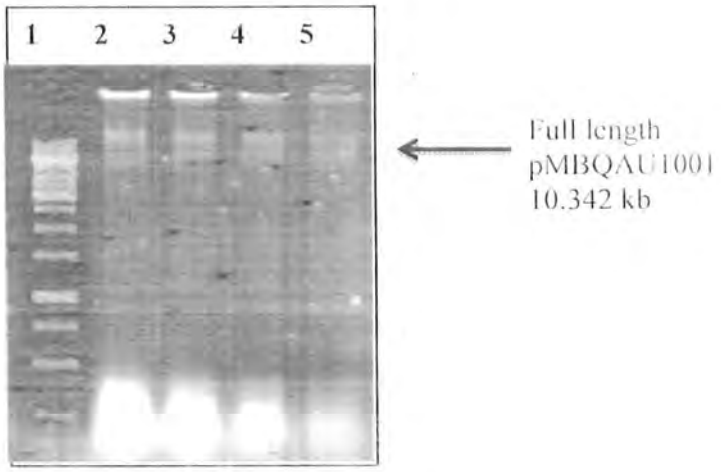


Figure 3.12 Recombinant plasmid pMBQAU1001 for qauntitation before restriction Lane 1 = molecular weight marker (1kb ladder): lane 2 = pMBQAU10014 ul: lane 3 pMBQAU1001 3ul; lane 4 = pMBQAU1001 2ul; lane 5 = pMBQAU1001 1ul.

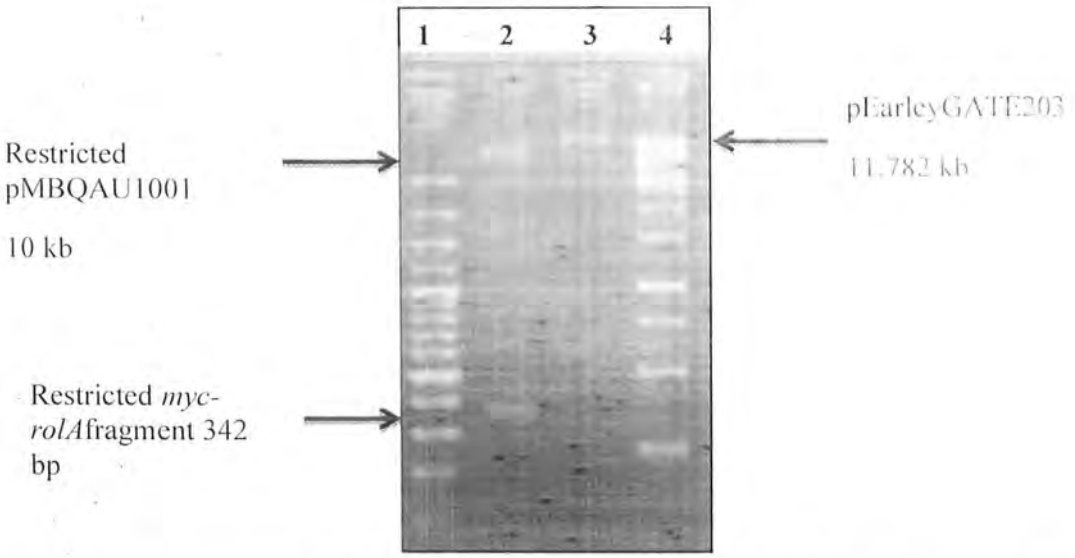


Figure 3.13 Restriction digestion of plasmid pMBQAU1001 for confirmation of cloning. Lane 1 = molecular weight marker (100bp ladder); lane 2 = pMBQAU1001 restricted with NcoI and XbaI (342 bp fragment of *myc-rolA* gene and 10000 bp pEarleyGate203 restricted): lane 3 = pEarleyGate203 unrestricted; lane 4 = molecular weight marker (1kb ladder).

3.2 Moving pMBQAU1001 in *Agrobacterium tumefacienes GV3101*

The electroporation ofpMBQAU1001was done after preparing Electro-competent *Agrobacterium tumefacienes GV3101*cells by Biorad micropulserTM using standard parameters (Sambrook et al., 1989). The recombinant *Agrobacterium tumefacienes GV3101*was incubated in shaker for 1 hr followed by spreading on LB agar at kanamycin selection 50mg/L and gentamycin 10mg/L selection and incubated.

About 20 colonies appeared on one petri plate after 2 days of incubation. A few of them were selected and screened for presence of *rolA* and *NPTII* gene by PCR (Figure 3.14).

3.2.1 Molecular analysis of clones by PCR

The confirmation of transgenic status of the bacteria was done by the PCR from plasmid DNA isolated after growing several colonies in Lb medium using forward and reverse cloning primers of *rolA* genes as well as simple primers of *NPTII* gene. *A rhizogenes* ARqua1 plasmid served as the positive control and pHarleyGate203 was used as the negative control for *rolA* gene while pHarleyGate203 served as positive control for *NPTII* gene. Presence of the amplified products of the expected size in the positive control and colonies, confirmed the transgenic status of the colonies and 5th colony was stored as glycerol culture (Figure 3.15 and 3.16).

Figure3.14 Colonies of recombinant *Agrobacterium tumifaciens GV3101* on kanamycin 50mg/L and gentamycin 10mg/L selection in LB agar plates

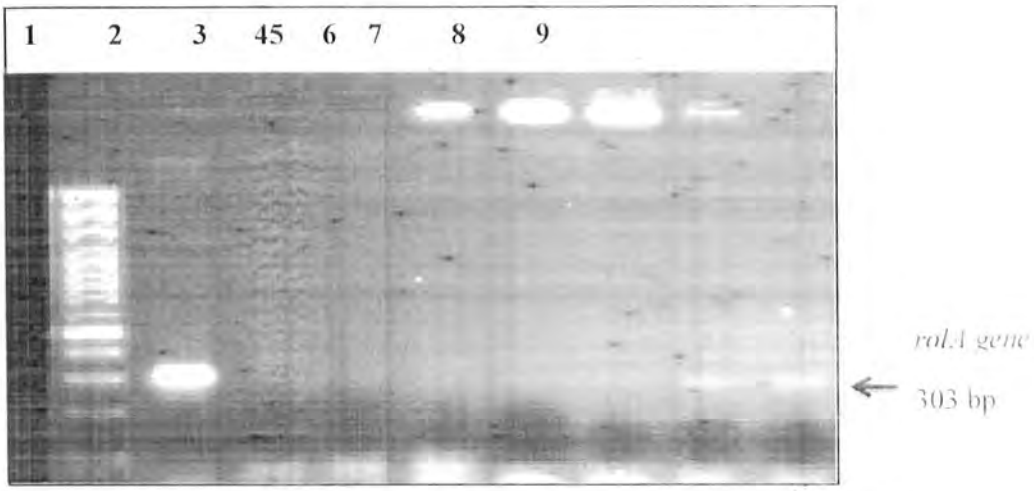


Figure 3.15 PCR amplification of 303bp fragment of the *rolA* gene. Lane 1 = molecular weight marker (100 bp ladder); lane 2 = *A. rhizogenes* ARqua1 DNA (positive control); lane 3 = plasmid DNA from non-transformed *A.tumifaciens GV3101* (negative control); lane 4 = colony 1; lane 5 = colony 2; lane 6 = colony 3; lane 7= colony 4; lane 8= colony 5; lane 9= colony 6.

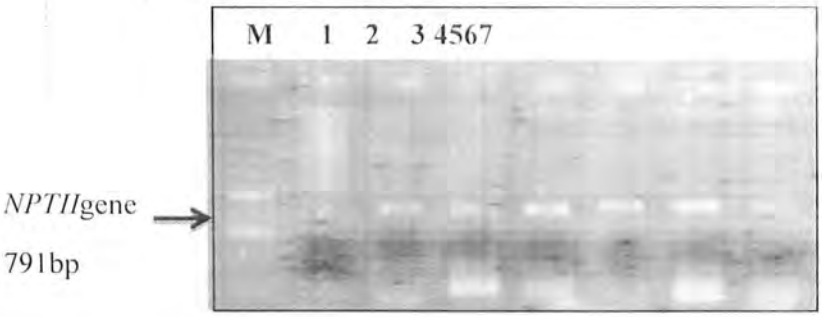


Figure 3.16 PCR amplification of 791bp fragment of the *NPTII* gene in pMBQAU1001 recombinant *A. tumifaciens GV3101* colonies. Lane 1 = molecular weight marker 100 bp; lane 2 = plasmid DNA from non-transformed *A.tumifaciens GV3101* (negative control); lane 3=colony 1; lane 4 = colony 2; lane 5 = colony 3; lane 6= colony 4; lane 7= colony 5.

3.3 Sequencing of *myc-rolA*

For sequencing *myc-rolA* was PCR amplified with pfu DNA polymerase to ensure high fidelity of replication (figure 3.17).

First purificationwas done by FermentasGeneJET^{TM} PCR purification kit according to protocol for collecting the purified product. 1st purification checking on gel before sequencing (figure 3.18), then Sequencing PCRwas run and stores the product in dark.For second Purificationsample was purified by ethanol precipitation protocol andthe pellet was suspended in 20 µl sample loading solution (SLS) then vortexed and spun for a few seconds. Sample loading tray was loaded with samples. Followed by their sequencing on DNA sequencer CEQ8800 (Beckman Coulter, USA).

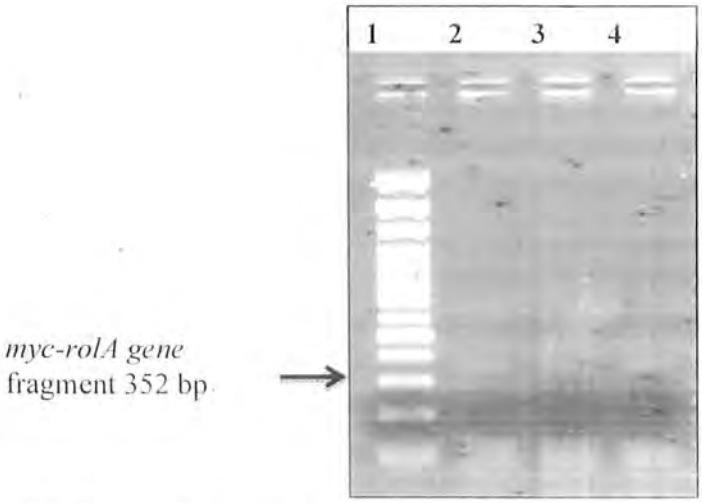


Figure 3.17*myc-rolA* gene PCR amplicon with pfu DNA polymerase for sequencing. Lane 1 = molecular weight marker (100 bp ladder); lane 2-4 = myc-rolA gene in triplicate.

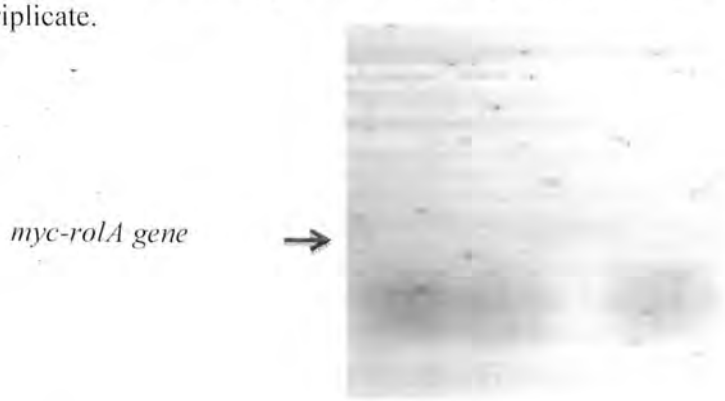


Figure 3.18 First purification during sequencing of *myc-rolA* gene.

2.9.3.1 Mutational analysis

DNA sequence and chromatograms were obtained from CEQ8800 DNA sequencer (Beckman Coulter, USA), which was compared with wild type rolA gene sequence from Arqua1 strain of *A. rhizogene* at NCBI database and it was validated that there is no frame shift or any other mutation in *rolA* gene along with bioedit, sequence alignment software version 6.0 as shown below.

ATTACCATGGAACAGAAACTGATC
GGACTAAACGTCGCCGGCATGGCC[illegible]
TGTTCTAAGC[illegible]
ATCCCCGG[illegible]
CTCCTTTGG[illegible]
AGTTGCTGCGAAGG[illegible]
GGGATTAA[illegible]TCTAGAGAGT

Chapter 4

Discussion

Discussion

Throughout the history plants and their secondary metabolites have been of great medicinal importance in treating a variety of ailments (Vormfelde and Paser 2000). One way of value addition in their medicinal significance is by exploiting the potential of plants genetic engineering which is called synthetic biology. Since 1980's plant genetic engineering have made great strides and now it is optimized for over 11 species (Primrose *et al.*, 2001). Now a days plant transformation not just a research curiosity but a commercial reality. Biolistic and *Agrobacterium*-mediated transformation commonest transgenic techniques for plant transformation although these techniques also have limitations. By large *Agrobacterium*-mediated plant transformation method is the most widely used method for several medicinal plants (Nin *et al.*, 1996; Nin *et al.*, 1997; Vergauwe *et al.*, 1998). For good transgenic practices, efficient, easy and reliable protocols involving cloning for vector generation as well as plant regeneration are prerequisite. Various parameter including suitable tools for gene insertion and choice of explant have major effect on transformation efficiency so they are being optimized. Over the past two decade, novel characteristics of transgenic plants have been studied including enhanced production of secondary metabolites and floricultural phenotypes by *rol* genes. It is known that the *rol* genes evade the cell's regulatory controls. The data shows that the *rol* genes suppress the ROS levels as well as modulate phytoalexin synthesis apart from ethylene, salicylic acid, methyl jasmonate-mediated mediated pathways and the NADPH oxidase pathway (Bulgakov *et al.*, 2004). But the ultimate result is independence of defense pathways from cellular control mechanisms. The *rolB* and *rolC* genes are extensively studied as regulators of growth, differentiation and secondary metabolites. But molecular function of *rolA* gene is yet to be explored (Shkryl et al., 2008). It is evident now that each *rol* gene has its independent role in plant metabolic pathways (Bulgakov, 2008). The *rolA* gene has emerged as stimulator of growth as well as secondary metabolites production (Bulgakov VP 2008; Chandra *et al.*, 2012). Previous studies performed to study the individual influence of *rolA* gene on secondary metabolism reported enhanced biosynthesis of nicotine (Palazon *et al.*, 1997) as well as of 2.8 fold higher amounts anthraquinones (AQs) in transformed plants (Shkryl *et al.*, 2008). The *rolA* is an interesting transgene because its expression

in *R. cordifolia* calli assured remarkably stable levels of AQs for over a period of 7 years. In addition it rendered conditions for robust growth of callus (Shkryl et al., 2007). The *rolA* gene only slightly decreases ROS levels. Because *rolA* protein has structural similarities with the E2 DNA binding protein of papillomavirus consequently it is suggested that *rolA* protein is a DNA binding protein (Rigden and Carneiro, 1999). This model anticipates that amino acids K 24 and R 27 are interacting directly with DNA bases in a sequence specific manner while it's eight other make nonspecific H-bonds with sugar phosphate backbone of the DNA (Matveeva and Lutova, 2014) in one of the studies on *rolA* protein. To date molecular function of *RolA* is generally not known. However, current experimental data does not provide any conclusive evidence concerning the effect of *rolA* on secondary metabolism. Production of transgenic plants with this gene and unraveling its exact mechanism seems to be the one of appropriate choices to improve production of secondary metabolites by synthetic biology approaches (Taneja *et al., 2010*). Rational designing, construction and selection of transformation vector are the most important steps in the *Agrobacterium* mediated transformation of plants.

As choice of transformation vector shapes the research goal and end points, the aim of present study was to generate construct for plant transformation for exploring the molecular role played by *rolA* gene. First PCR encoded epitope tagging of *rolA* gene was done followed by restriction digestion and forced cloning in pEarleyGate203 destination or expression vector to generate plant transformation vector. The pEarleyGate is a Gateway compatible binary vector series, for *Agrobacterium*-mediated plant transformation for epitope tagging of desired protein to enable their Immunoblot detection like AcV5, HA, FLAG, and cMyc tagging. The importance of these tags and vectors is increased as antibodies detecting these epitopes have little cross-reaction or affinity to endogenous proteins in a variety of dicotyledonous and monocotyledonous plants (Earley *et al.*, 2006). For example pEarleyGate203 binary vector with myc tag that is transnationally fused to protein of interest at N-terminus (Earley *et al.*, 2006). So it can be used for Immunolocalization as well as affinity purification of *rolA* protein for underpinning its biochemical role. This expression vector pEarleyGate203 is a contruct with N-terminal *Myc* tagged gateway cassette having *pVS1* origin of replication for *Agrobacterium* with 7-10 copies per cell due to

ColE1 ori in *E.Coli* and is basically derived from pCAMBIA vectors. It is a shuttle vector, so it can be propagated in *E. coli* or in *Agrobacterium*. In this case, there are two origins of replication. *Bar* (*Basta*^{R}) is Plant Selectable Marker gene of pEarleyGate203 derived from *Streptomyces hygroscopicus* (De Block *et al.*, 1989). The bacterial acetyltransferases that confer resistance to bialophos (consisting of two l-alanine residues and PPT) are used for achieving resistance against PTT herbicides, for variety of plant species like maize, rice and wheat (Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990; Vasil *et al.*, 1992; Rathore *et al.*, 1993). Many commercial broad spectrum herbicide formulations contain the L-phosphinothricin (PTT; gluphosinate ammonium) as active ingredients e.g. Basta™. It is a competitive inhibitor of an important enzyme that catalyze the detoxification of ammonia into glutamic acid known as glutamate synthetase (GS) ultimately leading to toxic levels of ammonia that results in plant cell death (OECD, 1999; Thompson et al., 1987; Wohlleben et al., 1988). In previous decade bar gene was one of the most extensively employed herbicide reistance selectable marker gene. It is broad spectrum, already marketed and available, as well as does not need additional marker genes. The most widely used bacterial Selectable Marker gene *NPTII or* Kan^{R} (kanamycin or neomycin phosphotransferase II gene) confer resistance to kanamycin antibiotic. Kanamycin is particularly widely used with dicotyledonous plants; but many cereals are tolerant to it. So in these cases G418 and hygromycin are often used. The use of markers conferring resistance to herbicides and antibiotics in transgenic crop plants has caused public concern, on the grounds that these genes may be transferred laterally. Two main approaches have been taken to deal with this. One has been to develop systems for the elimination of marker genes, in many cases by the Cre-lox system once stable transformation has been achieved. The other approach has been to use markers that do not require antibiotics for selection. One strategy exploits the fact that many plants are unable to use the sugar mannose and phospho-mannose isomerase gene allows plants to grow on mannose.

The CaMV 35S promoter of pEarleyGate203, having cis-regulatory elements involved in directing transcription initiation, spaning about 941 base pair (bp) upstream from the transcription start site has been shown to be active in various monocot and dicot cells and is one of the most widely used promoters. It is

constitutive with little tissue specificity and very active. Slight adjustments have been made to the sequence to improve expression. Moreover, pEarleyGate203 has left, right borders and 3' *UTRs* like poly A tail as well as *OCS* or octopine synthase enhancer elements that that facilitate the expression (Earley *et al.*, 2006).

The *myc tagged RolA* gene PCR amplified fragment by using Pfu DNA polymerase from pRiA4 was cloned by molecular copy paste in the gateway destination vector pEarlyGate203 to make the expression vector pMBQAU1001 with an N-terminal cMyc fusion tag for immunological protein detection (Chen *et al.*, 2013). Pfu DNA polymerase (Pyrococcus furiosus) is high fidelity polymerase best option for cloning purposes with exonuclease activity so it was given more time for amplification. the parameters like primer length, mismatches, internal secondary structures, primer primer annealing were considered using Vector NTI delux software before ordering the primers. This recombinant plasmid was electroporated into *E.Coli DH5α* that is used as holding strain because it is *recA* and *endA* knockout so transgene is less prone to recombination plus this strain is easy to transform and give good quality plasmid DNA after transformation for validation of cloning by PCR and restriction mapping. Then pMBQAU1001 was moved into disarmed *Agrobacterium* strain GV3101 having C58 chromosomal background with the disarmed Ti-plasmid pMP90 which is pTiC58 derivatives (Wood *et al* . 2001; Koncz and Schell, 1986) conferring gentamycin resistance plus rifampicin resistance is encoded by the chromosomal genes. Again cloning was validated through PCR using gene specific primers for both *rolA* and *NPTII* genes.

Conclusion and future prospects

Although, the some details about mechanism of how *rol* genes enhance secondary metabolism are emerging. So far, *RolA* is not yet epitope tagged or fluorescent tagged for its functional proteomics characterization. Unraveling the underlying mechanism would allow the engineering of plant with enhanced secondary metabolite contents and free of shortcomings inherent to the *rol* genes by controlling metabolic fluxes. The current study demonstrates the successful myc tagging of *rolA* gene in *Agrobacterium*-mediated plant transformation vector. Prospectively in near future various medicinal plants will be transformed and characterized by western blotting by using this novel construct in order to explore details of *rolA* gene mechanism of action.

Chapter 5

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