Molecular cloning of *korrigan* and *sucrose synthase* genes and genetic transformation of *Eucalyptus* for cellulose enhancement

A Thesis
Submitted in fulfillment of the requirements
for the award of degree of

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

DIWAKAR AGGARWAL
Regn. No. 90700006

Department of Biotechnology and Environmental Sciences

THAPAR UNIVERSITY

PATIALA-147004, INDIA

AUGUST 2013

CERTIFICATE

Certified that the thesis **"Molecular cloning of *korrigan* and *sucrose synthase* genes and genetic transformation of *Eucalyptus* for cellulose enhancement"** which is submitted by **Mr. Diwakar Aggarwal**, in fulfillment of the requirement for the award of the degree of *Doctor of Philosophy* in the Department of Biotechnology and Environmental Sciences (DBTES), Thapar University, Patiala, is a record of the candidate's own independent and original research work carried out by him under my supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree.

(Dr. Anil Kumar)
Assistant Professor and Supervisor
DBTES, Thapar University,
Patiala- 147004
Punjab

(Prof. M.S. Reddy)
Head, DBTES
Thapar University,
Patiala- 147004
Punjab

Countersigned
(Dean, Research and Sponsored Projects), Thapar University,
Patiala-147004, Punjab

DECLARATION

I hereby declare that the work which is being presented in this thesis **"Molecular cloning of *korrigan* and *sucrose synthase* genes and genetic transformation of *Eucalyptus* for cellulose enhancement"** submitted by me for the award of the degree of *Doctor of Philosophy* in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervision of Dr. Anil Kumar, Assistant Professor, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or Abroad.

(Diwakar Aggarwal)

ACKNOWLEDGEMENT

In pursuit of this academic endeavor, I feel that I have been singularly fortunate because inspiration, guidance, direction, cooperation, love and care - all came in my way in abundance and it seems almost an impossible task for me to acknowledge the same in adequate term.

Yes, I shall be failing in my duty if I do not record my profound sense of indebtedness and heart felt gratitude to my supervisor, **Dr. Anil Kumar**, Assistant Professor, Department of Biotechnology and Environmental Sciences (DBTES), Thapar University (TU), Patiala, who guided and inspired me in pursuance of this work. He nurtured my personality to adopt a socio-scientific temperament which enabled the metamorphosis of my raw skills into scientific expertise. It was his able supervision, advice, and guidance from the very early stage of this research as well as giving me extraordinary experiences through out the work which has resulted in fruitful outcome. Above all and the most needed, he provided me unflinching encouragement and support in various ways. His truly scientist intuition has made him as a constant oasis of ideas and passions in science, which exceptionally inspire and enrich my growth as a student and a researcher. I feel bereft of words to acknowledge his contribution to shape my academic perceptivity.

I wish to express my deep sense of gratitude with reverence towards my project supervisor and revered teacher, **Prof. M. Sudhakara Reddy**, Professor and Head, Department of Biotechnology and Environmental Sciences, Thapar University (TU), Patiala, for providing inspiration, motivation, encouragement and financial support for the present work. I am highly indebted to him for his support and thought provoking suggestions during many exigent circumstances. His systematic approach and unconditional co-operation made me work hard. Indeed, I shall remain grateful to him forever.

I am very thankful to **Dr. N. Das**, Associate Professor and Ex-Head, DBTES, TU, Patiala for providing suggestions, for thoughts and taking interest in the progress of this work since inception. I express my gratitude to Director as well as Dean (Research and Sponsored Projects), Thapar University, Patiala, for his encouragement and support during my research work in the Institute.

Constructive suggestions and critical remarks of all the faculty members from DBTES towards amelioration of this work have been of immense help in the progress of this work. I am highly obliged to them for their support in carrying out present research work.

I am thankful to Department of Biotechnology and Environmental Sciences (DBTES) and TIFAC-CORE at Thapar University, Patiala for providing necessary infrastructural support, assistance and co-operation.

Here the time for the acknowledging friends comes. I was not sure to write this page as I didn't want to end up with a long list of `cliché (which is, anyhow, exactly what I am going to do!). However, I decided to do it because there are plenty of people I want to thank and because I do think that most of the people, like me, enjoy reading their name in the acknowledgements. The cooperation received through my colleagues namely Pankaj Krishna, Santosh Karn, Achal, Giri, Balwant, Anshu, Raghu and other research scholars are thankfully acknowledged.

A special word of thanks to all the members of tissue culture unit (Mr. Rajesh, Mr. Khem Raj, Mr. Sandeep, Mr. Baljeet and Mr. Bhupinder) of TIFAC- CORE, Thapar University, Patiala. I would like to convey my thanks to Mr. Manmeet Singh, Mr. Harish Madan and Mr. Ashwani for their cooperation. I also owe a word of thanks to Mr. Babban (Ram Naval) and Lallan Yadav for their help. I also thank those who could not find a separate name but helped me directly or indirectly.

Financial Support in the form of SRF and Teaching Associateship from CSIR, New Dehli and Thapar University, Patiala is also duly acknowledged.

Above all it's my family members, who encouraged me to complete my Ph.D besides lots of odds and troughs in my life. I owe my personal reckoning of gratitude and benevolence to them for love, care, patience, inspiration, encouragement; motivation and constant driving force which has enabled me to complete my task.

Date : 08.0813
Place : Patiala

Diwakar Aggarwal

DEDICATION

I dedicate this thesis to my parents whom I have a deep respect, admiration and love for, and who taught me to be strong, to embrace life with enthusiasm, and to be generous and forgiving; to my wife who makes me a better person, gives me strength, and supports me all the way with his unselfish sacrifices and enduring love; to my son Krish who brings so much joy to my life. Thank you all!

The following publications are the outcome of the present research work:

- **Aggarwal D,** Kumar A and Reddy MS (2010): Shoot Organogenesis from elite plants of *Eucalyptus tereticornis*. ***Plant Cell Tissue and Organ Culture*** 102:45-52 **(IF: 3.63)**

- **Aggarwal D**, Kumar A and Reddy MS (2011): *Agrobacterium tumefaciens* mediated genetic transformation of selected elite clones of *Eucalyptus tereticornis.* ***Acta Physiologiae Plantarum*** 33:1603–1611 **(IF: 1.30)**

- **Aggarwal D**, Kumar A, Sharma J and Reddy MS (2012): Factors affecting micropropagation and acclimatization of elite clone of *Eucalyptus tereticornis* Sm. ***In Vitro Cellular and Developmental Biology-Plant*** 48:521-529 **(IF:1.13)**

CONFERENCE PRESENTATIONS

- **Aggarwal D**, Kumar A and Reddy MS (2010): **Studies on shoot regeneration and genetic transformation in elite clones of *Eucalyptus.*** National symposium on "Plant *Cell Tissue and Organ Culture: The Present Scenario."* Centre of advanced study, Department of Botany, University of Calcutta, Kolkata. 3-5 March, 2010

- **Aggarwal D**, Kumar A and Reddy MS (2012): **Micropropagation of commercially important elite clone of *E. tereticornis* Sm.** National symposium on "Emerging Trends in Biotechnology" Department of Biotechnology, MM Modi College, Patiala.24th Feb., 2012

- **Aggarwal D,** Kumar A and Reddy MS (2012)**: *Agrobacterium tumefaciens* *mediated genetic transformation of E tereticornis Sm*.** National seminar on "Plant *Cell Tissue and Organ Culture: Emerging Trends."* Department of Botany, Aligarh Muslim University, Aligarh. 10-11 March, 2012

- **Aggarwal D**, Kumar A and Reddy MS (2013): **Biotechnological Approaches for the improvement of *Eucalyptus.*** National Seminar on Plant Biotechnology "Plant Sciences: New Technologies, Conservation and Environment, Department of Botany, Aligarh Muslim University, Aligarh.23-24th February 2013.

Abbreviations

%	Percent
2,4-D	2,4-Dichlorophenoxy acetic acid
B5	Gamborgs medium (Gamborgs *et al.* 1968)
Abs	Absorbance
AFLP	Amplified fragment length polymorphism
BA	6-Benzyl adenine
bp	Base pair
CaMV	Cauliflower Mosaic Virus
cDNA	Complementary deoxyribonucleic acid
CFL	Cool Fluorescent Light
cm	Centimeter
CTAB	Cetyl Trimethyl Ammonium Bromide
Dia	Diameter
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	deoxynucleotide Triphosphates
EDTA	Ethylene Diamine Tetraacetic Acid
EGases	Endo- 1,4- β -glucanases
FAA	Formalin–Acetic acid–Alcohol solution
g	Gram
g	Gravitational constant
GUS	*β*-Glucuronidase
GA_3	Gibberellic acid
h	Hour
ht	Height
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IPA	Indole-3-propionic acid
IPTG	Isopropyl- β –thiogalactoside
ISSR	Inter-simple sequence repeat
kb	Kilobase
Kn	Kinetin (N^6-furfuryladenine)
l	Liter
M	Molar
m	Meter
mg	Milligram
min	Minute
ml	Mililitre
mM	Milli molar
mm	Millimeter
MMLV	Moloney murine leukemia virus
MOPS	3-(N-morpholino) propanesulfonic acid
MPa	Megapascals
mRNA	Messenger RNA

MS	Murashige and Skoog medium 1962
NAA	α-Naphthalene Acetic Acid
ng	Nanogram
nm	Nanometer
nmol	Nanomole
nos	Nopaline synthase
nptII	Neomycin phosphotransferase
ºC	Degree Celsius
OD	Optical density
ORF	Open reading frame
PAR	Photosynthetically Active Radiation
PCR	Polymerase chain reaction
PGRs	Plant growth regulator (s)
pI	Isoeletric point
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RAPD	Random amplification of polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Rotation per minute
rRNA	Ribosomal ribonucleic acid
RT	Room Temperature
RT-PCR	Reverse Transcriptase Polymerase chain Reaction
s	Second
S	Svedberg
SDS	Sodium Dodecyl Sulphate
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TDZ	Thidiazuron
TE	Tris-EDTA
Tris	Tris-(hydroxymethyl)- aminomethane
U	Unit
UV	Ultra Violet
V	Volt
v/v	volume by volume
w/v	weight by volume
WPM	Woody Plant Medium (Lloyd & McCown 1980)
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside
X-gluc	5-bromo-4-chloro-3-indolyl-*β*-D-glucuronic acid
YEP	Yeast Extract Peptone
µg	Microgram
µl	Microlitre
µm	Micrometer
µmol	Micrmole
µM	Micromolar

TABLE OF CONTENTS

Chapters **Page No.**

Chapter-1

Introduction

Eucalyptus (Family Myrtaceae) is among the most widely planted hardwoods in the world (Doughty 2000). These are generally long-lived, evergreen species (Ladiges *et al.* 2003). *Eucalyptus* is widely planted in the tropical and subtropical part of the world because of its superior growth, broader adaptability and multipurpose wood properties. Native to Australia, *Eucalyptus* includes over 700 species (Brooker 2000) and these fast growing trees were introduced into India, France, Chile, Brazil, South Africa, and Portugal in the first quarter of the 19th century (Doughty 2000) and were rapidly adopted for plantation forestry. The great economic interest in *Eucalyptus* species is due to versatility of their wood (Eldridge *et al.* 1993). Its timber has applications for many different purposes such as pulp and paper production, electric poles, charcoal, timber and furniture. *Eucalyptus* being a hardwood tree produces shorter fibers than softwoods like pines. Short fibers of this genus make it more reliable for use in paper and furniture industries due to its desirable surface characteristics, smoothness, brightness and low tensile strength (Lal *et al.* 1993). Nowadays, due to their good quality of wood fiber *Eucalyptus* and their hybrids are among the world’s leading sources of pulpwood and timber. Globally area under its plantation is estimated at 20 million ha (GIT Forestry 2008), spreading over 37 countries and accounting for 16% of worldwide forest area (FAO 2000). India has the largest area under *Eucalyptus* plantation (8 million ha), followed by Brazil (3 million ha) (Junghans *et al.* 2003). *Eucalyptus* plantations contribute 25% of total wood consumed in the developed countries and mainly used as source of wood and fiber for pulp and paper industry (Zhou 2005).

Eucalyptus tereticornis (Fig. 1.1) is a versatile member of the genus *Eucalyptus*, is also known as a forest red gum in Australia and Mysore gum in India and designated as one of the most productive forest crops of tropical countries (Sharma and Ramamurthy 2000). *E. tereticornis* is a fastest growing species and important source of raw material for the pulp and paper industry (Khan *et al.* 2002). It can reach up to 30 to 45 m in height and 1 to 2 m in diameter. It has a straight shaft with a big crown that is moderately dense. The trunk has a straight base and cylindrical shaft. The smooth, whitish bark comes loose in thin laminas or long strips, producing whitish, grey, or bluish spots in patches and leaving an accumulation of old bark (dark grey, wrinkled) at the base (Eldridge *et al.* 1993).

Figure 1.1 Elite plants of *Eucalyptus tereticornis* growing at Thapar Technology Campus, Patiala (Punjab, India)

The average annual yield from ordinary seed raised agro forestry plantations of *Eucalyptus* is in the range of 5–6 $m^3 ha^{-1} yr^{-1}$ by 3rd year and 10–15 $m^3 ha^{-1} yr^{-1}$ by 7th year of plant growth (Lal *et al.* 1993). Some selected clones showed mean annual productivity ranging between 16–20 $m^3 ha^{-1} yr^{-1}$ by 3rd year and 20–25 $m^3 ha^{-1} yr^{-1}$ by 7th year of plantation (Lal *et al.* 1993). *Eucalyptus* is traditionally propagated by seeds and interspecific hybrids are common in nature. In such genetically diverse stocks, trees with the better qualities, such as a straight clear bole, disease and pest resistance, drought tolerance, high productivity, fast growth, etc., occur at low frequencies. Due to extensive cross-pollination, seed progeny of superior trees fails to maintain their superior characteristics (Rao 1988). Thus, there is a felt need to develop faster methods of vegetative propagation of these promising elite clones. Although, multiplication through conventional vegetative propagation could be carried out (Jain 2006), yet it has several constraints such as poor rooting of stem cuttings, graft incompatibility (Vengadesan and Pijut 2009) and is further limited by the availability of propagules and season.

Micropropagation is an attractive alternative to conventional vegetative propagation with the advantage of enhancing the rate of multiplication of valuable clones from limited explant material (Beck and Dunlop 2001). Moreover, micropropagtion of these clones has led to the rapid genetic gains and higher returns from plantations due to increased productivity (Khuspe *et al.* 1987). Various *in vitro* propagation techniques, such as axillary and adventitious shoot multiplication, shoot organogenesis and somatic embryogenesis are currently employed in plantation forestry programmes for the large-scale multiplication of important tree species (Vengadesan and Pijut 2009; Zfira *et al.* 1998; Haines and Martin 1997). Micropropagation exploits the regeneration potential of the selected tissue and is preferred choice for the multiplication of difficult-to-root but economically or industrially important genotypes. It has been successfully used for rejuvenation and mass multiplication of many tree species (Yasodha *et al.* 2004) including *Eucalyptus* (Sharma and Ramamurthy 2000; Chang *et al.* 1992). Moreover, higher yield was reported from plantations raised from micropropagated plants as compared to seedling-derived plants (Khuspe *et al.* 1987). Thus, the potential impact of micropropagation on forest productivity, on forest based industry and global timber supplies are now well realized (Yanchuk 2001).

In spite of progress made in development of micropropagation protocol of various forest tree species, there is a limited use of this technology for clonal forestry programmes due to lack of awareness (Yasodha *et al.* 2004). Further, there is an urgent need to undertake micropropagation of these selected elite clones or genetically modified plants for the successful clonal forestry programmes.

In addition, to the development of micropropagation protocols for the superior clones, there is a need to undertake the trait specific improvement programme for elite clones using various biotechnological tools. There have been rapid developments in area of plant biotechnology in recent years; powerful tools that can enhance productivity and utilization are becoming available (Merkle and Dean 2000). Relevant methods include genetic manipulations to introduce exotic genes conferring resistance to biotic and abiotic stress including trait specific genetic modifications and marker-assisted breeding programmes (Teasdale 1995). Improvement of plants through transgenic technology enables introduction/improvement of specific trait(s) of interest in a selected genotype. The pre-requisites for plant genetic transformation are: (a) gene constructs carrying the polynucleotide sequences coding for desired proteins, (b) efficient methods to transform the explants, (c) procedures for selection of plant tissue harboring transgene and d) an efficient plant regeneration protocol form desired explants. In conventional breeding approach, the traits of interest have to reside within the same species. On the other hand, genetic transformation technology enables the scientists to transfer genes for selected traits across genera and kingdoms (Brunner *et al.* 2007). The transfer of selected genes is more important for tree species, as their improvement by conventional breeding programme is limited by long breeding cycles, high levels of heterozygosity and incompatibility barriers (Machado *et al.* 1997). However, due to herbaceous nature and ease of genetic transformation and subsequent regeneration of transformed tissue till now the major focus of genetic manipulations has been on crop species and there are many reports of successful introduction of foreign genes into crop plants (Girijashankar and

Swathisree 2009). Due to the difficulties in regeneration and genetic transformation, forests trees still remain a challenge to genetic manipulations.

Improvement of wood quality and biomass productivity are the major attractions of any wood based industries. During the last two decades, genetic transformation of tree species for modification of cellulose/lignin biosynthesis with the goal to modify wood quality for end use is an important area of research (Shani *et al.* 2004; Halpin and Boerjan 2003; Fenning and Gershezon 2002).

Quality of wood is determined by the strength and flexibility of the wood. Secondary cell wall consists of cellulose, lignin and other polysaccharides constituents 90% of the wood dry weight that provides support for the cell. Changes in the composition of these macromolecules, both qualitatively and quantitatively may lead to changes in cell wall properties, which are responsible for variations in wood properties observed within and across tree species (Spokevicius *et al.* 2007).

Importance of using *Eucalyptus* in the pulp and paper industry is due to its suitability for making high grade paper, which can be used for many important purposes. These characteristics are imparted by its fibers that are slender in nature, small and low coarse and resistant to collapse. The wood (raw material) is processed to obtain pulp (cellulose and hemi-cellulose) through the process called Kraft pulping, wherein many chemicals are used which are not only expensive but also cause serious environmental hazards. Since *Eucalyptus* contains higher quantity of lignin (22 to 25%) therefore, kappa number during Kraft pulping of its wood is very high, which is a serious concern of paper and pulp industry. A small increase in cellulose content or decrease in lignin content (about 5 % shift) in wood will not only result in reduction of cost during Kraft pulping but also results in increased yield and reduced use of hazardous chemicals.

Cellulose is a major structural component of plant cell walls and is the most abundant biopolymer on the earth. It is a major end product of photosynthesis that may constitute up to 50 % of plant biomass. Chemically, it is a linear polymer comprised of D-glucose residues linked by β-l,4-glycosidic bonds such that every glucose is rotated by approximately by 180 degree. The structural repeating unit in cellulose is cellobiose (Fig. 1.2), in contrast to many other glucan polymers where the repeating unit is glucose (Brown *et al.* 1996).

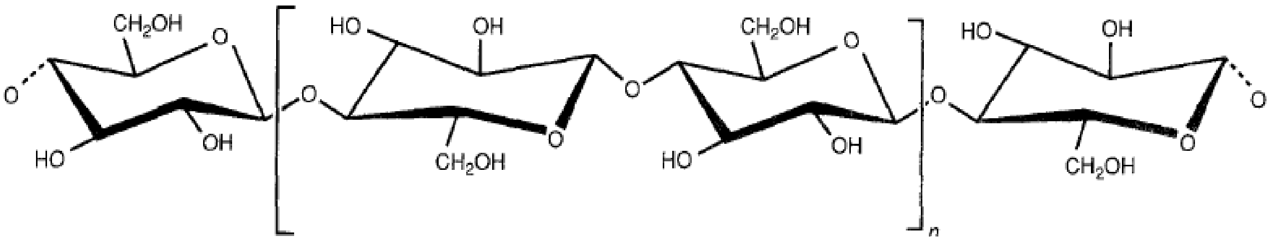


Figure 1.2 Structural formula of Cellulose (β-1,4- glucan polymer chain). The repeating unit cellobiose is indicated in brackets.

Because of its unique structure, cellulose chain can interact with each other and assemble into microfibrils containing an estimated 36 strands and these strands are aligned in a parallel fashion in cellulose (Reiter 2002). Microfibrils are further associated into macrofibrils or bundles. The formation of cellulose into microfibrils is associated with the structure of the cellulose synthase complex or ‘rosette terminal complex’. The rosette is an organized enzyme complex, hexagonal in structure, which produces cellulose microfibrils. It is believed to be composed of 6 units that contain a number of cellulose synthase subunits (possibly 6) and each produces a glucan chain (Delmer 1999). The resultant chain from each unit associates with adjacent chains of the same rosette to form a microfibril.

In recent years, due to the recent advances in plant genomics significant insight into the molecular details of cellulose biosynthesis has been gained. The plasma membrane rosettes contain the cellulose synthase catalytic subunit (CESA) proteins that are encoded by the *CESA* genes (Somerville 2006). Plant genome is reported to contain many *CESA* genes as part of a multigene family. For example, at least ten *CESA* genes are reported in *Arabidopsis*, rice has at least nine (Keegstra and Walton 2006) and poplar is reported to have about 18 (Djerbi *et al.* 2005). The idea that there are at least three different CESA proteins in a rosette comes from genetic evidence, which also supports the conclusion that the cellulose in primary cell walls is synthesized by a different set of CESA proteins than that in secondary cell walls (Joshi *et al.* 2004). In *Arabidopsis*, *CESA*1, *CESA*3, and *CESA*6 are required for cellulose biosynthesis in primary cell walls (Robert *et al.* 2004) whereas *CESA*4, *CESA*7, and *CESA*8 are required for cellulose biosynthesis during secondary wall deposition (Somerville 2006; Taylor *et al.* 2004). Similar conclusions have been reached in case of other plants (Nairn and Haselkorn 2005; Joshi *et al.* 2004).

CESA proteins in higher plants and their homologs in bacteria are reported to require additional proteins for cellulose biosynthesis (Matthysse *et al.* 1995). In addition to CESA proteins, cellulose biosynthesis requires the activity of other proteins, an endo-1,4-β-D-glucanase is shown to participate in cellulose synthesis (Matthysse *et al.* 1995), raising the possibility that other enzymes also play a role in cellulose synthesis. One such important gene is a plasma membrane bound endo-1,4-β-D-glucanase also known as *Korrigan (KOR*), which is required for normal cell wall assembly, cell elongation and cellulose synthesis (Nicol *et al.* 1998, Lane *et al.* 2001, Szyjanowicz 2004). *KOR* appears to be a member of the endo-1,4-β-D-glucanase family (EGase), a large, ubiquitous family of enzymes that hydrolyse 1,4-β-linkages adjacent to unsubstituted glucose residues (Brummell *et al.* 1994; Henrissat *et al.* 1989) and a class of proteins that have been reported to be involved in modifying plant cell walls (Zuo *et al.* 2000). All higher plant species has been reported to express EGases multiple protein family (Molhoj *et al.*

2002). The expression of most plant EGases is tightly regulated, some EGases are expressed specifically during fruit ripening or leaf abscission (Del Campillo and Bennett 1996; Lashbrook *et al.* 1994; Tucker and Milligan 1991; Cass *et al.* 1990) and the expression of other EGases is highest during cell expansion which suggest a role in cell wall polysaccharide assembly or rearrangements in the primary cell wall (Molhoj *et al.* 2002).

KOR was originally isolated from a mutant *Arabidopsis thaliana* plant (kor1-1) that showed pronounced architectural alterations in the primary cell wall when grown in the absence of light (Nicol *et al.* 1998). An additional *KOR* mutation (kor1-2) has been shown to cause the formation of abnormal cell plates, incomplete cell walls, and multinucleated cells, leading to abnormal seedling morphology (Zuo *et al.* 2000). In addition, the identification of irregular xylem mutants of *KOR*, such as irregular xylem mutant 2 (Szyjanowicz *et al.* 2004), suggests the requirement of *KOR* for normal xylem vessel development. The gene was found to be highly conserved between mono- and dicotyledonous plants. *KOR* is located primarily in the plasma membrane and presumably acts at the plasma membrane–cell wall interface (Nicol *et al.* 1998).

Sucrose, the major product of photosynthesis, can either be utilized directly through glycolysis or be translocated within the plant as a soluble carbohydrate by phloem. When imported into sink, sucrose is used for the maintenance of cellular metabolism, cell wall biosynthesis, and respiration or converted to starch as storage polysaccharide, which can be used at a later stage (Kutschera and Heiderich 2002; Tang and Sturm 1999). Cellulose is a major structural polymer in the plants, is an irreversible carbon sink. How plants control carbon partitioning to cellulose biosynthesis is a key question (Haigler *et al.* 2001). Recent models propose UDP-glucose as substrate for cellulose synthesis, which is channeled to the cellulose synthase complex by enzyme *Sucrose Synthase* (*SUS*, EC

2.4.1.13) (Haigler *et al.* 2001; Delmer *et al.* 1999). UDP-glucose has been suggested to be involved in cellulose biosynthesis by channeling glucose to cellulose synthase catalytic subunit(s) (Amor *et al.* 1995). *SUS* have been considered to be one of the most important players in cellulose synthesis process as it supplies the substrate UDP-glucose for cellulose biosynthesis (Delmer *et al.* 1999). Down regulation of *SUS* gene has been reported to suppress elongation of cotton fibre cells (Ruan *et al.* 2003).

SUS activity has been localized in different intracellular structures, such as cell membranes (Matic *et al.* 2004), the cytoskeleton (Winter *et al.* 1998), and the tonoplast (Extebberia and Gonzalez 2003). *SUS* also exists in two forms, the soluble form in the cytosol and the second form in association with the plasma membrane or cell walls (Carlson and Chourey 1996) with the latter proposed to be involved in the synthesis of cellulose required for cell wall formation by providing UDP-glucose directly to the cellulose synthases (Amor *et al.* 1995).

The overexpression of cotton *SUS* gene in tobacco transgenic plants increased the total biomass (Coleman *et al.* 2006). Overexpression of the cotton *SUS* gene in hybrid poplar promoted 2~6% of cellulose content and increased crystallinity in all transgenic lines with the increased SUS enzyme activity compared to controls (Coleman *et al.* 2009). However, the overexpression of the same cotton *SUS* construct into tobacco did not alter the cellulose content (Coleman *et al.* 2006). Recently Xu and Joshi (2010) reported that overexpression of aspen *SUS* gene promotes growth and development in transgenic *Arabidopsis* plants.

Overall, cellulose biosynthesis is considered to be a three step process: (1) plasma membrane-associated sucrose synthase (*SUS*) directly channelize UDP-glucose as a substrate to cellulose synthesizing machinery (2) coordinately expressed multiple cellulose synthase genes, organized in the form of hexagonal rosettes, polymerize glucose monomers into glucan chains while recycling liberated UDP back to *SUS* and (3) a membrane-associated cellulase β-1,4–glucanase; *korrigan* (*KOR*), acts as an editor of newly produced glucan chains (Joshi *et al.* 2004; Molhoj *et al.* 2002) (Fig.1.3).

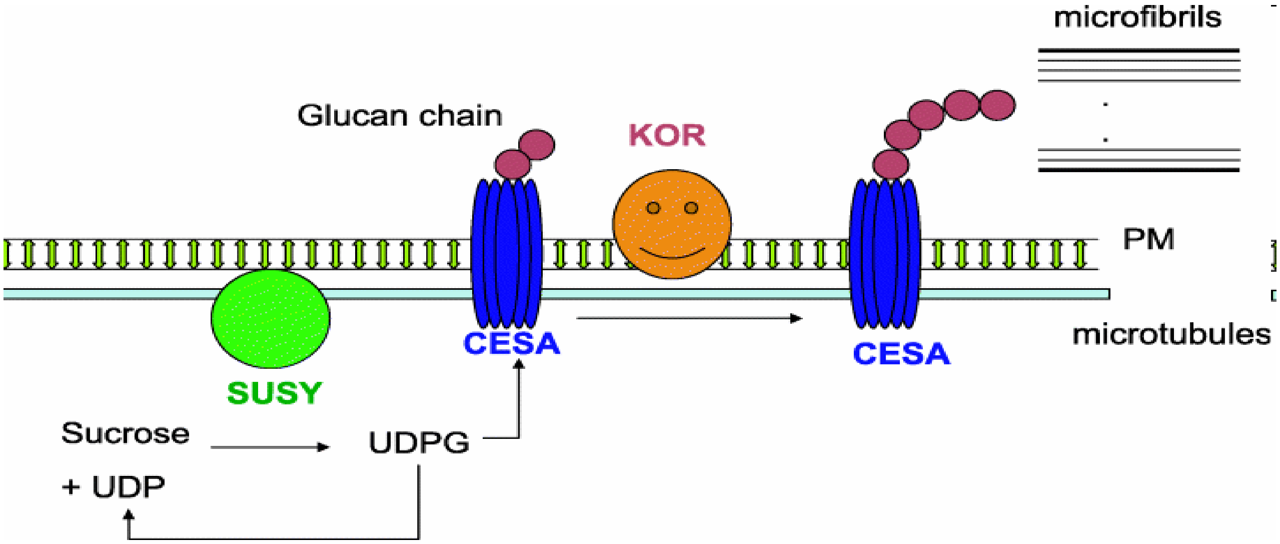


Figure 1.3 Plasma membrane (PM)-associated form of sucrose synthase (*SUS*) directly channels Uridine diphosphate glucose (UDPG) substrate to cellulose synthase (*CESA*) rosette complex that aid in glucan chain formation while recycling UDP back to *SUS*. Glucan chains self assemble into microfibrils and *korrigan (KOR)* acts as an editor/monitor of this process (Adapted from Joshi *et al.* 2004).

Understanding the biosynthesis of cellulose, the world's most abundant polymer, has been an interesting area of research for many years. However, in spite of the motivation, progress in studies on cellulose biosynthesis in plants has been slow. In plants the production of cellulose is as essential as photosynthesis. The deposition of cellulose in the plant cell wall can affect many aspects of plant growth and development. Eluciating even a small fraction of the biosynthetic mechanism of this polymer could be critical to future experiments aimed at increasing cellulose production in plants specially trees. Therefore, these two key genes (namely *korrigan* and *sucrose synthase*) which are to play key role in cellulose biosynthesis have been chosen for cloning and characterization in the present study. Further, there is a potential of genetic manipulations for improving woody plants but progress has been slow, because of recalcitrant nature of these plants for regeneration following transformation (Tournier *et al.* 2003). Therefore, it is important to develop regeneration and transformation protocol from the mature plants of *E. tereticornis*. Close perusal of literature reveals that no work has been undertaken for the up-regulation of cellulose biosynthesis in *E. tereticornis*. There is an urgent need for improvement of *Eucalyptus* with respect to reduced lignin content and/or higher cellulose content through plant genetic manipulation. To fulfill these objectives, a clone specific efficient regeneration protocol (organogenesis or somatic embryogenesis) and transformation protocol(s) are required to be developed and key genes involved in cellulose biosynthesis are required to be cloned for better understanding of the pathway.

1. Development of genetic transformation protocol from mature plants of *Eucalyptus*
2. Molecular cloning and characterization of cDNA clones encoding *korrigan* and *sucrose synthase* from poplar
3. Genetic transformation of *Eucalyptus* using the above genes

Chapter-2

Review of literature

Trees and forests are indispensable for human existence (Zhou 2005), as they are the source of wood and a range of other products required for daily livelihood. Wood has remarkable properties which have made it a priced commodity to humanity since centuries (Sutton 1999). Moreover, it is well known that actively growing plants are important sinks for atmospheric carbon dioxide. Wood is an important resource contributing to the world economy, but the pressures of human development and a growing demand for wood are contributing to the degradation of natural forests worldwide, creating a dilemma over its future supplies (Boyle 1999). The best way to meet the ever growing demand of wood is to grow trees as agro forestry. Plantation forests have the potential to supply the bulk quantities of wood for a long-term basis and so to reduce the harvest pressures on natural forests. However, if these forests are to be successful, these must have higher yield than their natural counterparts, on much shorter rotation times (Fenning and Gershezon 2002). Plantation forestry, either of introduced or indigenous species, involves the use of genetically improved planting materials. But, most of the planting stock material originates from unknown seed origin (Yasodha *et al.* 2004). Furthermore, traditional breeding approaches are a long-term process to produce propagules of high quality. In such genetically diverse stocks, trees with better combinations of characteristics occur at very low frequencies. Due to extensive cross-pollination, seedlings of these superior trees display a lot of heterogeneity amongst seed raised populations (Pena and Seguin 2001). Thus for further improvement of selected elite clones and subsequent multiplication of modified plants of tree species various modern biotechnological tools like micropropagation and genetic transformation of tree species need to be applied. By the use these technologies not only mass multiplication of selected plants can be taken up but, some major bottlenecks in tree breeding, such as juvenile phases and segregate evaluations, could be avoided (Pena and Seguin 2001).

Eucalyptus tereticornis is a versatile member of the genus *Eucalyptus* and the species ranks among the most extensively planted *Eucalyptus* in the tropics and subtropics, which includes most of the part of India as most productive forest crop (Sharma and Ramamurthy 2000; Evans 1992). Its popularity, as that of other widely planted eucalypts is attributed to its rapid growth and production of desirable wood when grown in a wide range of environmental and soil conditions (Zobel *et al.* 1987). It is grown over a several parts of the world to provide wood for fuel, poles, construction timber and raw material for pulp. During past twenty years, large-scale plantation of *Eucalyptus*, as a fast growing exotic, has been taken up in India, as part of a drive to reforest the subcontinent, and create an adequate supply of fuel and timber for rural communities under the augur of 'social forestry' (Chezhian *et al.* 2010; Lal *et al.* 1993). Besides, a source of fuel and timber, it is commonly used is as a raw material for pulp and paper industry. *Eucalyptus* pulp has been used for the manufacture of paper and fibre board, due to its suitability for making high grade of paper with high opacity, resistance to collapse and intrinsic fibre stiffness, smoothness, etc., which makes paper usable for many important applications (Santos 1997; Lal *et al.* 1993).

Due to production of high grade paper from *Eucalyptus* pulp, there is great demand of *Eucalyptus* by the pulp and paper industry. Moreover, *Eucalyptus* wood is cheap because of its higher growth rate and productivity. Furthermore, pulp yield is also higher as compared to other trees. For these reasons eucalypt pulps dominates the world hardwood pulp markets with the total production of 10 million tonnes/year with annual increment up to 6 % (twice as much as for pulp in general) (Patt *et al.* 2006). To meet this ever increasing demand of *Eucalyptus* pulp by the paper industry it is important to undertake clonal plantations of superior clones with a potential of higher yield. Worldwide, the estimated area covered under *Eucalyptus* plantation is ca. 20 million hectares (GIT Forestry 2008) and most of the forest plantation estates are for industrial end-use. The major problem of *Eucalyptus* wood for the pulp and paper industry is the presence of higher lignin content in its wood which is not only difficult to remove, during pulping, bust also cause a serious environmental threat due to heavy use of hazardous chemicals.

In vitro* propagation studies in *Eucalyptus

Although, most of the *Eucalyptus* species can be propagated vegetatively using traditional stem cutting techniques, yet it has several constraints such as poor rooting of stem cuttings and graft incompatibility problems (Vengadesan and Pijut 2009; Bennett *et al.* 1994). Therefore, micropropagation is becoming increasingly popular for the establishment of clonal plantations because of the prospects of rapid cloning. Importance of *in vitro* clonal propagation of *Eucalyptus* is evident from the amount of work carried out on the genus using various micropropagation techniques (Prakash and Gurumuthi 2005; Sharma and Ramamurthy 2000; Termignoni *et al.* 1996; Subbaiah and Minocha 1990; Das and Mitra 1990; McComb and Bennett 1986). Success has been achieved in raising micropropagated plants from different species of *Eucalyptus* through various methods (Dibax 2005; Mullins *et al.* 1997; Chang *et al.* 1992; Subbaiah and Minocha 1990), this opened up the possibility for large-scale clonal propagation of elite clones. Moreover, the establishment of a good regeneration system is fundamental for genetic transformation, which can only be achieved through in-*vitro* propagation (Kumar *et al.* 2004). For establishment of aseptic cultures, various plant parts such as cotyledons, hypocotyls and leaf fragments excised from *in vitro* seedlings have been used as explant

(Prakash and Gurumuthi 2005; Sharma and Ramamurthy 2000; Termignoni *et al.* 1996; Subbaiah and Minocha 1990). In some cases, aseptic cultures were also initiated from zygotic embryos (Serrano *et al.* 1996). Beside taking explants from these *in vitro* grown plantlets, explants such as coppiced shoots (Burger 1987), scion shoots (Franclet and Boulay 1982; Gonocalves 1980), epicormic shoots (Ikemori 1987) and young, vigorously growing shoots from mature trees (Rao 1988, Defossard *et al.* 1977) have also been used as explant material.

Disinfection of mature, field-grown material has proved difficult because of endogenous microbial contamination (Defossard *et al.* 1977). Age of material and season are important factors determining success in establishing aseptic cultures (Sharma and Ramamurthy 2000; Grewal *et al.* 1980). Sometime it is impossible to disinfect mature, field-grown shoots without severely damaging the tissues. But, the best sources of explants for the culture establishment are generally juvenile or rejuvenated tissues (Jones and Van Staden 1997). The organogenesis process generally easy from juvenile parts of the plant, such as cotyledons, hypocotyls and leaf fragments excised from young plantlets from germinating seeds (Groge 1996). Although, some attempts have been made to develop protocol for the shoot organogenesis/regeneration of *E. tereticornis*, clone specific work needs to be carried out as variations has been reported from one clone to another within same species (Mullins 1997). However, few reports are available on shoot regeneration studies of *E. tereticornis* (Subbaiah and Minocha 1990, Prakash and Gurumuthi 2005).

Gupta *et al.* (1983) has reported the protocol for micropropagation of *E. torelliana* and *E. camaldulensis* by taking explants from 12-15 yrs old trees. Multiple shoots were induced from nodal segments on MS (Murashige and Skoog 1962) medium supplemented with different concentration of kinetin (Kn), benzyl adenine (BA), calcium pantothenate and biotin. Incubation at 15°C with continuous illumination followed by growth in agitated liquid media was essential for shoot induction in case of *E. camaldulensis.* Whereas, in case of *E. torelliana*, culture in agitated liquid media alone was sufficient for induction of shoots. Rooting could be induced in microshoots of *E. torelliana* by treatment with α-naphthalene acetic acid (NAA) whereas treatment with a mixture of indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), indole-3-propionic acid (IPA) and NAA in dark for different time intervals was essential for *E. camaldulensis*. After auxin treatment, transfer of shoots to a charcoal-containing medium and incubation under light was necessary for root induction. Rooting occurred within 15-20 days in 70 % of the shoots of *E. torelliana* treated with NAA for 48 h. On the other hand, 50 % of *E. camaldulensis* shoots treated in dark for 72 h with a mixture of auxins containing IBA, IPA, IAA and NAA rooted within 20-25 days. Rooted plantlets thus obtained were successfully transferred to pots and field. Das and Mitra (1990) were able to achieve shoot multiplication on MS medium supplemented with 0.5 µM NAA and 4.4 µM BA using shoot tips as explants collected after coppicing of mature trees of *E. tereticornis*. These authors reported that the addition of charcoal and gibberellic acid (GA_3) to the medium was beneficial for shoot multiplication. Rooting was induced on Knop's medium (Knop 1884) supplemented with 4.9 µM IBA. The key factor in root induction was incubation in dark for a short period. Further, these authors have reported that during culture, genotypically different populations responded differently in spite of optimal growth conditions.

Subbaiah and Minocha (1990) were the first to report shoot regeneration from various explants of *E. tereticornis*. They reported the regeneration of adventitious shoots from leaf and stem callus. Callus was induced by incubating leaf or stem segments taken from seedlings on B5 medium (Gamborg *et al.* 1968) supplemented with 0.44 µM BA and 15-20 µM NAA in dark. They have used medium as reported by Smith and McCown (1983) with modifications; K_2SO_4 - omitted and containing myo-inositol -100 mg/l, thiamine - 0.1 mg/l, nicotinic acid - 0.5 mg/l), pyridoxine HCI - 0.5 mg/l and glycine - 2.0 mg/l; mWPM) containing 2.2 µM BA, 500 mg/l polyvinylpyrrolidone and 10% (v/v) coconut milk for shoot regeneration. Multiple shoots were regenerated directly from hypocotyl segments of 4 to 6 week old seedlings on B5 medium supplemented with 2.2 µM BA. Regenerated shoots were rooted on mWPM supplemented with 2.4 µM IBA with 100 % rooting efficiency and successfully transferred to soil.

Shoot regeneration protocol for *E. camaldulensis* using leaf explants taken from seedlings grown in culture have been reported (Mullins *et al.* 1997) on woody plant medium (Lloyd and McCown 1981) containing 1.0 g/l casein hydrolysate, 50 g/l sucrose, 0.5% (w/v) phytagar and supplemented with 16.1 µM NAA and 0.45 µM BA for the regeneration of shoots from leaf explants. Out of 24 clones used in the study, only 13 clones regenerated shoots. Subsequently, same protocol was used for the successful shoot regeneration from other species of *Eucalyptus* like *E. microtheca*, *E. ochrophloia*, *E. grandis* and *E. marginata*. However, frequency of regeneration varied from species to species and clone to clone within same species. The major finding of this study was the emphasis on the need to develop clone specific protocols.

Ho *et al.* (1998) were able to regenerate shoots from hypocotyls explants taken from 1-month-old seedlings of *E. camaldulensis* grown in culture. The B5 medium supplemented with 100 ml/l coconut milk, 200 mg/l glutamine and 100 mg/l casein hydrolysate was used as basal medium in this study. Successful shoot regeneration and multiplication of shoots was achieved on this modified B5 medium supplemented with 4.4 µM BA and 15 µM NAA via callusing. Microshoots were successfully rooted on modified MS medium, half-strength macronutrients and containing 4.9 µM IBA.

Barrueto Cid *et al.* (1999) has achieved shoot regeneration from *E. grandis x E. urophylla* using hypocotyls, cotyledons, cotyledonary nodes and primary leaves as explants obtained from 14-50 days old seedlings. These seedling-derived explants were incubated on a modified MS medium, supplemented with 2.0 µM thidiazuron (TDZ). The callus obtained on TDZ supplemented medium was transferred to modified MS medium supplemented with different concentrations of BA and NAA or zeatin and NAA. Shoots were induced from these calli at a high frequency on medium supplemented with 5.0 µM BA and 0.5 µM NAA. Shoot elongation was then achieved on medium supplemented with 1.0 µM BA, 0.5 µM NAA and 2.0 µM GA_3. For rooting, 50 mm long shoots were cultured on root induction medium containing 2.5 µM IBA for 5-15 days and then transferred to the basal medium for 30 days. Plantlets were then successfully transplanted to the greenhouse conditions.

Sharma and Ramamurthy (2000) have reported the micropropagation of elite clones of *E. tereticornis*. Explants (nodal segments) were collected from 4 years old trees. It was reported that March-April months were best periods for culture establishment as maximum explants showed shoot induction during this period. Shoot induction was successfully achieved on MS medium supplemented with 4.4 µM each of BA and NAA. Shoots were further elongated by lowering the concentration of both BA and NAA to 0.44 µM each. The phytagel was reported as better gelling agent then agar for shoot elongation but, induced hyperhydricity, which was controlled by increasing the concentration of calcium chloride to double. In this study better rooting response was observed on half strength MS medium supplemented with 4.9 µM IBA. Survival rate of 84 -100 % was obtained during acclimatization of plantlets in non sterile potting mix.

Parkash and Gurumurthi (2005) were able to induce indirect somatic embryogenesis from mature zygotic embryos of *E. tereticornis*. The MS and B5 basal media containing different concentrations of NAA, 2,4-dichlorophenoxyacetic acid (2,4-D) and BA were evaluated for callus induction and somatic embryogenesis. Higher frequency of both callus induction and somatic embryogenesis was observed on MS medium as compared to B5. Maximum frequency of friable callus (embryogenic callus) was obtained on MS medium supplemented with 10.74 µM NAA. When the callus was transferred to MS media containing various concentrations of BA, somatic embryos developed after 1–2 weeks with highest frequency (54%) on medium supplemented with 2.22 µM BA. The embryos were successfully germinated on basal MS medium.

Dibax *et al.* (2005) reported shoot regeneration from cotyledonary leaves of *E. camaldulensis*. Expalnts were cultured on MS medium supplemented with various combinations of NAA and BA. The best shoot regeneration frequency was observed on medium supplemented with 2.7 µM NAA and 4.44 µM BA. When explants were kept in the dark during the first 30 days, percentage of explants forming calli increased and explant necrosis was reduced. Regeneration frequency from calli further increased from 47 % to 54 % when strength of the basal medium was reduced to half. For shoot elongation, modifications were made only in basal medium by lowering the strength of ammonium nitrate and potassium nitrate to half. Addition of 0.2 % activated charcoal to above modified basal medium induced roots in microshoots after one month of culture.

Glocke *et al.* (2006) reported micropropagation of ornamental *Eucalyptus* hybrid (*Eucalyptus erythronema* var *erythronema x Eucalyptus stricklandii* cv. ‘Urrbrae Gem’) through enhanced axillary shoot proliferation. Shoot proliferation was higher on WPM and QL (Quoirin and Lepoivre 1977) medium supplemented with BA, NAA and GA_3 as compared to MS, B5, AP (Almehdli and Parfitt 1986) and TK (Tabachnik and Kester 1977) media supplemented with same composition of plant growth regulators (PGRs). In this study, WPM and QL were shown to provide better shoot growth than MS, AP, B5, and TK media. GA_3 was required for shoot elongation. Pulse treatment for 7 days at 20 µM IBA was required for induction of roots and rooting frequency further improved by lowering the strength of WPM medium to half.

Pinto *et al.* (2008) has described the factors affecting maintenance, proliferation and germination of somatic embryos in *E. globulus.* Somatic embryogenesis was recorded on MS medium supplemented with 15 µM NAA. Embryos were maintained on same medium up-to 2 years. The influence of basal medium (MS and B5), plant growth regulators (auxins and cytokinins), and light on induction of somatic embryos was studied. The MS medium without growth regulators was found to be more efficient for embryo formation and germination than the B5 medium. Reducing auxin levels increased the proliferation of globular somatic embryos. The addition of two cytokinins (BA and KIN) to the MS medium did not improve proliferation of globular secondary embryos.

Most of the above mentioned reports utilize seedlings as initial explant and no report on *in vitro* propagation of *E. tereticornis* through shoot regeneration or somatic embryogenesis from selected elite mature plants is available. It has been documented that shoot regeneration or somatic embryogenesis is difficult from mature plants as compared to juvenile tissue (Liu and Pijut 2008). However, it is important to develop direct regeneration protocol, which will help in taking up genetic manipulation work in this species. Moreover, many *Eucalyptus* species are still considered recalcitrant to tissue culture and genetic engineering. Further, reports exist on the variable regeneration ability of the different species and also amongst clones of the same species (Mullins *et al.* 1997). Therefore, there is felt need to develop clone specific micropropagation and regeneration protocols.

Clonal fidelity of micropropagted plants of *Eucalyptus*

Testing of clonal fidelity is one of the most important aspects of micropropagation industry. One of the problems encountered with the *in vitro* cultures is the presence of somaclonal variation arising as a direct consequence of *in vitro* culture of plant cells, tissues or organs (Kumar *et al.* 2010; Rani *et al.* 1995; Schoofs 1992; Swartz 1990). The extent of genetic variation resulting in tissue culture procedures depends upon the mode and length of culture. Regeneration through adventitious shoot differentiation is considered to be prone to genetic instability than axillary branching (Shenoy and Vasil 1992). However, the reliability depends upon the mode of regeneration, whether it is direct organogenesis from explants or indirect organogensis through callus phase (Rani *et al.* 1995). Moreover, the selection of the explant source has an influence on the genetic stability of regenerated plants (Kawiak and Lojkowska 2004).

Tissue culture-induced variations have been determined at the morphological, cytological, biochemical, and molecular levels with several techniques (Shenoy and Vasil 1992). Molecular markers suitable for generating DNA profiles have proved to be an effective tool in assessing the genetic stability of regenerated plants (Martins *et al.* 2004). These markers are not influenced by environmental factors and generate reliable, reproducible results (Li *et al.* 2011). DNA-based markers most frequently in use include: restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD, Williams *et al.* 1990)) and inter simple sequence repeats (ISSR, Zietjiewicz *et al.* 1994).

The RAPD and ISSR markers have proven to be efficient in detecting genetic variation. Both RAPD and ISSR markers have been successfully applied to detect the geneti€c similarities or dissimilarities in micropropagated material in many studies (Martin *et al.* 2006; Carvalho *et al.* 2004; Martins *et al.* 2004; Ramage *et al.* 2004; Sanchez *et al.*

2003). There are many reports highlighting the use of combination of two markers amplifying different regions of genome to study the genetic uniformity of *in vitro* propagated plantlets (Lattoo *et al.* 2006; Martin *et al.* 2006; Ray *et al.* 2006; Dhiman and Singh 2003; Palombi and Damiano 2002).

Perusal of literature shows that no serious attempt has been made so far in this direction although thousands of tissue-culture derived *Eucalyptus* plants have been produced for reforestation programmes and other economic benefits (Anonymous 1994; Keil and Griffin 1994; Rockwood and Warrag 1994; McComb and Bennett 1986). Their is only single report on genetic analysis of *E. tereticornis* using RAPD and ISSR markers to establish of clonal fidelity of in vitro raised plants (Rani and Raina 1998). The size of bands produced by these primers ranged from 350 to 1800 bp. Primers used produced fragments that were monomorphic across all the micropropagated plants and the corresponding single control indicating the clonal nature of plants. Tripathi *et al.* (2006) has reported the use of these markers to identity tissue culture raised *Eucalyptus* clones.

Genetic transformation studies in *Eucalyptus*

The basic approach of genetic modification in plants involves the modification of its genomic DNA by incorporation of new genes using genetic transformation protocols. Various transformation techniques have been developed to assist the transfer of recombinant DNA into recipient plant cells (Hansen and Durham 2000). These transformation techniques involve the insertion of a piece of DNA containing one or more genes from one organism into a chromosome/genome of another organism. In plants, it can be mediated either by a biological agents such as *Agrobacterium tumefaciens* (a common gram negative soil bacterium that have ability to transfer DNA fragment to host) (Tzfira and Citovsky 2006; Gelvin 2003) or by a direct gene delivery system (such as gene gun, electroporation, microinjection, lasers, polyethylene glycol

(PEG), silicon carbide fibers) that utilizes physical, electrical or chemical means to deliver gene of interest to a taget cells (Torney *et al.* 2007, Weir *et al.* 1998; Nehra *et al.* 1994).

The transfer of selected genes through transgenic technology is especially important for *Eucalyptus*, as its improvement by conventional breeding approach is limited by long breeding cycles, high levels of heterozygosity and incompatibility barriers (Machado *et al.* 1997). Literature review indicates that various methods have been tried for delivering foreign DNA into *Eucalyptus* like electroporation, biolistics and *Agrobacterium*-mediated transformations. But only few studies are successful on the development of genetic transformation protocol in *Eucalyptus* (Tournier *et al.* 2003; Ho *et al.* 1998). These studies mainly focused on the development of genetic transformation protocol using juvenile tissues of seed origin (Prakash and Gurumurthi 2009; Tournier *et al.* 2003; Ho *et al.* 1998).

The first attempt to optimize biological and physical parameters for particle gun mediated genetic transformation of *E. globulus* was carried by Rochange *et al.* (1995). Cultured zygotic embryos were used as the starting material for transformation. Based on transient GUS expression assay, they observed that both gunpowder apparatus and compressed-helium system exhibited similar transformation efficiency and reported that 6-day old cultured embryos as best explants for genetic transformation. These authors also highlighted the role osmotic pretreatment before the bombardment and were able to obtain up to 130 GUS expression events per embryo with a good distribution all over the tissue. Serrano *et al.* (1996) were the first to report successful regeneration from *E. globulus* following biolistic transformation of zygotic embryos. Histochemical GUS assay was used to confirm the presence of transgene and molecular analyses based on polymerase chain reaction and southern blot were used to confirm the integration of the corresponding DNA into the genome. Biolistic transformation was also carried on hypocotyls and cotyledons of *E. grandis* × *E. urophylla* hybrids (Sartoretto *et al.* 2002). All the above mentioned reports were able to obtain GUS-expressing calli but regeneration of transformed shoots from such calli could not be obtained. However, shoots were regenerated successfully in the control plants that were used for the biolistics based transformation but without bombardment of the tissue. Unfortunately, there are no reports of biolistic gun based transformation work on *Eucalyptus* where special efforts have been made for optimization of parameters for the successful genetic transformation.

So far, *Agrobacterium*-mediated transformation is the most adopted and successful method for genetic transformation of *Eucalyptus*. According to Mullins *et al.* (1997), this indirect gene transfer approach is preferred over biolistic mediated direct genetic transformation as it is known to reduce the insertion of multiple copies of the transgene, which can lead to multicopy gene silencing. Machado *et al.* (1997) evaluated the susceptibility of *E. grandis* × *E. urophylla* hybrids to twelve wild strains of *A. tumefaciens* and five strains of *A. rhizogenes*. Different degrees of virulence have been recorded using these strains, indicating the possibility of transforming *Eucalyptus* and its hybrids uisng *Agrobacterium*. Tumours obtained after infection showed autonomous growth when cultured on PGR-free medium and some tumours leads to the formation of shoots. This study suggested that *Agrobacterium* can be successfully used to transform *Eucalyptus* species. Further, Krimi *et al.* (2006) reported that *E. occidentalis* was more susceptible to *Agrobacterium* than *E. camaldulensis* and *E. cladocalyx*. The ability of *A. tumefaciens* to infect *Eucalyptus* was found to vary across species and genotypes thus suggesting the need to develop clone specific genetic transformation protocol.

Literature survey reveals that, *E. camaldulensis* was a favorite species for *A. tumefaciens* mediated genetic transformation (Chen *et al.* 2001; Ho et al 1998; Azmi *et al.* 1997;

Mullins et al 1997; Kawazu *et al.* 1996). The choice of this species is due to its good regeneration potential following genetic transformations. There are reports on genetic transformation of other *Eucalyptus* species like *E. globulus* (Serrano *et al.* 1996, Nugent *et al.* 2001) and *E. gunnii* (Chriqui *et al.* 1992).

Mullins *et al.* (1997) has reported the genetic transformation protocol for *E. camaldulensis* using cotyledonary leaves as explants. Five disarmed strains of *A. tumefaciens* (A6, LBA4404, GV3111, AGLI and GV3850) containing same binary vector were used in the study. These authors also studied the transformation efficiencies of different clones belonging to different species of *Eucalyptus.* They were able to obtain transformed tissue on kanamycin supplemented medium which was further confirmed by histochemical GUS assay and blotting techniques. Although, they were able to obtain transformed tissues from 5 clones of *E. camaldulensis,* but regeneration of transformed plants was achieved from one clone only. This report also highlighted the differences in transformation efficiencies among clones.

Ho *et al.* (1998) were perhaps the first to report the recovery of transgenic *Eucalyptus* plants using *A. tumefaciens*. Hypocotyl segments obtained from one month old seedlings of *E. camaldulensis* were used as explant material. Transformation was accomplished by using *A. tumefaciens* (CIB542 derived from EHA101 containing binary vector pBI121). The striking finding of this study was requirement of a prerooting treatment for successful rooting of transformed shoots as there was difficulty in inducing roots in transformed plants. This technique can also be used for other *Eucalyptus* species.

Harcourt *et al.* (2000) developed the insect and herbicide resistant *E. camaldulensis* plants These authors successfully transformed the seedling explants (cotyledons and hypocotyls) with cry3A gene from *Bacillus thruringiensis* and *bar* gene (conferring tolerance to herbicide glufosinate ammonium) using *A. tumefaciens* strain AGL-1.

Integration of transgene in transformed plants was confirmed through PCR and southern blot analysis and expression of transgene was checked using western blot. Transgenic plants thus obtained showed resistance towards ‘chrysomelid beetles’ and tolerance towards herbicide glufosinate ammonium.

Chen *et al.* (2001) were the first to report the production of transgenic *E. camaldulensis* plants carrying Cinnamate 4-Hydroxylase gene (C4H) from *Populus tremuloides*, a key enzyme involved in lignin biosynthesis. First, C4H gene was cloned in both sense and antisense orientation in binary vector pBI121 which was moved into *A. tumefaciens* strain CIB542 for transformation of plants. PCR based analysis of the genomic DNA performed to confirm the integration of the foreign gene.

Both Tournier *et al.* (2003) and Valerio *et al.* (2003) have reported the transformation of *E. grandis x E.* urophylla and *E. camaldulensis* respectively with construct carrying cinnamyl alcohol dehydrogenase (CAD) in antisense orientation. Tournier *et al.* (2003) has also highlighted the problem in regenerating the tissue following genetic transformation. To overcome this problem, they have selected the best genotype in terms of their regeneration potential out of the 250 tested clones of *E. grandis x E.* urophylla. In both the studies a strong inhibition of CAD activity was recorded. The CAD is a key enzyme involved in the lignin biosynthesis whose down regulation may result in lower lignin content in wood.

Sonication assisted *Agrobacterium* transformation (SAAT) system was used for the development of transgenic plants of *E. grandis* × *E. urophylla* hybrid (Gonzalez *et al.* 2002). The report indicated the higher percentage of transient GUS expression when explants were sonicated for 30 s and pre-sonication greatly enhanced the transformation efficiency of seedlings. Using this method, four stable transformants were generated and confirmed with southern blotting.

A close survey of literature could not reveal any report on development of genetic transformation protocol for the selected elite clone(s) of *E. tereticornis.* There is only one report on *A. tumefaciens* mediated genetic transformation of *E. tereticornis* using cotyledon and hypocotyl as explants (Prakash and Gurumurthi 2009). Precultured explants were co-cultured with *Agrobacterium tumefaciens* strain LBA 4404 harboring binary vector pBI121. Explants after infection were transferred toselection cum regeneration medium containing 2.2 µM BA, 0.5 µM NAA, 40 mg/l kanamycin and 300 mg/l cefotaxime. After two passages on the selection cum regeneration medium, the putatively transformed regenerants were transferred to MS liquid medium containing 2.2 µM BA and 40 mg/l kanamycin on paper bridges for further development and elongation. The elongated kanamycin-resistant shoots were subsequently rooted on the MS medium supplemented with 4.9 µM IBA and 40 mg/l kanamycin. Transformation was confirmed using PCR and southern blotting.

So, there is a need for the development of genetic transformation protocol (s) for the selected elite clones of *E. terticornis.* The transformation of selected clones will prove to be powerful tool for trait specific modification of existing clones with desirable traits, such as insect and herbicide resistance, male-sterility and reduction of lignin content (Haines 1994; Teulières *et al.* 1994).

Cellulose and its biosynthesis

A distinguishing feature of plant cells is the presence of cell wall, which is required not only for its structural traits but also for its many functional properties. Plant cell walls are not homogeneous - they are a complex mixture of polysaccharides and proteins (Brown *et al.* 1996). It consists mainly of lignin and polysaccharides like cellulose, hemicellulose and pectic substances. Cellulose has remarkable mechanical properties and shows a tensile strength comparable to that of steel. Cellulose is the most abundant polysaccharide in plants and provides the framework for all plant cell walls. It consists of β-1,4-linked glucan chains with consecutive glucose residues rotated 180° to the next, making the cellobiose as the repeating unit. The completed and extended glucan chains form a flat ribbon structure and these multiple parallel chains are held to form microfibrils by hydrogen bonds with higher degree of crystallization and increased insolubility in water, and to form structurally strong framework in the cell walls of plants. Cellulose is universally present and beneficial to human beings, either in their daily lives or in various industries. Its importance is reflected in its use as raw materials in textile, pulp-paper and forest product industries (Somerville 2006; Delmer 1999).

In plants cellulose has been reported to be synthesized at rosette like structures which consist of 36 hexagonally arranged protein subunits (cellulose synthases) embedded in plasma membrane (Williamson *et al.* 2001). Due to glycosyl transferase activity of cellulose synthase, the protein catalyzes the formation of β-1,4 bonds between the substrate molecules, UDP glucose (Saxena *et al.* 1995). After it is transported into the plant cell wall through the cell membrane, cellulose is reportedly assembled into microfibrils by association of different glucan chains due to inter and intra chain hydrogen bonds (Plomion *et al.* 2001). Cellulose synthase proteins have been identified only component of terminal enzyme complexes so far responsible for cellulose biosynthesis in plants (Mutwil *et al.* 2008; Taylor 2008; Joshi and Mansfield 2007; Somerville 2006; Delmer 1999). The first *CESA* gene from plants was identified from cotton fibre cDNA library through homology with that of bacterial *CESA* genes (Pear *et al.* 1996). The release of *Arabidopsis* genome showed that *Arabidopsis* has about 10 *CESA* genes encoding highly conserved proteins (Holland *et al.* 2000, Richmond 2000, Richmond and Somerville 2000). Poplar has 18 *CESA* genes (Kumar *et al.* 2009), Maize has at least 12 *CESA* genes (Ching *et al.* 2006) and barley has at least 8 (Burton *et al.* 2004).

The CESA proteins are ~1000 amino acids in length and found to carry eight putative transmembrane domains (Joshi *et al.* 2004). Two of these domains are near the N-terminus, and the others are clustered close to the C-terminus. The N-terminal region of each CESA protein has two zinc binding domains (Kurek *et al.* 2002), which have been implicated in regulating protein protein interactions in protein complexes (Saurin *et al.* 1996). The large central domain is highly conserved among all plant CESA proteins with signature motif D, D, D, Q/RXXRW (Kumar *et al.* 2009; Somerville 2006; Brown 1999; Delmer 1999). The linkage of cellulose synthases with termini of microfibrils in shown in freeze fractured samples of plasma membranes of many organisms with the help of electron microscope (Brown 1996, Brown *et al.* 1996) and this was further confirmed by immuno gold labeling methods (Kimura *et al.* 1999).

Recently, significant progress has been made in understanding the mechanism of cellulose biosynthesis, especially in the model plants *Arabidopsis thaliana*, *Populus trichocharpa* and some other plants (Mutwil *et al.* 2008; Taylor 2008; Joshi and Mansfield 2007; Somerville 2006; Doblin *et al.* 2002). It has now become more evident that the major components required for making cellulose are conserved in higher plants, especially from the results of those whole genomes sequenced in model plants including *Arabidopsis,* poplar and rice (Maloney *et al.* 2011). Due to the shortage of fossil fuels and the concerns of global climate change, increased attention has been focused on developing new and more cost-effective techniques to convert plant cell walls to alternative biofuels (Han *et al.* 2007; Ragauskas *et al.* 2006). Moreover, to meet the ever increasing demand of cellulose by pulp and paper industry a clear understanding of the biosynthetic pathway is required. However, the current major bottleneck in such efforts is our limited understanding of the entire process of cellulose synthesis including its deposition and regulatory mechanism. Nonetheless, the clear understanding of the mechanism of cellulose biosynthesis would facilitate the future genetic manipulation of cellulose synthesis aiming at its improved convertibility to biofules and the highest content in biomass.

Other proteins in cellulose synthase complex

Recent studies have highlighted the involvement of membrane-anchored endo-β-1,4-glucanases in cellulose biosynthesis in plants as earlier observed in *A. tumefaciens* and other bacteria which require endo-β-1,4-glucanases for cellulose synthesis (Molhoj *et al.* 2001). Genes encoding endo-β-1,4-glucanases (EGases, EC 3.2.1.4) have been found in many prokaryotic and eukaryotic organisms. EGases hydrolyse β-1,4-linkages behind unsubstituted glucose residues and was operationally defined by their ability to hydrolyse the artificial soluble cellulose derivatives carboxymethyl cellulose (CMC) or hydroxyethyl cellulose (HEC) (Brummell *et al.* 1994; Henrissat *et al.* 1989).

One such important endo-1,4-β- D-glucanase is thought to be a plasma membrane bound *Korrigan (KOR)*, required for synthesis of ordered, cellulose–hemicellulose network (Sato *et al.* 2001; Nicol *et al.* 1998). All higher plant species has been reported to express multiple EGases (Henrissat *et al.* 2001) and unlike microbial cellulases, plant cellulases act individually and catalyze limited hydrolysis of cellulose and sometimes, other non-crystalline glycans (Urbanowicz *et al.* 2007; Yoshida *et al.* 2006; Master *et al.* 2004; Ohmiya *et al.* 2003; Park *et al.* 2003; Molhoj *et al.* 2001; Ohmiya *et al.* 2000). Most plant cellulases belong to the glycoside hydrolase 9 (GH9) family, which play diverse roles in plants and have been found to be associated with wall disassembly, while others have been found in rapidly growing cells and are thought to promote wall loosening (del Campillo 1999, Rose and Bennett 1999, Molhoj *et al.* 2002).

Arabidopsis KOR gene encoded a unique membrane-anchored KOR protein of 622 amino acid residues containing eight potential *N*-glycosylation sites (Maloney and Mansfield 2010). The *N*-terminal stretch of 100 amino acids is uniquely conserved only in this subfamily of EGases from *Arabidopsis*, rice, *Brassica* and tomato (Molhoj *et al.* 2002). In a phylogenic tree of EGases, KOR enzymes was found to form a separate clade that lacks cleavable signal peptide needed for targeting to endoplasmic reticulum but possess a transmembrane domain at the *N*-terminus, suggesting that it might be an integral membrane protein (Molhoj *et al.* 2002). Further, several authors independently showed that, three allelic mutants of *KOR* gene (*acw*, *rsw2* and *kor*) were severely deficient in cellulose content of primary walls (His *et al.* 2001; Lane *et al.* 2001; Sato *et al.* 2001). Thus, the role of *KOR* gene in cellulose synthesis during primary cell wall formation was established.

Recently, a special co-operation between cellulose synthases that synthesise cellulose and cellulases that digest cellulose was discovered, which appears to be necessary for successful cellulose synthesis (Molhoj *et al.* 2002). It was speculated that a special type of cellulase, might be a part of the rosettes that make cellulose in plants (Read and Bacic 2002). The endo-1,4-β-D-glucanase *Korrigan*1 (KOR1) was found to be essential enzyme for cellulose synthesis, as shown by the severely cellulose-deficient phenotype (Molhoj *et al.* 2002). Despite the fact that an endo- 1,4-β-D-glucanase is also required for cellulose synthesis in bacteria (Molhoj *et al.* 2002), the exact function of KOR1 during cellulose synthesis in plants were not clear. On the other hand, KOR1 is thought to be involved in the removal of non crystalline glucan chains and/or in relieving tensional stress, which presumably is generated during the assembly of the large number of glucan chains into a microfibril (Joshi *et al.* 2004). In contrast to other endo-1,4-β-D-glucanases, KOR1 was reported an integral type II membrane protein.

Peng *et al.* (2002) has further, proposed that the role of KOR may be to cleave the sitosterol glucoside (SG) primer, which has been reported to act as primer for synthesis of new glucan chains. The only problem with this proposal is that the catalytic domain of KOR is predicted to be outside the plasma membranes while catalytic domains of CESA proteins are located towards cytoplasmic side (Somerville 2006). Doblin *et al.* (2002) have also discussed at length the speculative nature of SG primer model. Molhoj *et al.* (2002) proposed an alternative scenario in which KOR functions as an editor of elongating glucan chain outside the plasma membrane by removing defective glucan chains from the microfibril assembly. In either case, KOR does play some important role in cellulose biosynthesis, although the exact mechanism needs to be deciphered. Therefore, how KOR participates in cellulose biosynthesis is still an open question.

All *Arabidopsis* KOR mutations studied till date were associated with primary wall formation but, from the wood industry perspective, secondary wall is more important. Szyjanowicz *et al.* (2004) recently associated mutated *KOR* gene as a cause of *irx2* mutation in *Arabidopsis* that exhibits cellulose deficiency in the secondary wall of xylem cells, resulting in irregular xylem cells.

KOR was reported to play a critical role in the determination of crystallinity of the cellulose, a key property determining the quality of paper. Overexpression of the *PttKOR* (Hybrid of *Populus tremula* L *x tremuloides* Michx.) in *A. thaliana* led to lower cellulose crystallinity (Takahashi *et al.* 2009). Further, suppression of *KOR* gene by RNA interference (RNAi) in hybrid poplar (*Populus alba x grandidentata; PaxgKOR*) leads to increase in the crystallinity of cellulose in the plants (Maloney & Mansfield 2010).

Although a number of *KOR* genes from dicotyledon species have so far been cloned. Joshi *et al.* (2004) recently cloned the first *KOR gene* from aspen xylem cDNA library. The aspen *KOR*, *PtrKor1* cDNA is 2563-bp long with 263 bp at 5' and 423 bp at 3' are untranslated region (GenBank accession no AY535003). The coding region of 1860 bp encodes a protein of 620 amino acids which shared 74-90% sequence similarity with other known KOR proteins. It has one predicted transmembrane domain near the *N*-terminus and many conserved motifs such as two putative polarized targeting sequences, LL and YXXÖ in the cytoplasmic tail and two cellulase signature motifs near the C-terminus (Zuo *et al.* 2000). Park *et al.* (2003) has over expressed another poplar EGase gene in *Arabidopsis* that resulted in larger size transgenic plants. These authors have over expressed aspen *Kor* cDNA under constitutive promoter and xylem specific promoter in transgenic aspen and tobacco to study the effects of such genetic manipulation on biomass production as well as cellulose biosynthesis.

A number of studies examining the function of these membrane-bound endoglucanases have been undertaken in an attempt to elucidate the role(s) of these enzymes in cell wall assembly and more generally the overall physiology of plants. While none of these studies has been able to reveal the mechanism of *KOR* function, the results from these studies do indicate that plants, regardless of species, possess one particular membrane-bound endoglucanase that appeared to have similar functionality (Maloney & Mansfield 2010; Takahashi *et al.* 2009; Bhandari *et al.* 2006; Robert *et al.* 2005; Master *et al.* 2004). Cellulose, in contrast to starch, is essentially an irreversible sink (Haigler *et al.* 2001). Cellulose is produced from the precursor uridine diphospho-glucose (UDP-glucose), which can be formed via two potential pathways. UDP-glucose can be derived from the cleavage of sucrose in a reaction catalysed by sucrose synthase (SUS) yielding UDP-glucose and fructose. Alternatively, UDP-glucose can be generated from the phosphorylation of glucose-1-phosphate in a reaction catalysed by UDP-glucose pyrophosphorylase (UGPase) (Coleman *et al.* 2006). Sucrose synthase has been considered to be one of the most important player in cellulose synthesis process as it provides UDP-glucose as substrates. Further the reaction catalyzed by SUS has an energetic advantage over that of UGPase as it retains the glycosidic bond for use in cellulose formation (Coleman *et al.* 2009).

SUS has been characterized as existing both in the cytosol and in association with the plasmalemma (Carlson and Chourey 1996), with the latter membrane-associated form was hypothesized to provide UDP-glucose directly to the cellulose synthase complex (Amor *et al.* 1995) through metabolic channelling. Direct channelling would facilitate the recycling of UDP into UDP-glucose, as well as ensure the availability of UDP-glucose to the cellulose synthase complex despite demands from other pathways. Salnikov *et al.* (2001, 2003) showed that the SUS was specifically localized in and around the plasma membranes and the microtubules during the secondary wall thickenings, and thus establishing a spatial relationship and context with secondary wall cellulose synthesis. SUS enzyme activity was localized in roots of wheat in response to hypoxic conditions, and was linked with the secondary wall thickenings and the deposition pattern of cellulose (Albrecht and Mustroph 2003).

Cells expressing plant-specific sucrose synthase exhibit enhanced cellulose production in the bacterium *Gluconacetobacter* / *Acetobacter xylinum* (Nakai *et al.* 1999). Conversely, down regulation of sucrose synthase expression by antisense mRNA causes a reduction of both cellulose and starch biosynthesis in transgenic carrot plants (Tang and Sturm 1999). Similarly, initiation and elongation of cotton fiber cells were inhibited when sucrose synthase expression was repressed (Ruan *et al.* 2003). However, an *A. thaliana* quadruple mutant of *sucrose synthase* genes, in which sucrose synthase activity is down-regulated in soluble protein, shows no difference in cellulose content in stems when compared with wild-type *A. thaliana* plants (Barratt *et al.* 2009).

The model for SUS-mediated cellulose synthesis suggests that sucrose is the preferred substrate for cellulose synthesis, at least during secondary wall deposition, as was directly demonstrated in cotton fibers (Pillonel *et al.* 1980, Amor *et al.* 1995). Therefore, the availability of sucrose in the cell would affect the rate of cellulose synthesis. Down regulation of SUS has also been associated with the reduction in crystalline cellulose synthesis in transgenic plants strongly suggesting that it could be one of the key enzymes involved in cellulose biosynthesis (Haigler *et al.* 2001; Tang & Strum 1999).

In view of the importance of *KOR* and *SUS* in cellulose biosynthesis, simultaneous up regulation of *KOR* and *SUS* genes in transgenic trees may result in increased biomass as well as improved production of wood with better quality cellulose that is typically present in the secondary walls of xylem in woody tissues (Joshi *et al.* 2004).

Chapter -3

Materials and Methods

Plant Material

Elite clones of *Eucalyptus tereticornis* Sm. growing at Thapar University Campus, Patiala were used during this study. These plants were selected because of higher biomass productivity and self pruning ability. Aseptic cultures were established using nodal explants from freshly coppiced plants of *E. tereticornis* (10 years old). Actively growing shoots (30 – 40 days old) were the source of nodal segments.

Molecular cloning of *korrigan* and *sucrose synthase* genes was carried out from single plant of *Populus deltoides* W. Bartram ex Marshall (clone G-48) growing in the plantation area of Thapar Univresity Campus, Patiala. These plants were procured from Punjab Agriculture University, Ludiana and established in the plantation area of Thapar University, Patiala.

Chemicals, glassware and plasticware

All routinely used chemicals (AR Grade) were purchased from HiMedia Laboratories, Mumbai, India. Growth regulators, antibiotics and other fine chemicals like cetyl trimethyl-ammonium bromide (CTAB), tris base, EDTA, agarose etc. (Molecular Biology Grade) were procured from Sigma Chemical Co. (St Louis, MO, USA). Taq DNA Polymerase was procured from Larova (Teltow, Germany) and Restriction enzymes, T4 DNA Liagse other related products were procured from MBI Fermentas, India. Plasticware such as sterile disposable filter sterilization units were purchased from Merck Millipore (Merck Specialities Pvt. Ltd., Mumbai, India) and petri dishes, microfuge tubes, microtips, measuring cylinders, beakers etc. were procured from Tarsons Products Pvt. Ltd. (Kolkata, India). Glassware such as conical flask, measuring cylinders etc were procured from Borosil Glass Works Ltd. (Mumbai, India). Glass culture bottles of 300 ml capacity were procured from Kasablanka Corporation, (Mumbai, India).

Preparation of culture medium and stock solutions

Murashige and Skoog medium (Murashige and Skoog 1962) 58 mM sucrose and gelled with 0.7 % agar (w/v) (basal MS medium) was used for tissue culture experiments (Table 3.1). Various plant growth regulators (PGRs) like 6-benzyladenine (BA), 6-furfurylaminopurine (Kn), thidiazuron (TDZ), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), α-naphthalene acetic acid (NAA) and 2,4-dichlorophenoxy acetic acid (2,4-D) were added to the medium in various concentrations and combinations. The concentrated stock solutions of all the ingredients (macronutrients, micronutrients, vitamins) were prepared individually, which are then used to prepare the medium (Table 3.1). Stock solutions of all plant growth regulators (PGRs) in concentration of 2.5 mM were prepared by dissolving them in respective solvents (1N HCL, 1N KOH/NaOH, 70 % ethanol, DMSO etc.) and finally volume was made up using Milli Q water. All the stock solutions were kept under refrigeration (~ 4 ^{o}C). MS medium was prepared from the stocks and used for the tissue culture studies unless otherwise specified. The pH of medium was adjusted to 5.8 with 1N KOH or 1N HCl using pH meter (Cyberscan 510, Eutech Instruments, Singapore) before autoclaving. After preparation of medium it was dispensed (50 ml) into 300 ml glass culture bottles (Kasablanka, Mumbai). After dispensing the medium agar (0.7 %; w/v) was added to the individual culture bottle and medium was sterilized in an autoclave (121 °C; 15 psi; 20 min, Equitron, Mumbai, India). Stock solution of antibiotics such as kanamycin, cefotaxime etc. were prepared in required concentration and filter sterilized using disposable sterile filters of 0.22 µm pore size (Merck Millipore, India) and were stored at -20 ^{0}C in deep freezer (Vest frost, India).

Table 3.1 Composition of Murashige and Skoog (MS,1962) medium

Sr. No.	Components	Concentration (mg/L)
1.	KNO_3	1900.0
2.	NH_4NO_3	1650.0
3.	$MgSO_4.7H_20$	370.0
4.	$CaCl_2.2H_2O$	440.0
5.	KH_2PO_4	170.0
6	H_3BO_4	6.20
7	$MnSO_4.4H_2O$	22.30
8	$ZnSO_4.7H_2O$	8.60
9	$Na_2MoO_4.2H_2O$	0.25
10	$CuSO_4.5H_2O$	0.025
11	$CoCl_2.6H_2O$	0.025
12	KI	0.83
13	*Nicotinic Acid*	0.5
14	Pyridoxine HCl	0.5
15	Thiamine HCl	0.1
16	Glycine	2.0
17	Myo-inositol	100.0
18	FeEDTA. $2H_2O$ (sodium salt)	30.0
19	Sucrose	3000.0

(pH of the final medium was adjusted at 5.8 with 0.1N HCl or 0.1N KOH, pH was adjusted after addition of PGRs)

Culture/ growth conditions

Unless otherwise mentioned, cultures were incubated at 25±1 °C under cool white fluorescent lights (CFL) (Philips India Ltd, Mumbai) with the light intensity of 42 μmol m^{-2} s^{-1} inside the culture vessel in 16 h light/8 h dark cycle.

Preparation of explants and establishment of aseptic cultures

For establishment of aseptic cultures, nodal segments were collected after coppicing of elite plant of *E. tereticornis* (10 years old). First of all young actively growing freshly coppiced shoots (30 – 40 days old) were collected and leaves were removed from the shoots. Shoots were then excised into smaller segments (each piece with 2 nodes) to facilitate proper cleaning during the disinfection. After excision, the explants were washed thoroughly under running tap water for 20 min followed by washing with liquid detergent (Rankleen, Ranbaxy Lab. Ltd., India) for 10 min. The explants were again washed thoroughly under running tap to remove all traces of detergent. After initial washings explants were treated with Bavistin (50 %; w/w, Carbendazim wettable powder, BASF India Limited) solution (0.2 % w/v) for 30 min. These were then washed thoroughly with distilled water. Subsequent operations were carried out in a laminar flow cabinet under aseptic conditions. These were then taken for surface disinfection, which was carried out by treating explants with an aqueous solution of mercuric chloride (0.1 %, w/v) containing few drops of Tween-20 for 5-6 min depending upon the hardness/softness of explant. Explants were then washed with sterile distilled water (4 times equal volume) till the traces of disinfectant were removed. Following disinfection, the exposed ends of nodal segments were trimmed with the help of sterile forceps and scalpel fitted with sterile surgical blade on cool sterile glass plate (autoclaved and flamed with rectified spirit prior to use) and then the explants were placed vertically on MS medium variously supplemented with different concentration of BA (0.0-5.0 µM) and NAA (0.0-1.0 µM) as indicated in results. Initially cultures were sub-cultured on fresh medium at every seven days interval for three subculture cycles and subsequently these were subcultured on same medium at 4 weeks interval until emergence of shoots from explants. The actively growing shoot cultures were maintained on MS medium supplemented with 2.5 µM BA and 0.5 µM NAA and further used for micropropagation, regeneration and genetic transformation experiments.

Shoot proliferation and elongation

After successful establishment of aseptic cultures, shoot clumps of 15-20 shoots (0.3- 0.5 cm, three shoot clumps with 15-20 shoots per clump) were cultured on MS medium variously supplemented with (0.0-5.0 µM) BA, KIN or TDZ in combination with 0.5 µM NAA to study their effect on shoot multiplication and shoot elongation.

Effect of inoculum size on shoot proliferation and elongation

The effect of inoculum size (initial no. of shoots inoculated at sub-culturing stage) was studied taking two different size of shoot clumps (15-20 shoots per clump and 4-5 shoots per clump). These shoot clumps were cultured on MS medium supplemented with BA (2.5 µM or 0.1 µM) along with 0.5 µM NAA. Three shoot clumps per culture bottle were used in case of clumps size of 15-20 shoots and 10 shoot clumps per bottle were cultured in case of clumps size of 4-5 shoots. Total number of shoots cultured in each bottle was kept approximately same in both the cases.

Effects of light source

Shoot multiplication and growth

Shoot clumps of size 15-20 shoots were cultured on MS medium supplemented with BA (2.5 µM or 0.1 µM) along with 0.5 µM NAA for shoot proliferation and shoot elongation respectively and incubated either under photosynthetically active radiations (PAR, Lichtfarbe 77 fluora, Germany) or cool white fluorescent lights (CFL, Philips India Ltd, Mumbai). The light intensity obtained under both conditions was 42 µmol m^{-2} s^{-1} inside the culture vessel. The cultures were scored for average number of shoots proliferated, number of shoots elongated and shoot length.

Chlorophyll content and osmotic potential

The cultures were incubated under the respective light sources for four weeks and the samples were taken from the cultures and analyzed for chlorophyll content and osmotic potential.

Determination of Chlorophyll content and osmotic potential

<u>*Chlorophyll content:*</u> Tissue samples (200 mg) were ground with mortar and pestle in chilled aqueous acetone (80 %, v/v) and centrifuged (10000 x *g*, 20 min). The pellets were further re-extracted with 80 % (v/v) aqueous acetone and centrifuged (10000 x *g* for 20 min). Supernatants were combined, the volume was made up to 20 ml in glass test tube and absorbance was recorded at 663 nm and 645 nm using a UV-VIS spectrophotometer (U-2900, Hitachi, Tokoya, Japan). Total chlorophyll content was calculated following the method of Arnon (1949) as described below:

Chlorophyll a: [12.2 x (Abs. at 663) – 2.81 x (Abs. at 645)] x 50

Chlorophyll b: [20.13 x (Abs. at 645) – 5.03 x (Abs. at 663)] x 50

Total chlorophyll: Chlorophyll a + Chlorophyll b

<u>*Osmotic potential:*</u> Osmotic potential (Ψs) was measured with vapour pressure osmometer (Wescor 5500, Logan, Utah, USA) following the method of Hernandey-Sebastia *et al.* (1999). In brief, fresh tissue (500 mg) was placed in a 5 ml syringe and immediately frozen at -20 ^{0}C. Samples were thawed and about 50-60 µl of sap was collected by squeezing the tissue inside the syringe. The sap was centrifuged (10000 x g; 30 min at 4 ^{0}C) and 10 µl was used for each measurement. The values of Ψs in molarity (obtained from osmometer) were transformed to MPa using Van't Hoff empiral relationship Ψs = -CiRT, where C is the concentration of the solution expressed in morality (moles of solute per kg of water), i is an ionization constant which is assumed to

be one, R is a gas constant (0.00831kg MPa mol^{-1} K^{-1}) and T is absolute temperature (K = ^0C + 273).

Rooting of microshoots and acclimatization of plantlets

Elongated shoots were excised from clumps just below the node, leaves were removed from lower nodes and microshoots were cultured on MS medium (Full strength, 1/2 strength and 1/4 strength) supplemented with different concentrations of NAA, IAA or IBA (0-5 µM). Similarly, effect of PAR and CFL light source was also evaluated on rooting efficiency of microshoots cultured on 1/4 MS medium supplemented with 5 µM IBA. Acclimatization of plantlets was carried out in polyhouse with controlled temperature (25-28 °C) and humidity (90-95 %). Plantlets were planted in a mixture of soil and agropeat (3:1 ratio w/w) in polythene bags and kept in polyhouse. During the initial periods 90 % relative humidity was maintained and slowly it was reduced to 40 % over a period of one month.

Biological hardening

Two antagonistic bacterial isolates, *Bacillus subtilis* and *Pseudomonas corrugata* isolated from the rhizosphere of tea plants growing in different geographical locations in India, were tested as microbial inoculants for hardening of microshoots of *E. tereticornis*. Broth cultures of bacterial isolates were raised on Luria broth. Mixture of autoclaved soil and agropeat (3:1 w/w) was filled in polythene bags of 7 cm dia, 8 cm ht. Tissue culture raised *E. tereticornis* plants were then transplanted (one per bag) in polythene bags. After transplantation, bags were inoculated with 1.0 ml of bacterial suspension of *B. subtilis* and *P. corrugate* containing 10^4 to 10^5 cells ml^{-1}. Both bacterial isolates were obtained form Dr. Anita Pandey, Scientist- E, G. B. Pant Institute of Himalayan Environment and Development Kosi-Katarmal, Almora 263 643, India. A total of three treatments were considered, (1) control: plants transferred to soil without bacteria and inoculated with broth only, (2) plants transferred to soil inoculated with *B. subtilis*, (3) plants transferred to soil inoculated with *P. corrugate.*

Development of shoot organogenesis protocol for *E. tereticornis*

Development of an efficient regeneration protocol (through shoot organogenesis) is a prerequisite for the development of an efficient genetic transformation protocol. Following factors were studied to obtain successful shoot regeneration in *E. tereticornis*.

Effect of plant growth regulators

Young, expanded leaves (1.5–2 cm) from microshoots maintained on MS medium supplemented with 2.5 μM of BA and 0.5 μM of NAA were taken as explants to induce shoot organogenesis. Leaves were cut transversely along the midrib and 3-4 mm segments were inoculated on MS medium supplemented with various concentrations of BA (0.0-12.5 μM) and/or 2,4-D (0.0-5.0 μM) or NAA (0.0-0.5 μM) with their adaxial surface facing the medium. In another experiment, leaf explants were inoculated on MS medium medium supplemented with NAA (0- 20 μM).

Effect of leaf maturity

The shoot organogenesis response was compared amongst leaves of different maturity level. Leaves of different maturity level (3rd to 7th leaf from top) were taken and inoculated with their adaxial surface down on MS medium supplemented with BA (5.0 μM) and 2,4 – D (1.0 μM).

Effect of antibiotics on shoot regeneration

The effect of various antibiotics namely, cefotaxime, carbenicillin and cephalexin (0-500 mg/l) was also tested on shoot organogenesis from leaf segments. Antibiotics (Filtered sterilized, Stock 200 mg/ml) were added to the medium supplemented with BA (5.0 μM) and 2, 4 – D (1.0 µM) after autoclaving. Leaf segments (5th leaf from top) were used as explants for this experiment and inoculated with their adaxial surface facing the medium.

Clonal variation in shoot regeneration

Shoot organogenesis potential of three different selected clones of *E. tereticornis*, namely 'T1', 'CE2' and 'Y8' was also compared. The segments of the 5th leaf (explant/s) with their adaxial surface facing the medium were inoculated on MS medium supplemented with BA (5.0 µM) and 2,4 – D (1.0 µM).

Histological analysis/studies

Histological studies was carried out by fixing the plant tissues (after 7-8 weeks of incubation on MS medium supplemented with 5.0 µM BA and 1.0 µM 2,4 – D) in FAA (formaldehyde acetic acid 50 % alcohol; 5:5:90; v/v) for seven days. Thereafter, the specimens were washed for 3-4 times with distilled water. Dehydration of the explants was carried out by passing them through *t*-butyl alcohol series. This was followed by embedding in paraffin wax (melting point 58-60 °C). Sections of 10 µm thickness were cut using a rotary microtome (RP-30, Radical Instruments, Ambala Cantt, India)**.** The slides were then stained with a Safranine and Fast Green combination and mounted in D.P.X following the method of Johansen (1940). Histological sections mounted on slides were observed and photographed under a microscope (Nikon Eclipse 50 i, Nikon, Japan).

Procedure for slide preparation for histological studies (Johansen 1940)

<u>*Fixing and preservation of tissue:*</u>

Plant material was fixed in FAA (formaldehyde acetic acid 50 % alcohol; 5:5:90; v/v) for one week and subsequently tissue can be stored in 70 % ethyl alcohol. Dehydration was carried out in tertiary butyl alcohol (TBA) series as given below

Sr. No.	Rectified alcohol (ml)	t- butyl alcohol - TBA (ml)	Water (ml)	Time (h)
A	30	20	50	3-4
B	50 + few drops of Eosin	20	30	3-4
C	50	35	15	3-4
D	45	55	-	3-4
E	25	75	-	3-4
F	-	100	-	3-4

To each grade material is kept for 3-4 h, at C material can be kept for maximum of 24 h

Waxing

Material was kept in oven at 60 ºC and slowly added pellets of wax (Himedia Laboratories Pvt. Ltd., Mumbai) to the material in TBA. So that, TBA is replaced with wax, material is kept in the oven till the smell of TBA gets disappeared.

Block Making

Block of the material is made in wax in proper orientation.

Section cutting

10 µM section s were cut with help of microtome

Mounting and stretching

Sections were stretched in water over a hot plate at 60 ºC and allowed to dry at room temperature for 5-6 days.

Dewaxing

Slides were dipped in pure xylol for 1-2 h (till the wax is dissolved) and then theses were kept in each of the following grade for 2-3 min unless otherwise specified

Sr. No	xylol (%)	Alcohol (%)	Clove oil (%)	Water (%)
1	75	25	0	0
2	50	50	0	0
3	25	75	0	0
4	0	100	0	0
5	0	90	0	10
6	0	75	0	25
7	0	50	0	50
8	0	25	0	75
9	--	Safranin (2%, w/v) in 25 % ethanol	0	--
10	0	25	0	75
11	0	50	0	50
12	0	75	0	25
13	0	95	0	05
14	0	Absolute-I	0	0
15	0	Absolute-II	0	0
16	0	75	25	0
17	0	50	50	0
18		Fast green (0.1-0.5 %, w/v) in 50 % ethanol and 50 % clove oil (a dip)		
19	50	0	50	0
20	75	0	25	0
21	100	0	0	0
22	100	0	0	0

After dewaxing, sections were mounted in DPX and observed under microscope.

Testing clonal fidelity using RAPD and ISSR markers

Clonal fidelity of both micropropagated and regenerated plants were checked using RAPD and ISSR markers. For establishment of clonal fidelity following procedure was followed:

Isolation of Plant Genomic DNA

Genomic DNA was extracted from leaves of mother plant and randomly selected micropropagated and regenerated plants following 12 weeks of acclimatization using CTAB method (Doyle and Doyle 1990):

- 3.0 g of fresh tissue (Leaves) was taken, cleaned by washing with sterile distilled water; Leaves were cut into smaller pieces and grounded in liquid nitrogen in an autoclaved pestle mortar to make fine powder. Powder was immediately transferred to 50 ml centrifuge tubes containing pre heated CTAB DNA buffer (2 % CTAB, 20 mM EDTA, 100 mM Tris HCl, 1.4 M NaCl, 0.2 % beta-mecaptoethanol, pH- 7.5- 8.0) to make slurry and added 400 µl 2-mercaptoethanol. Tubes were than incubated at 65 ºC for 1 h in water bath with intermittent shaking.
- To extract added equal volume of chloroform: iso-amyl alcohol (24:1), mixed for about 3 min and samples were centrifuged (5000 x *g*; 10 min; RT).
- After centrifugation, aqueous phase was removed with wide-bore pipette (cut off blue tip) to the clean fresh centrifuge tube and DNA was precipitated with 0.66 vol. of cold isopropanol.
- DNA precipitates were sedimented by centrifugation (10000 x *g*; 15 min; RT)
- Supernatant was discarded and pellet was dissolved in 1 ml TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) and solution was shifted in 2.0 ml capacity microfuge tube.

- To DNA solution, added 2 µl RNase solution (10 mg/ml stock) and incubated at 37 ºC for 1 h for removal of any contaminating RNA.
- After RNase treatment, to the extract added equal volume of phenol: chloroform (1:1, v/v) and samples were mixed slowly and again centrifuged (10000 x *g*; 10 min) and aqueous phase was retained in fresh microfuge tubes.
- DNA was then precipitated by adding 0.3 volume of 3 M sodium acetate (For 50 ml stock, 12.3 g of Sodium acetate was dissolved in water and adjust the pH with glacial acetic acid , pH-5.2) and 0.6 volume of chilled isopropanol.
- Finally, samples were centrifuged (10000 x *g*; 10 min; RT) and pellet was retained after removal of supernatant. Pellet was washed with 70 % ethyl alcohol (500 µl per sample) and air-dried.
- Pellet was then dissolved in TE buffer (100 µl per sample) and stored at –20 ºC.

Electrophoresis of DNA on agarose gels

In order to check quality of DNA, 0.8 % agarose gel (w/v) was prepared by adding 0.32 g of agarose (Life Technologies India Pvt. Ltd.) in 40ml of 1X TAE (Tris–Acetate-EDTA) buffer (10X TAE buffer- 24.2 Tris base, 5.71 ml glacial acetic acid, 3.72 g Na_2EDTA.2H2O, make up the volume (1 liter) with double distilled water); the agarose was melted in microwave oven (LG Electronics, India) until dissolved completely. The molten agar was cooled, added 1 µl of Ethidium bromide from the stock of 10 mg/ml and poured into casting trey inserted with combs and allowed to solidify at room temperature. Disposable surgical gloves were used while handling Ethidium bromide. 5 µl of DNA sample were mixed with 0.2 volume of 6X gel loading buffer (Bromophenol blue 0.25 %, Xylene cyanol FF 0.25 %, Glycerol in water 30.0 %) and loaded into well. The gel was electrophoresed on horizontal electrophoresis apparatus (Amersham Bioscience, U.S.A) in TAE running buffer at 50 volts for one hour and visualized on a U.V. transilluminator (Vilber Loumart, France).

Spectrophotometric quantification of DNA

The concentration of extracted DNA in solution was estimated by spectrophotometric measurement using NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) at A260. The quality of the DNA was evaluated by measurement of the A_{260}/A_{280} and the A_{230}/A_{260} ratios. Ideally, the A_{260}/A_{280} ratio should be 1.8-2.0 while the A_{230}/A_{260} ratio should be 0.3-0.9. Ratios (A_{260}/A_{280}) less than 1.8 indicate protein or phenol contamination, while ratios greater than 2.0 indicate the presence of RNA.

PCR amplification using RAPD and ISSR markers

PCR amplification was performed in 20 µl volume using 20 random amplified polymorphic DNA (RAPD) decamer primers (OPD1–OPD 20; Operon Technologies, Alameda, CA**,** Table 3.2) and 20 inter simple sequence repeat (ISSR) (16-20 nucleotide) primers (Table 3.2). The reaction mixture consisted of 40 ng of genomic DNA, 1.0 U Taq DNA polymerase (Larova, Teltow, Germany), 100 µm dNTPs mixture, 2.0 µl reaction buffer (10X), and 10 nmol primer, Mill-Q water (Millipore India, Bangalore, India) was added to make up the final volume to 20 µl. Amplifications were performed in thermal cycler model Gene Amp 9700 (Applied Biosystem, San Francisco, USA). Amplification conditions were initial denaturation 94 ºC 5 min; 41 cycles of : 94 °C 1 min, 35 °C 45 sec for RAPD and 55 °C in case of ISSR and 72 °C 1.5 min; with final extinction at 72 °C 5 min. The amplified products were separated on a 1.5 % (w/v) agarose gel at 50 volts on horizontal midi gel electrophoresis system (Life technologies, USA) and viewed under UV transilluminator (Vilber Loumart, France) following ethidium bromide staining.

Table 3.2 Sequence of ISSR and RAPD primers used in the study

PRIMER NO. (ISSR)	PRIMER SEQUENCE (5'-3')	PRIMER NO. (RAPD)	PRIMER SEQUENCE (5'-3')
ISSR-1	$(CA)_8CG$	OPD-1	ACC GCG AAG G
ISSR-2	$(GA)_8CG$	OPD -2	GGA CCC AAC C
ISSR-3	$(GA)_8TC$	OPD -3	GTC GCC GTC A
ISSR-4	$(AC)_8GCGC$	OPD -4	TCT GGT GAG G
ISSR-5	$(AC)_8$	OPD -5	TGA GCG GAC A
ISSR-6	$(CA)_8TG$	OPD -6	ACC TGA ACG G
ISSR-7	$(CA)_8GC$	OPD -7	TTG GCA CGG G
ISSR-8	$(GA)_8TA$	OPD -8	GTG TGC CCC A
ISSR-9	$(GC)_8T$	OPD -9	CTC TGG AGA C
ISSR-10	$(GC)_8A$	OPD -10	GGT CTA CAC C
ISSR-11	$(GC)_8AT$	OPD -11	AGC GCC ATT G
ISSR-12	$(CT)_8G$	OPD -12	CAC CGT ATC C
ISSR-13	$(CT)_8A$	OPD -13	CTT CCC CAA G
ISSR-14	$(CT)_8AG$	OPD -14	CAT CCG TGC T
ISSR-15	$(GT)_8A$	OPD -15	AGG GCG TAA G
ISSR-16	$(GT)_8C$	OPD -16	TTT CCC ACG G
ISSR-17	$(AT)_8C$	OPD -17	GAG AGC CAA C
ISSR-18	$(AT)_8G$	OPD -18	CTG GGG ACT T
ISSR-19	$(AT)_8GC$	OPD -19	CTG CGG TCA G
ISSR-20	$(AT)_8$	OPD -20	ACC CGG TCA C

Agrobacterium mediated genetic transformation of elite clones of *E .tereticornis*

Agrobacterium strain (s) and plasmid vector

The genetic transformation experiments were conducted using *Agrobacterium tumefaciens* strains EHA105 and LBA4404 harboring the binary vector pBI121 (Clontech, San Diego, CA, USA). The *A. tumefaciens* strain EHA105 (Hood *et al.* 1993) and *A. tumefaciens* strain LBA4404 (Hoekema *et al.* 1983) were used the study. The map of binary vector pBI121 as shown in Fig. 3.1 is carrying *uidA* gene (β-glucuronidase) (GUS) as a reporter gene under the control of CaMV 35S promoter and NOS terminator and *nptII* (neomycin phosphotransferase II) gene used as selection marker gene is under the control of NOS promoter and terminator within the t-DNA region. The plasmid was introduced into *A. tumefaciens* disarmed strains EHA105 (provided by Prof. S.B. Gelvin, Purdae University, Purdae, USA) and LBA4404 (provided by Dr. Nirinjan Das, Thapar University, Patiala) by the freeze-thaw method (Holsters *et al.* 1978) as briefly described below:

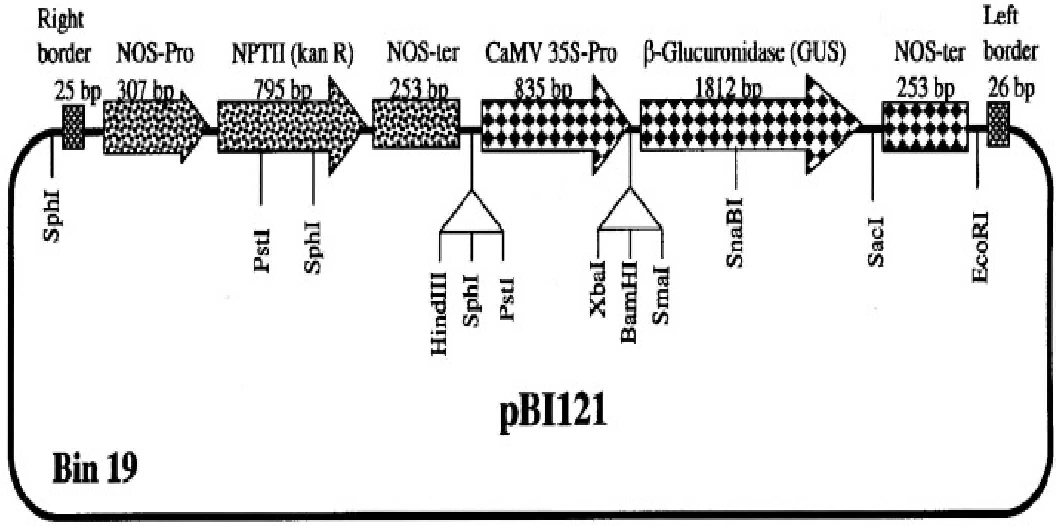


Figure 3.1 Diagrammatic representation of the binary plasmid vector pBI121, T-DNA region indicating the localization of the *nptll* gene, encoding neomycin phosphotransferase (*nptll*) and the *uidA* gene, encoding β-Glucuronidase (GUS), NOS ter (nopaline synthase terminator), NOS pro (nopaline synthase promoter), CaMV35S pro (cauliflower mosaic virus 35S promoter

Preparation of competent cells of *A. tumefaciens*

A single colony of *A. tumefaciens* (strains EHA105 or LBA4404) from a freshly grown plate was inoculated into 25 ml of Yeast Extract Peptone (YEP) broth (10 g/1 bacto peptone, 10 g/l yeast extract and 0.5 g/l NaCl, pH-7) containing 15 µg/ml rifampicin in a 250 ml flask and incubated the culture for 36 h at 28 °C under shaking condition (180 rpm). Aseptically 200 µl of the above culture was transferred into 50 ml of fresh YEP broth in a 250 ml flask. The culture was then incubated with vigorous shaking (250 rpm) at 28 °C for 3-4 h until the culture grows to OD_{590} (optical density at 590 nm) of ~ 0.5 to 0.8. To monitor the growth of the culture, OD_{590} was determined hourly. Above culture (OD_{590} ~ 0.5 to 0.8) was transferred to sterile, ice-cold 50 ml polypropylene tubes. The culture was cooled to 0 °C by storing the tubes on ice for 10 min. The cells were harvested by centrifugation (3000 x g; 5 min; 4 °C). The pellet was resuspended in 1 ml of ice-cold solution of 20 mM $CaCl_2$ and store on ice for 15 min.

Transformations of competent cells using freeze thaw method

100 µl of the suspension of competent cells was transferred to a sterile and prechilled microfuge tube (1.5 ml capacity). The plasmid DNA sample (100 to 300 ng in 5 µl volume) was added to each tube. The contents of the tube were mixed gently and snap freezed in liquid nitrogen for 5 min. The cells were thawed in a circulating water bath (37 °C for 5 min). One ml of YEP broth was added to each tube and incubated the cultures for 4 h at 28 °C to allow the recovery of bacteria from shock treatment and to express the antibiotic resistance marker encoded by the plasmid. Tubes were centrifuged (3000 x *g*; 5 min) and pellet was resuspended in 100 µl of fresh YEP broth. Putative transformed cells was spread on each 90 mm YEP agar-plates containing 15 µg/ml rifampicin and 50 µg/ml of kanamycin and incubated at 28 °C. Transformed colonies were observed after 36 h. The presence of plasmid pBI121 was confirmed in the antibiotic resistant bacterial

colonies by PCR using *nptII* gene specific primers. The transformed *A. tumefaciens* strains were maintained at 28 °C on YEP agar medium (10 g/1 bacto peptone, 10 g/l yeast extract, 0.5 g/l NaCl and 1.5 g/l agar pH-7) containing 15 µg/ml rifampicin and 50 µg/ml kanamycin and used for the genetic transformation experiments.

Explant Source

Leaves taken from actively growing microshoot cultures of *E. tereticornis* (maintained on MS medium supplemented with 2.5 µM BA and 0.5 µM NAA) were used as starting material for genetic transformation experiments.

Determination of antibiotic sensitivity

The sensitivity of the leaves of *E. terticornis* for kanamycin was determined by culturing leaf segments on shoot induction medium (MS+ 5.0 µM BA + 1.0 µM 2, 4 – D) supplemented with different concentrations of kanamycin (0, 10, 20, 30, 40, 50, 70 and 100 mg /l).

Selection of *A. tumefaciens* strain

For the selection *A. tumefaciens* strain, leaf segments of *E. tereticornis* were infected with both strains i.e. EHA105 and LBA4404 harboring binary vector pBI121 and scored for transient GUS expression. Based on transient GUS expression analysis of leaf segments, *A. tumefaciens* EHA105 was selected for further work on development of *Agrobacterium*-mediated genetic transformation protocol of *E. tereticornis.*

Agrobacterium-mediated transformation

The protocol for *Agrobacterium*-mediated genetic transformation of *E. tereticornis* with *A. tumefaciens* EHA105 harboring binary vector pBI121 was developed. A single bacterial colony of *A. tumefaciens* EHA105 containing plasmid pBI121 was inoculated in 10 ml liquid yeast extract peptone medium supplemented with 50 µg/ml kanamycin and 15 µg/ml rifampicin (hereafter referred as YEP medium) and grown overnight at 28 ^{o}C on a gyratory shaker (250 rpm). From the overnight grown culture, 0.5 ml was freshly inoculated to 50 ml of YEP medium and grown for 24 h. Bacterial cells were pelleted by centrifugation (4000 x *g*; 2 min) and suspended in YEP medium supplemented with 100 μM acetosyringone (Sigma Chemical Co., St Louis, MO) to attain the desired OD_{590}.

Factors effecting *Agrobacterium*-mediated genetic transformation of *E. tereticornis*

Effect of pre-culturing

To study the effect of pre culturing of explants prior to infection, leaf explants were cultured on pre-culture medium (PCM) as mentioned in table 3.3 for different interval of times ranging form 0–5 days.

Effect of method of injury

To find out the effect of method of injury to tissue prior to infection with *Agrobacterium*, leaf segments were injured following different procedures namely, pricking with hypodermic needle, cutting with surgical blade, rubbing with carborundum paper and glass beads. Following these injuries leaves were co cultivated for two days, washed and the transient GUS expression was scored.

Effect of bacterial concentration and infection time

Leaf segments were infected with suspension of *A. tumefaciens* with different OD_{590} values (0.2, 0.4, 0.6, 0.8 and 1.0) for different time periods (10 ml, 0–30 min) in Petri plates. Following infection, leaves were co cultivated for two days, washed and the transient GUS expression was scored for both the parameters separately.

Effect of co-cultivation period and pH of co-cultivation medium

To find out the effect of co-cultivation period on transient GUS expression, leaf segments were blotted with sterile filter paper to remove the excess of bacterial cells and medium. These were then cultured on antibiotic-free co-cultivation medium (CCM; Table 3.3) with varying pH (5.2, 5.5 and 5.8) for different time intervals (1–5 days) and scored for transient GUS expression.

Effect of Photoperiod

To find out the effect of photoperiod, cultures were sealed with cling film and incubated under different photoperiods viz continuous light, 16-h light/8-h dark or continues dark. After incubation under different photoperiods leaves were scored for transient GUS expression.

Selection of transformed tissue

Following co-cultivation, leaf explants were washed 4-5 times with sterile distilled water containing 500 mg/l cefotaxime, blotted on sterile filter paper and transferred to MS medium supplemented with 5.0 µM BA, 1.0 µM 2,4-D, 50 mg/l kanamycin and 500 mg/l cefotaxime (selection-*cum*-shoot induction medium: SM1 Table 3.3) and incubated at 25±1°C under cool white fluorescent lights with the light intensity of 42 µmol m^{-2} s^{-1} inside the culture vessel in 16- h light/8h dark cycle. Despite repeated transfer of leaf segments in SM1 medium, leaf segments fail to regenerate. So therefore, to overcome these problems certain changes were made in the SM1 and a new medium was designed (SM2) as mentioned in table 3.3. The cultures were sub-cultured on same medium at every 20 days interval for shoot regeneration and putatively transformed regenerated shoots were multiplied on MS medium supplemented with 2.5 µM BA and 0.5 µM 2,4-D.

Table 3.3 Composition of media used in *Agrobacterium* mediated transformation of *E. tereticornis*

Culture medium	Composition
Shoot induction medium (SIM)	MS medium + 5.0 µM BA +1 µM 2,4-D + 0.7 % agar + 30 g/l sucrose, pH 5.8
Pre culture medium (PCM)	MS medium+ 5.0 µM BA +1 µM 2,4-D + 0.7 % agar + 30 g/l sucrose $_+$100 µM Acetosyringone, pH 5.8
Co-cultivation medium(CCM)	MS medium + 0.7 % agar + 30 g/l sucrose $_+$100 µM Acetosyringone, pH 5.2
Selection medium-l (SM-l)	MS medium + 5.0 µM BA +1 µM 2,4-D + 0.7 % agar + 30 g/l sucrose + 50 mg/l kanamycin + 500 mg/l cefotaxime, pH 5.8
Selection medium-ll (SM-ll)	MS medium – KNO_3 + NH_4NO_3 = 391.8 mg/l + K_2SO_4 = 990 mg/l + NH_4SO_4 =323 mg/l + Inositol = 200mg/l + 5.0 µM BA +1 µM 2,4-D + 0.7 % agar + 30 g/l sucrose + 50 mg/l kanamycin + 500 mg/l cefotaxime, pH 5.8

Histochemical GUS assay

GUS assay was carried out using regenerated kanamycin resistant shoots and freshly infected explants after 2 days (transient assay) of incubation for scoring transient as well as stable GUS expression following the method of Jefferson *et al.* (1987). Tissues were incubated overnight at 37 °C in 100 mM sodium phosphate buffer (pH 7.0), containing 1 mM X-Gluc. Details of the GUS histochemical solution are given in following Table 3.4:

Table 3.4 Composition of GUS histochemical solution

Stock solution	Final Concentration	Reagent Mix µl/ml
1.0 M $NaPO_4$ buffer, pH 7.0	0.1M	100
0.25 M EDTA, pH 7.0	10 mM	40
0.005 M K-ferricyanide pH 7.0	0.5 mM	100
0.005 M K- ferrocyanide pH 7.0	0.5 mM	100
0.002 M X-glucuronide	1.0 mM	50
10 % triton X-100 (optional)	0.1 %	10
Subtotal		400 ml
Distilled water		600 ml
Final volume		1000 ml

After staining, the tissues were fixed by dipping the samples in fixative solution (100 ml- Formaldehyde 10 % v/v - 10 ml, Ethanol - 20 ml, Acetic Acid - 05 ml, Distilled water – 65 ml) for 4 h as per the method of Jefferson *et al.* (1987).

The procedure for GUS histochemical assay is described as follows**:**

1. Leaves and/or small plant portions were cut from putative transgenic plants and placed in the 2 ml microfuge tube containing histochemical staining solution.
2. Tube was subjected to vacuum treatment for 1-2 min.
3. After vacuum treatment, tubes were incubated at 37 °C overnight.
4. Next day, staining solution was removed from the tube and tissue was dipped in fixative solution and incubated at room temp for minimum of 4 h.
5. After that, fixative solution was removed and tissue was again dipped in 50 % solution of ethanol, to remove chlorophyll for better visualization of stained tissue. At this stage 50 % ethanol was changed intermittently.
6. Over the period of time (2 h) the concentration of ethanol solution was slowly raised to 100 % by changing the grades of alcohol for complete removal of chlorophyll.
7. After complete removal of chlorophyll, the tissue was observed for the presence of blue color.

Molecular analysis of putative transgenic plants

PCR amplification of nptll and uidA genes

In order to confirm the insertion of T-DNA in putative transformed plants, molecular analysis was carried out by performing PCR amplifications using primers specific for *nptll* and *uidA* genes. Genomic DNA was isolated from transformed as well as untransformed shoots following CTAB method as described previously (Section: Testing of clonal fidelity using RAPD and ISSR markers). The PCR amplification of DNA fragments specific to *nptll* and *uidA* genes was carried out using genomic DNA isolated from the leaves of GUS positive putative transgenic shoots and untransformed plants. Primer pair used for *nptll* gene was 5'-GAGGCTATTCGGCTATGACTC-3' (forward) and 5'-ATCGGGAGAGGCGATACCGTA-3' (reverse) and for *uidA* was 5'-GGTGGGAAAGCGCGTTACAAG-3' (forward) and 5'- GTTTACGCGTTCCGCCA-3' (reverse). The PCR reaction mixture consisted of 20 ng of genomic DNA, 1.0 U of *Taq* DNA polymerase (Larova, Teltow, Germany), 100 µmol dNTPS mixture, 2.0 µl reaction buffer (10X), 10 nmol each primer and sterile Milli-Q water (Millipore India, Bangalore) was added to make up the volume to 20 µl. Amplification conditions were initial denaturation 94 ºC for 5 min; 31 cycles of 94 °C for 1 min, 58 °C for 45 sec and 72 °C for 1.5 min with final extension at 72 °C for 5 min. A fragment of about 1500 bp in case of *uidA* gene and a fragment of about 720 bp specific to *nptII were* amplified. Plasmid DNA of pBI121 was used as a positive control and untransformed shoots of *E. tereticornis* were the source of DNA for negative control. The amplified products were separated on 1.2 % (w/v) agarose gel and viewed under UV transilluminator (Biorad, CA, USA) following ethidium bromide staining.

16S rRNA analysis

To rule out possibility of any possible bacterial contamination due to the presence of residual *Agrobacterium* in the transformed tissue, PCR amplification of 16S rRNA fragment of about 1500 bp was carried out using DNA extracted from putative transformed and untransformed shoots using primer pair 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'- ACGGGCGGTGTGTTC-3' (Weisberg *et al.* 1991). Bacterial genomic DNA was used as positive control. Amplification conditions were same as mentioned above (Annealing temp – 55 °C). The amplified products were separated on 1.2 % (w/v) agarose gel and viewed under UV transilluminator (Biorad, CA, USA) following ethidium bromide staining.

Reverse Transcriptase-PCR analysis

To check the expression of *nptII* and *uidA* genes at transcription level in the transgenic plants, reverse transcriptase polymerase chain reaction (RT-PCR) analysis was carried out using cDNA as template, synthesized from total RNA isolated from different independent transgenic lines and control untransformed plants.

<u>RNA Isolation</u>

Total RNA was isolated as per the method described by Cheng *et al.* (1993). The detailed procedure is given below:

1. 100 mg of tissue (Leaves/shoots) was taken, cleaned by washing with sterile distilled water, cutted into small pieces and ground in liquid nitrogen in autoclaved mortar and peastle to make fine powder. Powder was Immediately transferred to 50 ml cautoclved centrifuge tubes conatining preheated 15 ml of CTAB RNA extraction buffer (2 % CTAB, 2% PVP, 100 mM Tris HCl, 25 mM EDTA, 2 M NaCl, 0.2 % beta- mercaptoethanol) to make slurry and added 400 µl 2-mercaptoethanol, mixed well and kept at 65 °C for 10 min.
2. 15 ml of Choloroform: Isoamylalcohol (24:1 v/v) was added and centrifuged (5000 x *g*; 20 min; RT).
3. The upper phase was transferred to a new tube with 15 ml of Choloroform: Isoamylalcohol (24:1 v/v) and mixed followed by centrifugation (5000 x *g*; 20 min; RT).
4. The upper aquoues phase (~ 12 ml) was again transferred to a new tube and to it added 3 ml of 10 M LiCl and incubated overnight at 4 °C.
5. Sample were centrifuged (10000 x *g*; 30 min; 4 °C) and supernatant was removed.

6. Pellets were resuspended in 700 μl SSTE buffer (1 M NaCl, 0.5 % SDS, 1mM EDTA) and then transferred to a 2 ml eppendorf tube and kept at 60 °C for few min.
7. 700 μl of chloroform was added to the pellets, mixed properly and centrifuged (10000 x *g*; 10 min; 4 °C).
8. The upper phase was transferred to a new tube with 700 μl of chloroform and again centrifuged (10000 x *g*; 10 min; 4 °C).
9. The upper phase was than taken in new 2 ml capacity centrifuge tubes and 1.2 ml of absolute ethyl alcohol was added to precipitate RNA and incubated for 2 hour at -80 °C.
10. After incubation samples were centrifuged (13000 x *g*; 30 min; 4 °C) to precipitate the RNA.
11. Washed the RNA pellet twice with 1ml of 70 % ethanol.
12. The RNA pellet was briefly dried in air and dissolved in minimal amount RNase-free autoclaved water and stored at -80 °C.

Electrophoresis of RNA in denaturing conditions

RNA samples (20-40 μg) in 5-6 μl were denatured for 5 min at 65 ^{0}C in 0.5 x 3-(N-morpholino) propanesulfonic acid (MOPS), 17.5 % (v/v) formaldehyde and 50 % (v/v) formamide in a final volume of 25 μl. Specially prepared 5x loading dye and ethidium bromide (1 mg/ml) were added to the samples prior to loading onto a 1 % (w/v) agarose gel dissolved in 1x MOPS buffer (10X (200 ml)- 41.9 g MOPS, 6.8 g Sodium Acetate, pH 7.0) and 9 % (v/v) formaldehyde. Samples were fractionated in 1x MOPS running buffer at 60 V. The RNA was then visualized under U.V. light.

cDNA synthesis and PCR analysis

The cDNA was synthesized from total RNA by reverse transcription using The Reverse AID^{TM} First Strand cDNA Synthesis Kit (MBI Fermentas Life Sciences, USA) as per manufactures instructions. 2 µg of total RNA was denatured at 65 °C for 5 min in the presence of 0.5 µg of oligodT$_{18}$ primer and immediately cooled on ice. The first strand cDNA was synthesized in the presence of 200 U MMLV reverse transcriptase, 20 U ribonuclease inhibitor, 10 mM of each dNTPs and 5X reaction buffer supplied by the manufacturer. The reaction was carried out at 42 °C for 1 hour. The enzyme was inactivated by incubating at 70 °C for 10 min and immediately placed on ice and then it was stored at -20 °C for further use. The presence of *nptll* and *uidA* genes in the cDNA population was confirmed by using gene specific primer by PCR. The amplified products were separated on 1.2 % (w/v) agarose gel and viewed under UV transilluminator (Biorad, CA, USA) following ethidium bromide staining.

Gene expression analysis using quantitative Real Time–PCR (qRT–PCR)

Quantitative real-time polymerase chain reaction (*qRT-PCR*) analysis of *nptll* gene was conducted using the Real Master Mix SYBR ROX Master Mix (5 prime, GmbH, Hamburg) on Realplex 2.2 real-time PCR system (Eppendorf AG, Hamburg) to determine critical thresholds (Ct), using gene-specific primers (forward primer, 5'-GAATGAACTGCAGGACGAG-3' and (reverse primer, 5'-ATACTTTCTCGGCAGGAGCA-3'). For quantification of gene expression in transgenic lines, actin-9 (forward primer 5'-CTATTCTCCGCTTTGGACTTGGCA-3' and reverse primer 5'- AGGACCTCAGGACAACGGAAACG -3') was used as an endogenous control (Volkov *et al.* 2003). Conditions for the RT-PCR reactions were as follows: 95 ^{o}C for 2 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s, and 68 °C for 20 s. Gene expression was quantified using the comparative method Ct: $2^{-\Delta\Delta Ct}$ method.

Molecular cloning of *Korrigan* and *Sucrose Synthase* genes from *Populus deltoides*

The reported sequences of *KOR* and *SUS* genes were retrieved from the NCBI database and aligned using ClustalW. In the first instance, primers were designed from the conserved region of these genes to amplify partial fragments (1800 bp fragment in case of *KOR* and 1200 bp in case of *SUS*). Total RNA was isolated from the young shoots of *Populus deltoides* following CTAB method. cDNA was synthesized from RNA and used as template for amplification of partial fragments of DNA specific to these genes. The amplification of 1800 bp *KOR* fragment and 1200 bp *SUS* fragment was carried out using following primers (Table 3.5).

Table 3.5 Primer sequences used for amplification of partial *KOR* and *SUS* genes from *P. deltoides* cDNA

Symbol	Primer sequence (5'-3')
KOR	Forward- AAGCATAATAACGTGTCATGG
	Reverse- TTGCAAATCCAGTAATGAGATGA
SUS	Forward-TACATTTTGGATCAAGTTCGT
	Reverse-TCCATGGTAAGGATCAATATG

The PCR reaction mixture consisted of 20 ng of template cDNA (*Populus deltoides* cDNA), 1.0 U of XT-20 *Taq* DNA Polymerase with proof reading ability (Banglore genei, India), 100 µmol $dNTP_S$ mixture, 2.0 µl reaction buffer (10X), 10 nmol each primer and sterile Milli-Q water (Millipore India, Bangalore) was added to make up the volume to 20 µl. The amplification of both the fragments was carried out with the GenAmp 2700 thermocycler (Applied Biosystem, USA). Amplification conditions were initial denaturation 94 ºC for 5 min; 31 cycles of 94 °C for 1 min, 50 °C for 45 sec (in case of *sucrose synthase*) and 55 °C (in case of *korrigan*) and 72°C for 1.5 min with final extension at 72°C for 5 min. Successful amplifications were confirmed by agarose gel (1.0 % w/v) electrophoresis and ethidium bromide staining.

Purification of PCR products

Amplified fragments of both genes were purified using the QIAquick PCR purification kit (Qiagen, USA), following the instructions of the manufacturer. Purified PCR products were eluted from the purification columns by the addition of 50 µl 10 mM Tris buffer (pH 8.0). PCR products were electrophresed on agarose gel (0.8 %) prior to cloning. After staining with ethidium bromide, a defined band of required size was visualized under UV irradiation and excised. Besides removing surplus primers, nucleotides, and salts, this method possessed the advantage that incomplete (shorter) amplification fragments are also removed prior to cloning. Subsequently, the DNA was extracted from the gel matrix material, using the QIAquick gel extraction kit (Qiagen, USA) as per the manufacturer's instructions. PCR products were eluted with 30 µl TE buffer (pH 8.0) and used for the cloning.

Ligation of amplicons in T-vectors

The amplicons were ligated into pGEM®-T easy vector (Promega, Madison, Wisconsin, USA) (Fig. 3.2) as per manufactures instructions. The ligation reaction mixture consisted of 3 µl of plasmid pGEM®-T easy vector (55ng/µl), 4 µl of Insert (75ng/µl), 6 µl of Buffer (5×) , 1 µl of T4 DNA Ligase (5U/ µl) and sterile Milli-Q water (Millipore India, Bangalore) was added to make up the volume to 20 µl. The reaction mixture was incubated overnight at 4 °C. The ligation reaction was heat inactivated at 65 °C for 10 min and cooled at room temperature. The ligation mixture was stored at -20 °C.

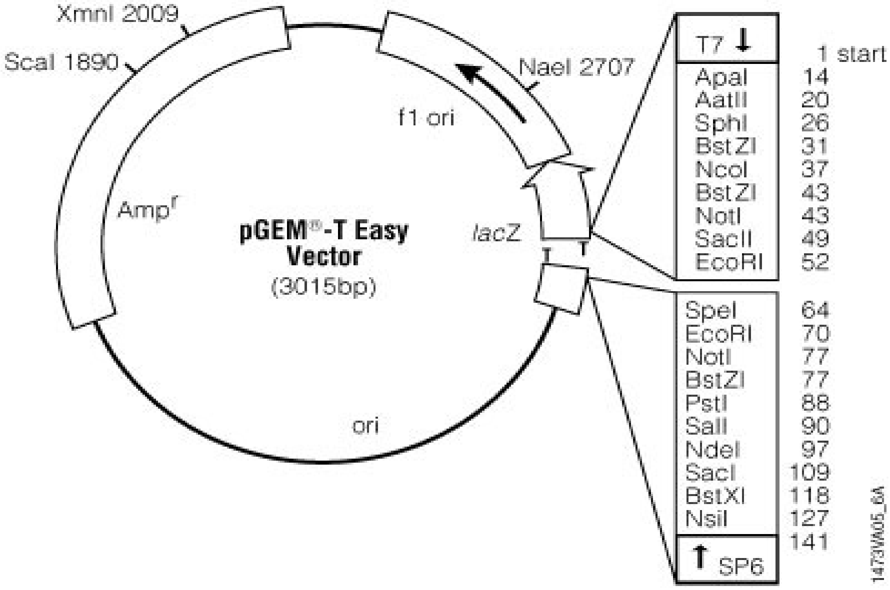


Figure 3.2 Restriction map of pGEM®-T Easy Vector

Preparation of competent cells and genetic transformation of *E. coli* DH5α using $CaCl_2$ method

Competent cells of *E. coli* DH5α were prepared follwing the method of Mandel and Higa (1969) and plasmid transformation was carried out following the method of Singh *et al.* (2010). A single colony of *E. coli* DH5α from a freshly grown plate was inoculated into 25 ml of LB broth in a 250 ml flask and incubated for 16-20 h at 37 °C on gyratory shaker (120 rpm). 200 µl of the above culture was aseptically transferred into 25 ml of fresh LB broth in a 250 ml flask. The culture was further incubated with vigorous shaking (180 rpm) at 37 °C for 2-3 h. To monitor the growth of the culture, OD_{590} was determined at every one-hour (OD_{590} should be ~ 0.5). The above culture was transferred to sterile, disposable, ice-cold 50 ml polypropylene tubes. The culture was cooled to 0 °C by storing the tubes on ice for 10 min. The cells were harvested by centrifugation (8000 x g; 10 min at 4 °C). The pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and store on ice for 15 min. Further the cells were recovered by centrifugation (8000 x *g*; 10 min at 4°C). The cell pellet was resuspended in 1 ml of ice-cold 0.1 M $CaCl_2$ and incubated under ice for minimum of 3-4 h. 100 µl suspension of competent cells was transferred to a sterile and prechilled microfuge tube (1.5 ml capacity). The ligated product sample (~100 ng in a volume of 5 µl or less) was added to each tube. The content of the tubes were mixed gently and kept on ice for 30 min. The tubes were incubated in a circulating water bath that has been preheated to 42 °C for exactly 2 min without shaking. The tubes were rapidly transferred to an ice bath to chill the cells for 1-2 min. One ml of LB was then added to each tube and incubated for 45- 60 min at 37 °C to allow the bacteria to recover the shock and to express the antibiotic resistance marker encoded by the plasmid. 100 µl of transformed cells was spreaded on each 90 mm Luria agar-Ampicillin-X-Gal/IPTG plates and incubated at 37 °C. Colonies of transformed cells were observed between 12 - 16 h.

Blue/white screening for recombinant plasmids

After transformation of the ligated product, the *E. coli* DH5α (Lac Z^-) bacterial host cells were plated on Luria agar medium containing 50 µg/ml ampicillin, for selection of transformants. X-Gal and IPTG were used to screen for colonies containing a recombinant plasmid. The cloning site in the pGEM®-T easy vector is located in the multiple cloning sites (mcs) of the plasmid's lacZα gene; if no insert is present, functional β-galactosidase is produced, and the bacterial colony gets blue colour. However, if the host cell receives a recombinant plasmid (containing a fragment of interest) insert in the lacZα gene, the resulting transformant colony is white.

Isolation and purification of plasmid DNA from recombinant bacteria by alkaline lysis method

The plasmid DNA was isolated based on the alkaline lysis method (Bimboim and Doly 1979). A single transformed *E. coli* DH5α colony was transferred into 3 ml of Luria broth medium containing appropriate antibiotic (Ampicillin, used in a final concentration of 50 µg/ml) in a loosely capped 15 ml tube and incubated the culture overnight at 37 °C with shaking (120 rpm). 2.0 ml of the above culture was transferred into a microfuge tube and cells were harvested by centrifugation (8000 x *g*; 5 min). The bacterial pellet was resuspended in 200 µl of ice-cold Solution I (Glucose 50 mM; Tris.HCl 25 mM (pH 8.0); EDTA 10 mM, pH 8.0) by vigorous vortexing to ensure that the bacterial pellet is completely dispersed in this solution. Further 200 µl of freshly prepared Solution II (NaOH 0.2 N; SDS 1.0 %) was added and the contents were mixed by gentle inversion of the tubes avoiding vigorous shaking, five to ten times. The tubes were kept on ice for 5 min. Finally 300 µl of ice-cold Solution III (3 M Potassium acetate) was added and mixed by inversion to disperse Solution III through the viscous bacterial lysate. The tubes were kept on ice for 10 min. The tubes were centrifuged (12000 x *g*; 10 min). The upper aqueous phase was then extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). Plasmid DNA was precipitated by adding 0.7 volumes isopropanol to the aqueous phase and incubated at -20 °C for 10 min, followed by centrifugation (12000 x *g*; 10 min). The DNA pellets were washed with 500 µl EtOH (70%) and centrifuged (10000 x *g*; 10 min). Finally, the pellets were resuspended in 40 µl TE buffer/milli-Q water and stored at 4 °C for further use.

Size screening for recombinant plasmids

Clones containing approximately size as that of original cloned fragments were identified by PCR amplification from plasmid DNA using T7 universal primer (5'-AATACGACTCACTATAGGG-3') SP6 universal primer (5'-ATTTAGGTGACACTATAG -3') plasmid primers. The PCR reaction mixture consisted of 20 ng of template DNA (plasmid from transformed colony), 1.0 U of *Taq* DNA polymerase (Larova, Teltow, Germany), 100 µmol dNTPS mixture, 2.0 µl reaction buffer (10X), 10 nmol each primer and sterile Milli-Q water (Millipore India, Bangalore) was added to make up the volume to 20 µl. The amplification of both the fragments was carried out using GenAmp 2700 thermocycler (Applied Biosystem, USA). Amplification conditions were initial denaturation 94 ºC for 5 min; 31 cycles of 94 °C for 1 min, 55 °C for 45 sec and 72 °C for 1.5 min with final extension at 72°C for 5 min.. Successful amplifications and size of amplified products were confirmed by agarose gel (1.0 % w/v) electrophoresis following ethidium bromide staining.

Sequencing

The fragments of genes were sequenced for both strands using T7 and SP6 universal primers. The sequence was generated by chain termination method (Sanger *et al.* 1977) using an Applied Biosystems automatic sequencer (DNA Sequencing Facility, Department of Biochemistry, South Campus, Delhi University, New Delhi, India).

Analysis of sequence data

The sequence received was cleaned by 'Vec Screen' (to remove vector sequences) on NCBI (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). The sequences of the genes thus obtained were compared with reported nucleotide and protein sequences those available in GenBank/ EMBL databases using BLAST program (Altschul *et al.* 1997) accessible through NCBI (National Centre for Biotechnology Information – http://www.ncbi.nlm.nih.gov). Multiple alignments were performed using *Multalin* program (http://multalin.toulouse.inra.fr/multalin/). The nucleotide and deduced amino acid sequences of the cloned gene fragments were also subjected to global alignment using ClustalW 1.8 tool.

Molecular cloning of full length *Korrigan* and *Sucrose Synthase* genes from *Populus deltoides*

Subsequently, on the basis of percentage similarity of partial sequences of *KOR* and *SUS*; primers for full length amplification of these genes (*KOR* and *SUS)* along with overhangs of restriction enzymes *BamH1* and *Sac1* in case of *KOR* and *BamH1* and *Sma1* in case of *SUS* were designed for the directional cloning of these genes in the transformation vector pBI121 (Table 3.6). Using these primers; fragments of 1860 bp: full length coding sequence of *KOR* with overhangs of *BamH1* and *Sac1* and 2400 bp: full length coding sequence of *SUS* with overhangs of *BamH1* and *Sma1* were amplified from *P. deltoides* cDNA following the strategy mentioned below:

Table 3.6 Primer sequences used for amplification of full length *KOR* and *SUS* genes from *P. deltoides* cDNA

Symbol	Primer sequence (5'-3')
KOR FL	Forward-CGGGATCCATGTACGGAAGAGATCCATGGGGAG
	Reverse- CCGAGCTCTCATGGTTTCCAAGGTGCTGGTGGTG
SUS FL	Forward- CGGGATCCATGACTGAACGTGCTCTTACTCGTG
	Reverse- TCCCCGGGTTACTCCTTGGTCAAAGGAACAGACTC

Recognition sequences of respective restriction endonuclease are underlined. *Bam H1*: GGATCC, *Sac 1:* GAGCTC, *Sma 1*: CCCGGG

Strategy followed for the molecular cloning of full length fragments of *KOR and* SUS genes from *P. deltoides*

1. First of all RNA was isolated from *P. deltoides* following CTAB method (Cheng *et. al.* 1993).
2. After analyzing the quality and quantity of RNA, cDNA was synthesized using Reverse AID^{TM} First Strand cDNA Synthesis Kit (MBI fermentas, USA) using manufactures instructions.
3. PCR amplification of full length fragments of *KOR* and *SUS* from cDNA template (*P. deltoides* cDNA) using gene specific primers (Table 3.6).
4. Visualization of PCR products on 0.8 % agarose gel following ethidium bromide staining.

5. Purification of PCR fragments using the QIAquick PCR purification kit (Qiagen, USA) following manufactures instructions.
6. Ligation of purified products into pGEM®-T easy vector system (promega, Madison, Wisconsin, USA).
7. Transformation of ligated product in competent cells of *E. coli* DH5α using $CaCl_2$ method.
8. Blue/white screening for recombinant plasmids followed by isolation and purification of plasmid DNA from recombinant bacteria by alkaline lysis method (Bimboim and Doly 1979).
9. Size screening for recombinant plasmids with help of PCR using T7/SP6 universal primers followed by sequencing of positive clones.
10. Sequence analysis using various bioinformatics tools.

Characterization of *korrigan* and *sucrose synthase*

The sequence received was cleaned by 'Vec Screen' (to remove vector sequences) on NCBI (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). The sequences of the genes thus obtained were compared with reported nucleotide and protein sequences those available in GenBank/ EMBL databases using BLAST program (Altschul *et al.* 1997) accessible through NCBI (National Centre for Biotechnology Information – http://www.ncbi.nlm.nih.gov). The identification of the ORF and amino acid sequences coded by these fragments was deduced using the ORF finder program provided by the NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Homologues amino acid sequences corresponding to these two genes were retrieved from databases using BLAST P. These sequences were aligned using MAFFT and edited using BIOEDIT program (Hall 1999). Potential transmembrane segments were identified using TMHMM-2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0) programme. The glycosylation and phophorylation sites were identified using NetNGlyc 1.0 and NetPhos 2.0 Server from technical University of Denmark (http://genome.cbs.dtu.dk//cgi-bin/webface?jobid=netNglyc and http://genome.cbs.dtu.dk//cgi-bin/webface?jobid=netphos). Molecular mass and isoelctric point of both proteins were computed using Compute pI/Mw tool from ExPASy (Expert Protein Analysis System) bioinformatics resource portal (http://web.expasy.org/compute_pi/) (Bjellqvist *et al.* 1993). Similarly for calculating the amino acid composition of the predicted amino acid sequences, the ProtParam tool of ExPASy proteomics server of the Swiss Institute of Bioinformatics (SIB; http://expasy.org/tools/) was used. Domains and family of both proteins were identified using interproscan tool of European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/pfa/iprscan/) (Quevillon *et al.* 2005).

Cloning of *korrigan* and *sucrose synthase* genes in binary vector pBI121

After characterization of both the gene fragments, these genes were directionally cloned into binary vector (pBI121) as described below:

1. PCR amplification of full length fragments of *KOR a*nd *SUS* from plasmid isolated from positive clones using gene specific primers (Table 3.6).
2. Restriction digestion of both, full length gene fragments of *KOR* and *SUS* and binary vector pBI121 with respective restriction enzymes.
3. Visualization and subsequent purification of digested gene fragments and binary vector pBI121 using QIAquick PCR purification kit (Qiagen, USA) following manufactures instructions.
4. Ligation of digested gene fragments into digested binary vector pBI121 using T4 DNA ligase (16 °C/ overnight).
5. Transformation of ligated product into competent cells of *A. tumefaciens* strain EHA105 using freeze thaw method (Holsters *et. al.* 1978).

6. Selection of positive colonies of A. *tumefaciens* strain EHA105 on kanamycin supplemented YEP medium, followed by isolation and purification of plasmid DNA from recombinant bacteria by alkaline lysis method (Bimboim and Doly 1979).
7. Screening for recombinant plasmids with help of PCR using gene specific primers. After, confirmation of positive clones, *A. tumefaciens* strain EHA105 carrying gene of interest were used for genetic transformation of *E. tereticornis* clone 'CE2'.

Transformation of *Eucalyptus tereticornis* with *korrigan* and *sucrose synthase*

The A. tumefaciens strain EHA105 transformed with pBI121 carrying *KOR and SUS* genes driven under the CaMV 35S promoter was used for the transformation studies as mentioned below:

Agrobacterium mediated genetic transformation of *E. tereticornis* with *KOR*

Leaves were taken from actively growing microshoot of *E. tereticornis* clone CE2 maintained on MS medium supplemented with 2.5 µM BA and 0.5 µM NAA and used as explant material for genetic transformation of *E. tereticornis.* Pre-cultured (2 days) Leaf explants precultured for 2 days on PCM medium (Table 3.3) were injured by pricking with hypodermic needle and then these were infected with suspension of *A. tumefaciens* grown at a OD values of 0.8 for 10 mins in petri plate. After infection, tissues were blotted with sterile filter paper to remove the excess of bacterial cells and medium. These were then co- cultivated (2 days) on antibiotic-free co-cultivation medium (CCM, Table 3.3). Cultures were sealed with cling film and incubated under 16-h light/8-h dark cycle. Following co-cultivation, leaf explants were washed 4-5 times with sterile distilled water containing 500 mg/l cefotaxime, blotted on sterile filter paper and transferred to SM-ll medium (Table 3.3) and incubated at 25±1 ^{o}C under cool white fluorescent lights with the light intensity of 42 µmo lm^{-2} s^{-1} inside the culture vessel in 16- h light/8h dark cycle. The cultures were sub-cultured on same medium at every 20 days interval for shoot regeneration and putatively transformed regenerated shoots were multiplied on MS medium supplemented 2.5 µM BA and 0.5 µM NAA. Roots were induced on PGR free MS medium after treament of elongated microshoots on MS medium supplemented with 50.0 µM of IBA for 36 hrs.

Molecular analysis of transformants

In order to confirm the insertion of transgene in putative transformed plants, analysis was carried out by performing PCR amplification reaction using primers specific for *nptll* and *KOR* genes (described previously). Genomic DNA was isolated following CTAB method as described previously. The PCR amplification of DNA fragments specific to *nptll* and *KOR* genes was carried out using genomic DNA isolated from the leaves of putative transgenic shoots and untransformed shoots.

Reverse Transcriptase-PCR analysis

To check the expression of *nptII and KOR* genes at transcription level in the transgenic plants, reverse transcriptase polymerase chain reaction (RT-PCR) analysis was carried out using cDNA as template, synthesized from total RNA isolated from different independent transgenic lines and control plants following methodology as described previously.

Gene expression analysis using quantitative Real Time–PCR (qRT–PCR)

Quantitative real-time polymerase chain reaction (*qRT-PCR*) analysis of *KOR* gene was conducted using the Real Master Mix SYBR ROX Master Mix (5 prime, GmbH, Hamburg) on Realplex 2.2 real-time PCR system (Eppendorf AG, Hamburg) to determine critical thresholds (Ct), using gene-specific primers (forward primer 5’-CTCTGTCAAGGCCATTGGAT-3’ and reverse primer 5’-

TCGTTGCTGCTGGTCTATTG -3'). For quantification of gene expression in transgenic lines, actin-9 (forward primer 5'-CTATTCTCCGCTTTGGACTTGGCA-3' and reverse primer 5'- AGGACCTCAGGACAACGGAAACG -3') as described previously by Volkov *et al.* (2003) was used as an endogenous control. Conditions for the RT-PCR reactions were as follows: 95°C for 2 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s, and 68 °C for 20 s. Gene expressions was quantified using the comparative method Ct: $2^{-\Delta\Delta Ct}$ method.

Cellulose estimation

For estimation of cellulose standard method given by Updegraff (1969) was followed:

1. 0.2 g of sample [oven dried (105 ^{0}C/6 h)] was taken in test tub, added 10 ml of Acetic/Nitric reagent. Sample was mixed on cyclo mixer and placed on water bath at 100 °C for 30 mins.
2. Test tubes were cooled and contents were shifted to centrifuge tube and centrifuged for 15 mins at 12000 rpm. Supernatant was discarded and residue was washed 4-5 times with distilled water.
3. Added 10 ml of 67 % sulphuric acid to the residue mixed properly and allowed to stand for 1 hour and total vol. is measured.
4. Diluted 1 ml of above solution to 100 ml with 67 % sulphuric acid.
5. 1 ml of above solution was taken in test tube and cooled on ice for 5 mins. Then added 5 ml of chilled Anthrone reagent mixed and again kept on ice for 5 mins.
6. Tubes were heated on boiling water bath for 10 mins and again cooled on ice for 5 mins and colour intensity was measured at 630 nm. (Distilled water was taken as blank.)
7. <u>Preparation of standard curve:</u> 50 mg of cellulose powder (predried in an oven at 1050C for 6h) was taken in test tube and added 10 ml of 67 % sulphuric acid,

mixed properly and allowed to stand for 1 hour and volume is made up to 500 ml with distilled water. Than a series of volumes were taken from above solution in separate test tubes (0.2 ml -1.0 ml) and made up to with distilled water. Colour was developed with Anthrone reagent as mentioned in step 5 and standard curve was drawn after taking the absorbance at 630 nm.

8. Alternatively amount of cellulose can be calculated using following formula:

$$\text{Cellulose \%} = \frac{W1 \times A2 \times D \times 100}{A1 \times 1000^{*} \times W2}$$

W1= Weight of Standard (μg), A1= Absorbance of standard, A2= Absorbance of sample

* = conversion factor (μg to mg), D = dilution factor, W2 = Weight of raw material

Statistical analysis

Unless otherwise stated, all experiments were conducted using four replicates with three explants in each culture vessel and repeated four times. The data were recorded after 4 wk of subculture. Osmotic potential and chlorophyll contents were estimated three times for each vessel, and repeated three times. Data were analyzed by analysis of variance and the means were compared with Duncan's multiple range test (Duncan 1955) using GraphPad Prism 4 software.

Chapter-4

Micropropagation and shoot organogenesis of selected clones of *Eucalyptus tereticornis*

Establishment of aseptic cultures

Following surface disinfection with mercuric chloride, nodal explants were inoculated on MS medium variously supplemented with BA (0.0-5.0 µM) and NAA (0.0-1.0 µM) (Table- 4.1). Browning of medium at the base of explants was observed after 6-7 days of inoculation, possibly due to leaching of phenolics, leading to the death of the explants. To avoid browning, explants were sub-cultured with in 3-4 days of inoculation in to fresh medium until the process of leaching of phenolics slows down. After 6-8 weeks about 30 % of the explants showed sprouting (Table 4.1) from the nodes which later on resulted in the formation of shoot clumps after 2-3 cycles of sub-culturing on MS medium supplemented with PGRs (Fig. 4.1C). Whereas explants cultured on PGR-free medium did not sprout and died after 6-8 weeks. The sprouting was observed from nodal segments taken from freshly coppiced shoots, whereas nodes taken from mature plants did not sprout. Experiments were carried out to study the effect of different concentrations of BA and NAA on sprouting of explants. The concentration of cytokinin influenced sprouting from nodal explant, higher concentrations of BA (more than 2.5 µM) did not favour sprouting from nodal explants (Table-4.1). Better response was observed on medium supplemented with 2.5 µM BA and 0.5 µM NAA (Table 4.1). Following successful initiation of culture, newly formed shoots were excised from the explant and further cultured on MS medium supplemented with 2.5 µM BA and 0.5 µM NAA to increase the number of shoots for further work.

Table 4.1 The effect of different concentrations of BA and NAA on shoot induction from nodal explants taken from coppiced shoots of *E. tereticornis* on MS medium

Combinations of plant growth regulators (µM) supplemented to culture medium (a + b)		**Percent explants showing shoot induction**
BA (a)	NAA (b)	
0.0	0.0	0.0 ± 0.00 h
0.5	0.0	8.8 ± 0.11 g
1.0	0.0	11.1± 0.18
2.5	0.0	17.7 ± 0.19 d
5.0	0.0	13.3 ± 0.17 f
0.5	0.5	13.1 ± 0.16 f
1.0	0.5	23.2 ± 0.16 c
2.5	0.5	30.5 ± 0.14 a
5.0	0.5	26.4 ± 0.09 b
0.5	1.0	15.5 ± 0.12e
1.0	1.0	21.2 ±0.10 c
2.5	1.0	26.1 ± 0.11 b
5.0	1.0	22.8 ± 0.13 c

Data was recorded after 6-8 weeks of inoculation of nodal explants. Means with the same letter within a column are not significantly different according to Duncan's multiple range test ($P < 0.05$), values are mean ± standard deviation

Effect of cytokinines on shoot proliferation and shoot elongation

The effect of different concentrations of BA, KIN and TDZ in combination with 0.5 µM NAA was examined on shoot proliferation and elongation of microshoots. Maximum numbers of 342 shoots per culture vessel were observed on MS medium supplemented with 2.5 µM BA (Fig. 4.1D, Table 4.2), whereas maximum number of elongated shoots per culture vessel (54, Fig. 4.1E-F) along with maximum shoot length (4.17 cm) was observed on MS medium supplemented with 0.1 µM BA. Out of the three tested cytokinins namely BA, KIN and TDZ; BA was found to be best for both shoot proliferation and shoot elongation followed by KIN and TDZ (Table 4.2).

Table 4.2 The effect of different cytokinins on proliferation and elongation of microshoots of *E .tereticornis* on MS medium

Cytokinin (μM)	Average no. of shoots proliferated/culture vessal	Average no. of shoots elongated/culture vessal	Average shoot length (cm)
BA 0.0	75±0.82ef	33 ± 1.23b	2.8 ± 0.21bcd
0.1	84±1.47def	54 ±1.18a	4.17 ± 0.38a
0.5	124 ± 1.11cd	25 ±1.08d	2.08 ± 0.26d
1.5	235 ± 1.66 b	00± 0.00f	00.00 ± 0.00h
2.5	342 ± 1.58a	00 ± 0.00f	00.00 ± 0.00h
KIN 0.1	89 ± 1.11def	35 ±1.11b	3.7 ± 0.21a
0.5	101 ± 1.21cdef	30 ± 1.10bc	3.4 ± 0.18ab
1.5	115 ± 1.43cde	28 ± 0.82cd	2.8 ± 0.16cd
2.5	142 ± 1.47c	24 ± 0.78d	2.7 ± 0.29cd
TDZ 0.1	73 ± 1.53ef	24 ± 0.86d	3.6 ± 0.23a
0.5	99 ± 0.82cdef	10 ± 0.56e	2.8 ± 0.32bc
1.5	134± 1.09c	9 ± 0.81e	2.4 ± 0.33bc
2.5	143 ± 1.22c	0 ± 0.00f	0.00 ± 0.00h

All media combinations were supplemented with 0.5μM NAA. Data was recorded after four weeks of incubation. Means with the same letter within a column are not significantly different according to Duncan's multiple range test ($P< 0.05$), values are mean ± standard deviation Initial number of shoot buds at the time of sub-culturing was kept to 15-20 per clump and 3 three shoot clumps were used per culture bottle, shoot height at the time of sub-culturing was 0.5–0.8 cm

Figure 4.1 A. Elite plants of *E. tereticornis* used in the present study for established of aseptic culture and subsequent experiments **B.** Actively growing fresh coppiced shoots of *E. tereticornis* used for collection of nodal explants **C.** Newly formed shoots of *E. tereticornis* on MS medium supplemented with 2.5 µM BA and 0.5 µM NAA **D.** Shoot multiplication on MS medium supplemented with 2.5 µM BA and 0.5 µM NAA **E - F.** Shoot Elongation on MS medium supplemented with 0.1 µM BA and 0.5 µM NAA

Effect of inoculum size on shoot proliferation and elongation

Effect of initial shoot clump size on shoot proliferation and elongation was evaluated. Average number of elongated shoots per culture vessel was significantly higher (65) when the initial shoot clump of size 4-5 shoots was cultured on MS medium containing 0.1 µM BA along with 0.5 µM NAA as compared to larger shoot clumps of 15-20 shoots per clump (54). Average number of shoots proliferated per culture vessel were significantly lower (245 shoots per culture vessel) when smaller shoots clumps of size (4-5 shoots per clump) were cultured as compared to larger shoot clumps (15- 20 shoots per clump) were cultured (342 shoots per culture vessel) (Table 4.3).

Table 4.3 The effect of size of initial shoot clump on proliferation and elongation on microshoots of *E. tereticornis* on MS medium

Initial shoot clump size	**Average no. of shoots proliferated/culture vessel**	**Average no. of shoots elongated/culture vessel**	**Average shoot length (cm)**
Clump with 15-20 shoot buds	342 ± 1.58a	54 ± 1.23b	4.17 ± 0.30b
Clump with 4-5 shoot buds	245 ± 1.33b	65 ± 1.32a	5.23 ± 0.41a

Means with the same letter within a column are not significantly different according to Duncan's multiple range test ($P < 0.05$), values are mean ± standard deviation. Medium used for shoot proliferation was MS medium supplemented with 2.5 µM BA and 0.5 µM NAA and medium used for shoot elongation was MS medium supplemented with 0.1 µM BA and 0.5µM NAA

Effect of light source on shoot multiplication and growth

The effect of two light sources i.e. photosynthetically active radiations (PAR) or cool white fluorescent lights (CFL) was evaluated on shoot multiplication and growth. Both, number of shoots proliferated and elongated increased significantly in cultures incubated under PAR light as compared to those incubated under CFL (Table 4.4).

Table 4.4 The effect of light source on growth and morphogenesis of microshoots of *E. tereticornis*

Response	**Light Source**	
	PAR	**CFL**
Average no. of shoots proliferated/culture vessal* [1]	378 ± 1.51 a	342 ± 1.58 b
Average no. of shoots elongated/culture vessal* [2]	65 ± 1.35 a	54 ± 1.47 b
Average shoot length [2]	5.19 ±0.43 a	4.17± 0.30 b

Means with the same letter within a each row are not significantly different according to Duncan's multiple range test (P< 0.05). Values are mean ± standard deviation **,*** Each treatment consisted of four culture vessels containing three shoot clumps of 15-20 shoot buds and was repeated four times 1. Medium used for shoot proliferation was MS medium supplemented with 2.5 µM BA and 0.5 µM NAA. **2.** Medium used for shoot elongation was MS medium supplemented with 0.1 µM BA and 0.5 µM NAA

Effect of light source on chlorophyll content and osmotic potential

Effect of light source on was also evaluated on chlorophyll content in leaves and osmotic potential of sap of plants. Both, chlorophyll content and osmotic potential was higher in cultures incubated under PAR light as compared to CFL. The total chlorophyll content was 0.853 mg/g in case of cultures incubated under PAR light whereas chlorophyll content was 0.786 mg/g in case of cultures incubated under CFL. Similarly, osmotic potential of sap was also found to be higher (-0.9071) in cultures incubated under PAR light as compared to those incubated under CFL (-1.0119, Table 4.5).

Table 4.5: Chlorophyll content and osmotic potential of microshoots of *E. tereticornis* inoculated under different light source on MS medium

Light Source	**Chlorophyll content (mg/g)**	**Osmotic potential (MPa)**
PAR	0.853 ± 0.33	-0.9071± 0.23
CFL	0.786 ± 0.54	-1.0119± 0.34

The cultures were incubated under the respective light sources for four weeks and the samples were taken from the cultures and analyzed for chlorophyll content and osmotic potential. Three estimations were carried out from each culture vessel and experiment was repeated thrice. Medium used for shoot proliferation was MS medium supplemented with 2.5 µM BA and 0.5 µM NAA

Effect of PGRs on shoot organogenesis

The fully expanded leaves (fig) from elongated shoots cultured on MS medium supplemented with 0.1 µM BA and 0.5 µM NAA, were used as explants for the induction of shoot organogenesis. The effect of BA either alone or in combination with 2,4-D or NAA was examined to study the effect of different PGRs on shoot organogenesis from segments of expanded leaves taken from microshoots cultured on MS medium. Callus formation was observed in all explants cultured on all of the different PGR combinations. However, shoot organogenesis was observed in only some of the PGR combinations (Table 4.6, Fig.4.2 A-B) Explants cultured on medium containing 2,4-D showed a higher frequency (%) of shoot organogenesis than those cultured on MS medium supplemented with NAA. Maximum shoot organogenesis (29.6 %) was observed on medium supplemented with 5.0 µM BA and 1.0 µM 2,4-D with an average of 14.6 shoots per explant (Table 4.6).

Effect of leaf maturity

Leaf maturity was also found to influence the shoot organogenic response of leaf segments (third to seventh leaf from top of microshoot). The maximum shoot organogenic response was found when fifth leaf from the top was used as explant (14–16 days old), 40.5% of explants showed shoot organogenesis and a mean of 16.0 shoots per explant were found to diffrentiate. In comparison, the organogenic response decreased in both mature and younger leaves (Table 4.7).

Table 4.6 The effect of plant growth regulators on shoot organogenesis from leaf segments taken from microshoots of *E. tereticornis* on MS medium

Plant Growth Regulator (µM)		Percent explants showing shoot regeneration	Average no. of shoots / explant
BA	**2,4-D**		
0.0	0.0	00.0 ± 0.00 d	00.0 ± 0. 00 d
1.0	1.0	12.3 ± 1.06 b	06.3 ± 0.09 b
1.0	5.0	09.4 ± 0.18 c	05.0 ± 0.41 c
5.0	1.0	29.6 ± 1.42 a	14.6 ± 0.71 a
5.0	5.0	00.0 ± 0.00 d	00.0 ± 0.00 d
12.5	1.0	13.8 ± 0.89 b	05.4 ± 0.53 c
12.5	5.0	00.0 ± 0.00 d	00.0 ± 0.00 d
BA	**NAA**		
1.0	0.1	00.0 ± 0.00 d	00.0 ± 0.00 d
1.0	0.5	00.0 ± 0.00 d	00.0 ±0.00 d
5.0	0.1	00.0 ± 0.00 d	00.0 ± 0.00 d
5.0	0.5	00.0 ±0.00 d	00.0 ± 0.00 d
12.5	0.1	13.5 ± 0. 85 b	05.3 ± 0.41 c
12.5	0.5	00.0 ± 0.00 d	00.0 ± 0.00 d

Cultures were sub-cultured on same medium at 4-week interval. Data were scored after 8 weeks of inoculation. Values sharing a common letter within the column are not significant at $P < 0.05$
Values are mean ± standard deviation

Table 4.7 The effect of leaf maturity on shoot organogenesis from leaf segments taken from microshoots of *E. tereticornis* on MS medium supplemented with 5.0 µM BA with 1µM 2,4-D

Leaf number (from top to bottom)	Percent explants showing shoot regeneration	Average no. of shoots / explant
3rd	33.2 ± 1.23 bc	15.1± 0.83 bc
4th	36.5 ± 1.11 b	15.3 ± 0.83 b
5th	40.5 ± 1.29 a	16.0 ± 0.90 a
6th	30.0 ± 1.21 c	14.8 ± 0. 78 c
7th	30.3 ± 0.91 c	14.8 ± 1.11 c

Data were scored after 8 weeks of inoculation and sub-culturing was carried out at 4 weeks interval on same medium. Values sharing a common letter within the column are not significant at $P < 0.05$, values are mean ± standard deviation

Effect of antibiotics on shoot organogenesis

Effect of antibiotics namely cefatoxime, carbenicillin and cephalexin was evaluated on shoot organogenesis potential of leaves of *E. tereticornis*. Incorporation of cefotaxime in the medium increased the response of explants in terms of both frequency of explants (%) resulting in shoot organogenesis and the number of shoots differentiated per explant. Increase in concentrations of cefatoxime resulted in increased higher number of explants showing shoot organogenesis and an increased number of shoots per explant were also recorded. Maximum shoot organogenesis occurred in explants cultured on medium containing 500 mg/l cefotaxime (44. 6%) in comparison, 29.6% of explants cultured on medium devoid of cefotaxime resulted in shoot organogenesis. Carbenicillin and cephalexin inhibited shoot organogenesis from leaf explants. A complete inhibition of shoot organogenesis was observed when 500 mg/l cephalexin was added to the culture medium (Table 4.8).

Table 4.8 The effect of different antibiotics on shoot organogenesis from leaf segments taken from microshoots of *E. tereticornis* on MS medium supplemented with 5.0 µM BA with 1µM 2,4-D

Antibiotic	Concentration (mg/l)	Percent explants showing shoot regeneration	Average no. of shoots/ explant
Cefotaxime	0	29.6 ± 1.33 cd	13.9 ± 0.29 c
	100	29.9 ± 1.28 cd	14.0 ± 0. 45 c
	300	38.0 ± 1.48 b	14.6 ± 0.44 b
	500	44.6 ± 1.41 a	15.2 ± 0.90 a
Carbenicillin			
	100	31.2 ± 1.23 c	13.8 ± 0.86 c
	300	28.2 ± 1.63 cd	13.5 ± 0.78 d
	500	23.7 ± 1.41 de	12.6 ± 0.91 e
Cephalexin			
	100	21.6 ± 1.09 e	10.9 ± 0.91 f
	300	10.8 ± 1.11 f	07.1 ± 0.93 g
	500	0.0 ± 0.00 g	0.0 ± 0.00 h

Data were scored after 8 weeks of inoculation and sub-culturing was carried out at 4 weeks interval on same medium. Values sharing a common letter within the column are not significant at P< 0.05, values are mean ± standard deviation

Histological studies

Histological examination revealed that some of the cells in the surface layer of the callus showed intense cell division and were organized into meristematic zones. These meristematic zones grew into shoot buds that showed a vascular connection with the parent tissues (Fig. 4.2 C-D).

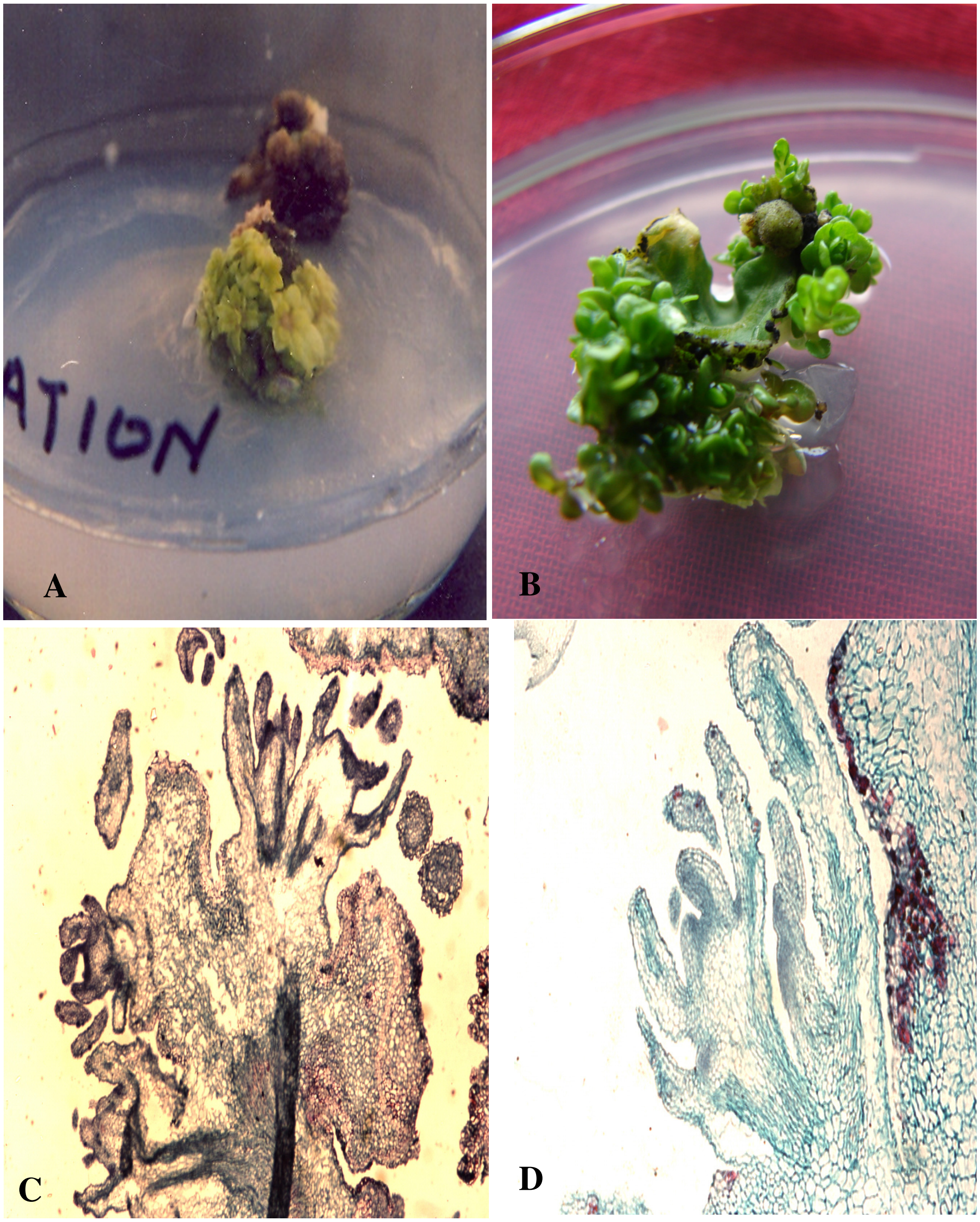


Figure 4.2 Shoot organogenesis and histological analysis of *E. tereticornis* **A-B.** Shoot organogenesis from leaf explants of *E. tereticornis* on MS medium supplemented with 5.0 µM BA and 1.0 µM 2,4-D **C-D.** Section of explant (10 µm thick) showing shoots organogenesis

Effect of clonal variations

The shoot organogenic potential varied amongst different clones of *E. tereticornis*. A higher frequency of explants resulted in shoot organogenesis and callus formation when the 'T1' clone was used as compared to the 'CE2' and 'Y8' clones. In terms of shoot organogenesis and callus formation, clone 'CE2' performed better than clone 'Y8' (Fig 4.3).

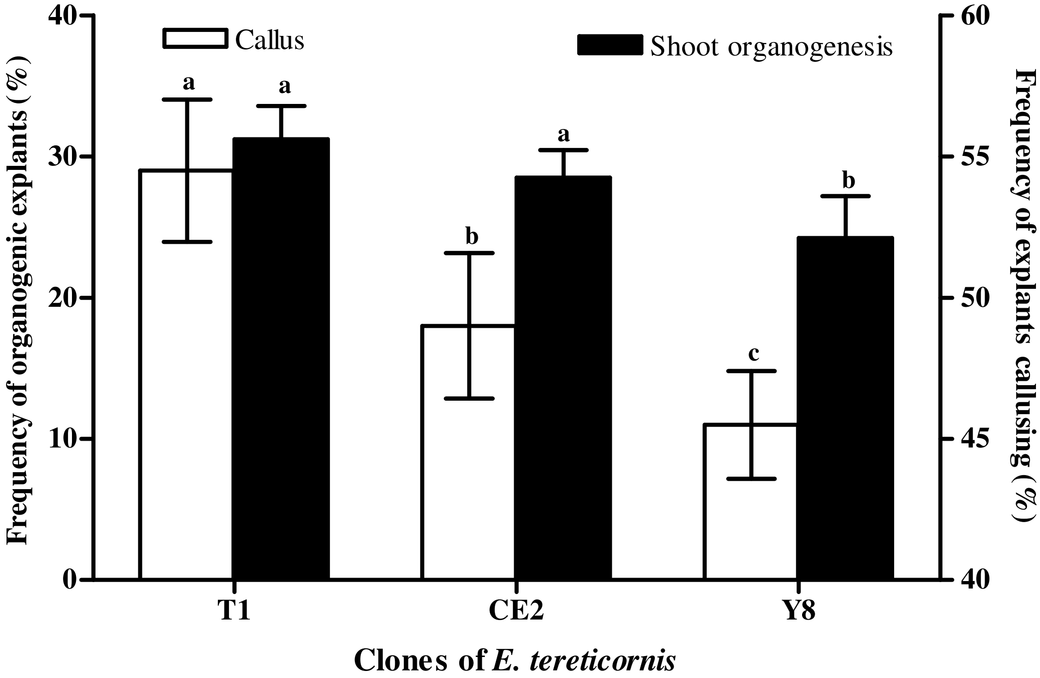


Figure 4.3 Shoot regeneration potential of different clones of *E. tereticornis* from leaf segments taken from microshoots (fifth from top) on MS medium supplemented with 5.0 μM BA and 1.0 μM 2,4-D. Data were scored after 8 weeks of culture and sub-culturing was carried out at 4-week intervals on the same medium. Values are given as the mean ± standard deviation

Rooting of microshoots

The effect of various auxins i.e. NAA, IBA and IAA and medium strength (MS, 1/2 MS and 1/4 MS) was examined on rooting efficiency of microshoots. Maximum rooting of shoots (80.66 %) was observed on 1/4 MS medium supplemented with 5.0 μM IBA. Maximum numbers of roots (4.25) per rooted shoot was also observed on same medium, whereas maximum root length (2.90 cm) was recorded on full strength MS medium supplemented with 5.0 µM IBA (Table 4.9, Fig. 4.4 A-B).

Table 4.9 The effect of MS concentration (full-strength MS, 1/2 MS and 1/4 MS) and auxins on rooting of microshoots of *E. tereticornis*

Medium	Auxin (µM)	Percentage of shoots showing rooting	Average no. of roots per shoot	Average root length (cm)
MS	0.0	00.00 ± 0.00 g	00.00 ± 0.00 g	0.00 ± 0.00 i
MS	NAA 1.0	60.8 ±1.65 d	2.64 ± 0.06 d	1.01 ± 0..06 e
MS	2.5	55.53 ± 1.87ef	2.13 ± 0.11 e	0.84 ± 0.04 f
MS	5.0	57.0 ± 1.91 e	2.97 ± 0.10 d	1.33 ± 0.08 d
MS	IBA 1.0	54.46 ± 1.81 f	2.19 ± 0.13 e	0.64 ± 0.04 g
MS	2.5	63.0 ± 1.98 c	2.92 ± 0.09 d	1.30 ± 0.08 d
MS	5.0	72.0 ± 2.01 b	3.8 ± 0.11 b	2.90 ± 0.11 a
MS	IAA 1.0	55.6 ± 2.02 ef	1.38 ± 0.08 f	0.54 ± 0.06 h
MS	2.5	57.13 ±1.78 e	1.36 ± 0..08 f	0.74± 0.07 g
MS	5.0	62.80 ± 1.25 c	1.56 ± 0.06 f	1.05 ± 0.06 e
1/2 MS	IBA 5.0	73.66 ± 1.19 b	3.4 ± 0.10 c	2.49± 0.04 b
1/4 MS	5.0	80.66 ± 1.89 a	4.25 ± 0.08 a	2.01 ± 0.11 c

Means with the same letter within a column are not significantly different according to Duncan's multiple range test ($P< 0.05$). Values are mean of 3 experiments, each treatment consisted of 15 shoots (in triplicate) and experiment was repeated thrice, values are mean ± Standard deviation

Effect of light source on rooting

The effect of two light sources i.e. photosynthetically active radiations (PAR) or cool white fluorescent lights (CFL) was evaluated on rooting. Percent shoots rooting, root length and number of roots per rooted shoot, increased significantly in cultures incubated under PAR. Cultures incubated under PAR light showed root emergence on 5th day, whereas 8 days were required for root emergence from cultures incubated under CFL (Table 4.10).

Table 4.10 The effect of light source on rooting of microshoots of *E. tereticornis*

Response	Light Source	
	PAR	CFL
Rooting of microshoots (%) $^{\# 3}$	84.66 ± 2.11 a	80.66 ± 2.08 b
No. of roots per rooted shoot $^{\# 3}$	5.02 ± 0.66 a	4.25 ± 0.78 b
Average root length $^{\#}$	3.40 ± 0.81 a	2.01 ± 0.88 b
Days required for emergence of first root	5	8
Plant Survival (%) **	74.6 ± 1.44 a	70.4 ± 1.45 b

Means with the same letter within a each row are not significantly different according to Duncan's multiple range test ($P< 0.05$), values are mean ± standard deviation. # Each treatment consisted of 15 shoots (in triplicate) and experiment was repeated thrice. 3. Medium used for rooting was ¼ MS medium supplemented with 5.0µM IBA. ** Values are average of 100 plants and data were recorded 6 weeks following transfer of plantlets into polythene bags and maintained polyhouse conditions.

Acclimatization of plantlets

In order to achieve the maximum ex- vitro survival of micropropagted plants and to get the quality planting material special efforts were made for acclimatization of plantlets. Effect of light source (plantlets proliferated and rooted under PAR and CFL) was also checked on the survival of plantlets. It was observed that number of plants survived was significantly higher (74.6 %) when these were propagated under PAR light as compared to plantlets propagated under CFL (Table 4.10, Fig 4.4C). Further, the survival rate was increased when plantlets were inoculated with bacterial isolates (at the time of transfer to soil) tested for their fungal antagonistic properties namely *Bacillus subtilis* and *Pseudomonas corrugate* (isolated from Himalayan region). With this treatment, plant survival was increased to 84 % when inoculated with *Bacillus subtilis* followed by plantlets inoculated with *Pseudomonas corrugata* (80.8%), whereas 74.6 % survival was recorded in plants which were inoculated with no bacteria (Table 4.11). Moreover, the plants inoculated with *B. subtilis* showed faster growth and more number of leaves.

Table 4.11 The effect of bacterial inoculations on survival and growth of plantlets of *E. tereticornis*

Treatment	**Survival of plants (%)**	**Increment in plant height over initial* (mm)**	**Increment in leaf no. per plant over initial***
Control	74.6 ± 1.14 c	11.06 ± 0.08 c	4.86 ± 0. 09 c
Bacillus subtilis	84 ± 1.48 a	19.26 ± 0.11 a	9.26 ± 0.10 a
Pseudomonas corrugate	80.8 ± 1. 56 b	16.13 ± 0.12 b	6.42 ± 0.09 b

Means with the same letter within a column are not significantly different according to Duncan's multiple range test ($P < 0.05$).Values are average of 100 plants and data were recorded 6 weeks following transfer of plantlets into polythene bags and maintained under similar polyhouse conditions.* At the time of transfer the approximate length of plantlets was 40 mm with four leaves, values are mean ± Standard deviation

Clonal fidelity of *in vitro* raised plants

The RAPD and ISSR profiles of plants derived from newly formed shoots and those of the mother plant were similar, indicating the clonal nature of these plants (Fig.4.5). Of the 40 primers used (20 each of RAPD and ISSR), 16 RAPD and 12 ISSR primers resulted in the amplification of DNA fragments. These primers produced a maximum of seven and a minimum of two bands. Of the total of 133 markers obtained, 58 markers were scored with using ISSR primers (Table 4.11) and 75 markers were scored using RAPD primers (Table 4.12). The size of the amplified markers ranged from 200 to 2,500 bp. This similarity in the banding profiles of the RAPD (Fig.4.5 A-B) and ISSR (Fig.4.5 C-D) markers in plants derived from newly differentiated shoots and the mother plant established the clonal nature of these shoots.

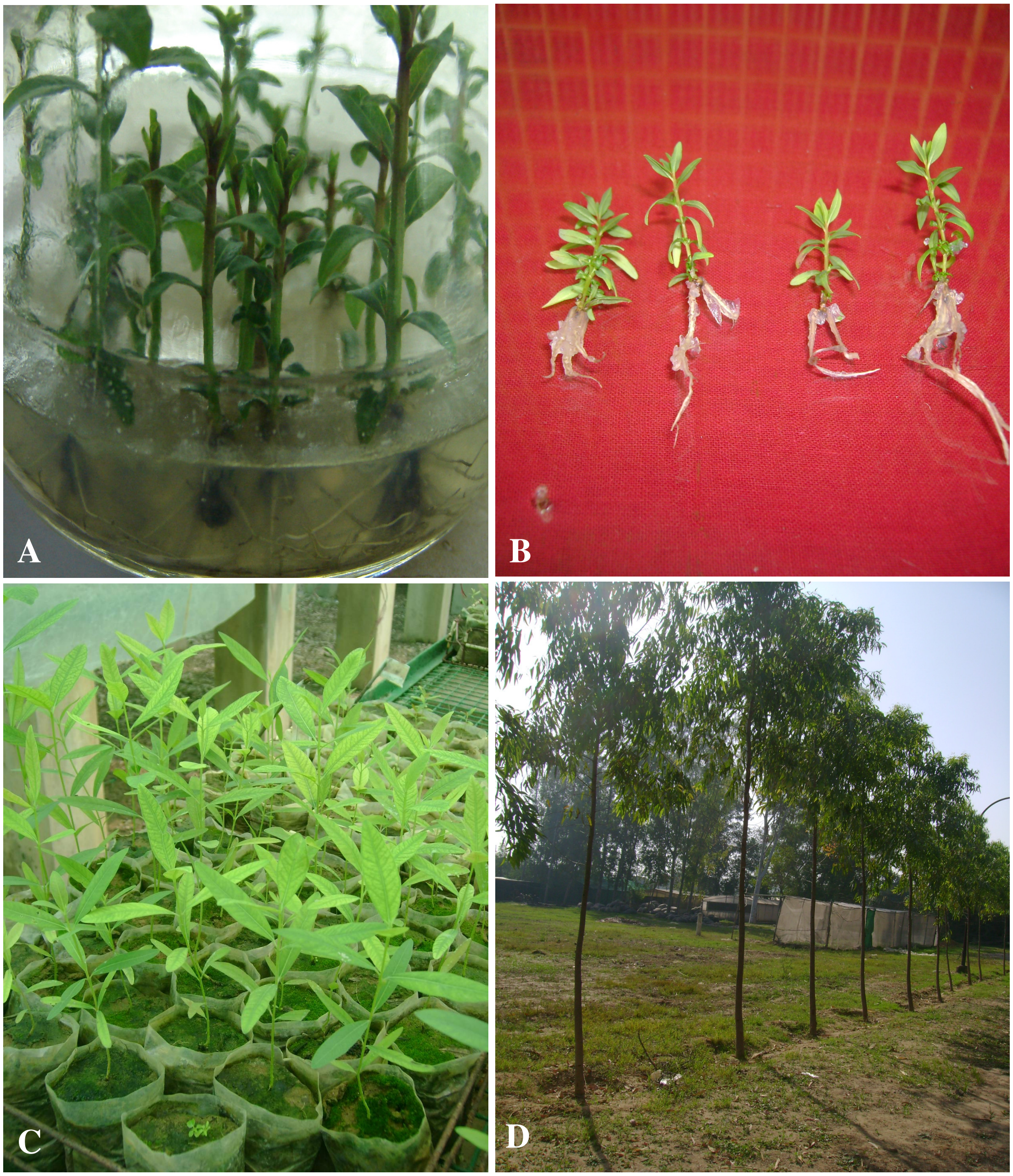


Figure 4.4 Rooting and acclimatization of *E. tereticornis* **A-B.** Microshoots rooted on one fourth-strength MS medium supplemented with 5.0 µM IBA **C.** Acclimatized plantlets under green house conditions **D.** 1 year old tissue culture raised plants growing in the field

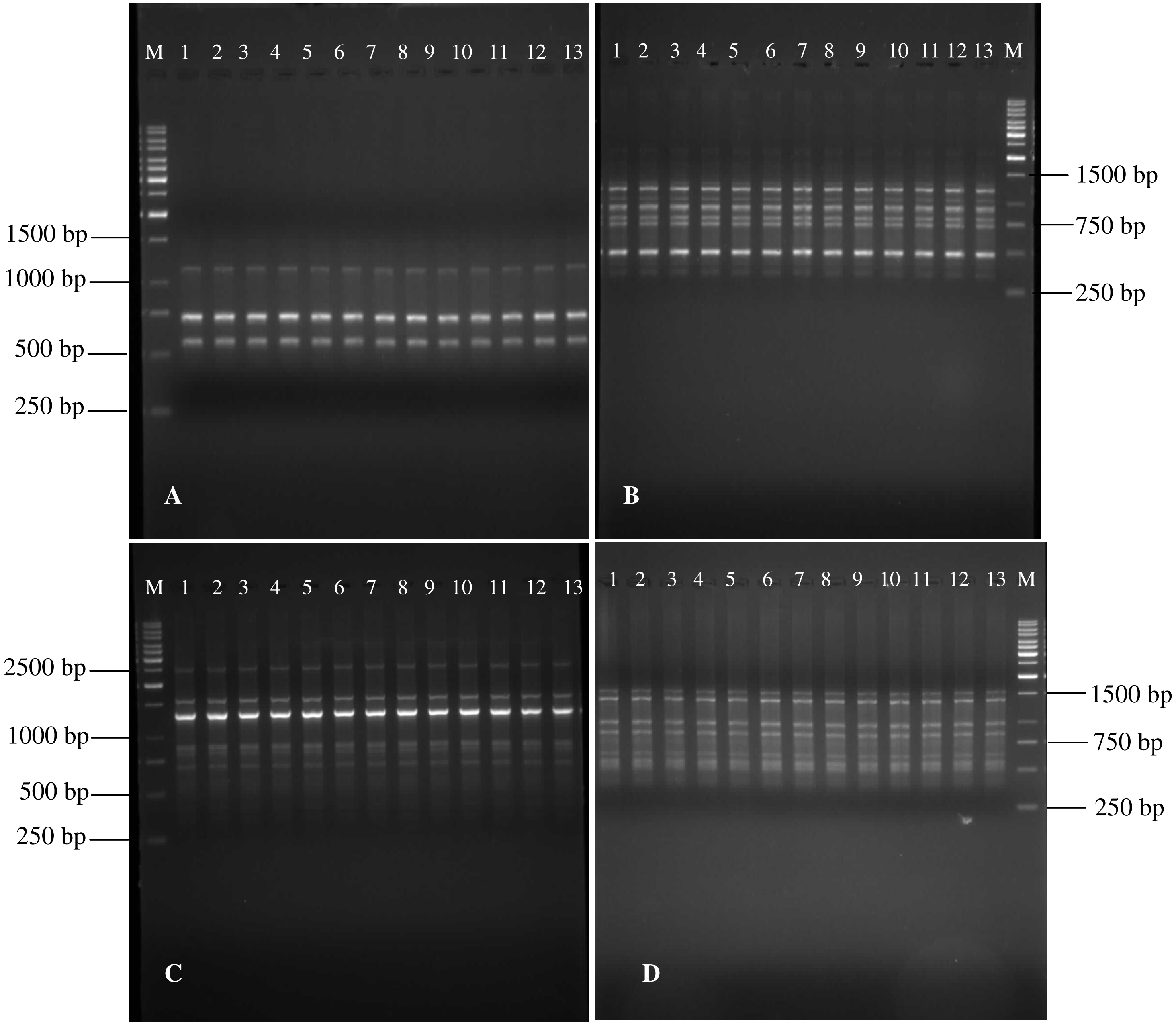


Figure 4.5 RAPD and ISSR profiles of micropropagated plantlets and mother plant of *E. tereticornis* plants using RAPD primers **A:** OPD-6; **B**: OPD – 20 and ISSR primers **C:** ISSR-2; **D:** ISSR-13. Lane 1: Mother Plant; Lane 2-13: micropropagated plants; Lane M: 1 kb molecular weight markers

Table 4.11 Sequence of various primers used in ISSR profiling of regenerated plants and the number of bands amplified and their size range (bp).

PRIMER NO.	PRIMER SEQUENCE (5'-3')	NO. OF BANDS	SIZE RANGE [BP]
ISSR-1	$(CA)_8CG$	6	500-2000
ISSR-2	$(GA)_8CG$	5	250-1500
ISSR-3	$(GA)_8TC$	5	250-1000
ISSR-4	$(AC)_8GCGC$	--	--
ISSR-5	$(AC)_8$	5	250-1000
ISSR-6	$(CA)_8TG$	7	500-1500
ISSR-7	$(CA)_8GC$	5	750-1500
ISSR-8	$(GA)_8TA$	6	500-1500
ISSR-9	$(GC)_8T$	--	--
ISSR-10	$(GC)_8A$	--	--
ISSR-11	$(GC)_8AT$	--	--
ISSR-12	$(CT)_8G$	6	500-2000
ISSR-13	$(CT)_8A$	3	500-2500
ISSR-14	$(CT)_8AG$	2	500-1000
ISSR-15	$(GT)_8A$	3	500-1500
ISSR-16	$(GT)_8C$	5	500-2500
ISSR-17	$(AT)_8C$	--	--
ISSR-18	$(AT)_8G$	--	--
ISSR-19	$(AT)_8GC$	--	--
ISSR-20	$(AT)_8$	--	--

Table 4.12 Sequence of various primers used in RAPD profiling of regenerated plants and the number of bands amplified and their size range(bp).

PRIMER NO.	PRIMER SEQUENCE (5'-3')	NO. OF BANDS	SIZE RANGE [BP]
OPD-1	ACC GCG AAG G	5	500-1500
OPD -2	GGA CCC AAC C	5	500-2500
OPD -3	GTC GCC GTC A	4	250-1500
OPD -4	TCT GGT GAG G	--	--
OPD -5	TGA GCG GAC A	3	250-1000
OPD -6	ACC TGA ACG G	3	500-1500
OPD -7	TTG GCA CGG G	--	--
OPD -8	GTG TGC CCC A	3	250-1500
OPD -9	CTC TGG AGA C	4	500-1500
OPD -10	GGT CTA CAC C	3	250-1000
OPD -11	AGC GCC ATT G	--	--
OPD -12	CAC CGT ATC C	7	500-2500
OPD -13	CTT CCC CAA G	5	250-1500
OPD -14	CAT CCG TGC T	5	250-1500
OPD -15	AGG GCG TAA G	4	250-1000
OPD -16	TTT CCC ACG G	6	250-1500
OPD -17	GAG AGC CAA C	5	250-2500
OPD -18	CTG GGG ACT T	6	250-2500
OPD -19	CTG CGG TCA G	--	--
OPD -20	ACC CGG TCA C	7	250-1500

Discussion

Micropropagation, at present is one of most efficient and reliable method for the mass multiplication of true to type plants (Altman and Loberant, 1998). The number of species cultured *in vitro* has been steadily increasing over the last two decades (Hartman *et al.* 1990; Bonga and Durzan 1987; Vasil and Vasil 1980). However, studies on tree tissue culture have received little attention, consequently the success in micropropagting forest trees has been rather slow and limited (Bisht *et al.* 1998; Bajaj 1989). Nevertheless, micropropagation protocols for several trees species have been developed (Vengadesan and Pijut 2009; Sood *et al.* 2002; Sharma and Ramamurthy 2000; Kumar *et al.* 1999; Shekhawat *et al.* 1993; Rai and Chandra 1989) but reproducibility has been achieved only in a few cases. Therefore, the present study was focused to optimize and standardize each step in micropropagation protocol of selected elite clones of *E. tereticornis*, so that large number of plantlets can be obtained in shorter time frames. The factors affecting micropropagation and subsequent acclimatization of elite clone of *E. tereticornis* have been investigated. Further, shoot organogenesis/somatic embryogenesis are the prerequisite for the clonal propagation.

One of the major problems with miropropagation is the establishment of aseptic cultures from mature tissue, as mature tissue often turns recalcitrant to vegetative propagation because of problem of loss of juvenility (Jones and Van Staden 1997). In the present study, induction of rejuvenality by coppicing the mature trees was beneficial for establishment of cultures from nodal segments. Therefore, it may be necessary to overcome the problem of loss of juvenility, a condition closely associated with a reduced capacity for clonal propagation. It is always easier to propagate juvenile tissues. Establishment of juvenile characteristics prior to *in vitro* culture has proved to be important for the establishment of aseptic cultures from mature tissue (Thorpe *et al.*

1991). Coppicing has been shown to increase juvenile characteristics of adult tissue and allows mature tissue to be easily propagated *in vitro* (Jones and Van Staden 1997). Therefore in present study, explants have been collected after coppicing of the elite plant of *E. tereticornis* (10 years old) and these explants proved to be beneficial for culture establishment from such mature tissue.

The effect of different cytokinins was tested on shoot multiplication and elongation. Earlier, many studies have focused on study the effect of a cytokinin and their concentrations in different plant species (Kaur *et al.* 1999; Hutchinson and Zimmerman 1987). In general, different concentrations of BA in combination with the auxins like NAA and/or IAA were beneficial for the induction of shoot buds *in vitro* (Kaur *et al.* 1999). In the present study also BA, KIN and TDZ in combination of NAA were tested for shoot multiplication and elongation in *E. tereticornis*. All the three tested cytokinins promoted shoot multiplication. However, BA was found to be more effective than KIN and TDZ (Table 4.2). Higher concentrations (> 2.5 μM) of BA promoted shoot multiplication whereas lower concentrations (< 1.0 μM) were beneficial for shoot elongation (Table 4.2). Earlier, beneficial effect of BA over other cytokinins for shoot multiplication/organogenesis was reported (Vengadesan and Pijut 2009; Rout *et al.* 2008). Cytokinins were the first compounds recognized for their ability to induce cell division in certain plant tissues, are now known to evoke a diversity of responses in plants (Letham 1978). Roots are considered as site of cytokinins biosynthesis and known to move to xylem to the shoot where these are known to control of both development and senescence (Letham and Palni 1983). Moreover, higher concentrations cytokinins are known to suppress apical dominance and thus stimulate shoot multiplication (George 1996). The requirement of NAA for shoot multiplication/elongation could be due to its reported role in elimination of phenolic substances by competing for the active sites of auxin oxidase enzyme involved in oxidization of phenols (Perez-Tornero *et al.* 2000), thus helping BA in production of multiple shoots (Sugimura *et al.* 2005). Earlier, presence of NAA along with BA in the medium has been reported to improve shoot induction and multiplication (Saritha and Naidu 2008; Thomas 2007; Kaur *et al*. 1999).

Effect of initial shoot clump size on shoot proliferation and elongation was also evaluated. Smaller shoot clumps (4-5 shoots per clump) led to rapid shoot elongation, while larger shoot clumps (15-20 shoots per clump) showed poor shoot elongation and better shoot multiplication (Table 4.3). The effect of initial inoculum size on shoot multiplication/elongation has been related to activity of enzymes involved in different metabolic pathway influencing plant growth (Contin *et al.* 1998). Plant cells required a critical minimum inoculum density for growth. However, the optimum inoculum size is highly variable among different plants species (Figueiredo *et al.* 2000). The effect of inoculum size on shoot multiplication of *Eucalyptus* seems to be noval observation.

Effect of two light sources i.e., PAR and CFL on shoot multiplication, growth and rooting was also investigated. PAR light was found to be useful both for shoot multiplication and elongation (Table 4.4). Light quality is a critical factor influencing plant growth and development (Lee *et al.* 2007). Tanaka *et al.* (1998) reported that the mixture of red and blue light enhance plant growth and development by increasing the rate of photosynthesis because of the fact that spectral energy distribution of red and blue light coincided with that of chlorophyll absorption (Kumar *et al.* 2003; Goins *et al.* 1997). The emission spectra of PAR light used in this study has two major peaks i.e. at 410-450 nm and 650-680 nm, while emission was in a broad range from 380-660 nm in case of CFL (Kumar *et al.* 2003). Significant increase in chlorophyll content was also observed in cultures incubated under PAR light as compared with cultures incubated under CFL (Table 4.5). These results are in line with earlier findings of Lee *et al.* (2007) and Kumar *et al.* (2003).

Cultures incubated under PAR light had higher osmotic potential as compared to cultures incubated under CFL (Table 4.5). Osmotic potential is an important physiological parameter, which is reported to influence growth of cultures and subsequent survival of plants during acclimatization (Hernandey-Sebasita *et al.* 1999). Light is reported to control expression and post translational regulation of nitrate reductase gene which is known to influence water relations and growth of plants (Neill *et al.* 2003; Appenroth *et al.* 2000; Sharma *et al.* 1999a). Kumar *et al.* (2003) also reported the increase in osmotic potential of plants incubated PAR light.

In this study, shoot organogenesis is reported from selected elite clones of *E. tereticornis*. It has been reported that the lack of an efficient shoot organogenesis/somatic embryogenesis protocol is a bottleneck for undertaking the genetic manipulation of selected clones of *Eucalyptus* spp., including *E. tereticornis* (Tournier *et al.* 2003). The shoot organogenesis protocol reported here may provide the experimental means for developing an efficient genetic improvement programme for these clones. There are successful reports of shoot organogenesis (Tournier *et al.* 2003; Prakash and Gurumurthi 2005; Arezki *et al.* 2001; Ho *et al.* 1998; Mullins *et al.* 1997; Muralidharan and Mascarenhas 1987) and somatic embryogenesis (Pinto *et al.* 2008) in a number of *Eucalyptus* species. However, a literature search was unable to find a single report on shoot organogenesis from explants taken from selected clones of *E. tereticornis*, although shoots organogenesis (Prakash and Gurumurthi 2005; Subbaiah and Minocha 1990) and somatic embryogenesis (Prakash and Gurumurthi 2005) has been reported from juvenile tissues such as embryos on young seedlings. Subbaiah and Minocha (1990) were able to achieve direct multiple shoot organogenesis from hypocotyl segments cultured for 4–6 weeks on medium supplemented with BA. The formation of somatic embryos and subsequent conversion of these into plants has been reported from mature zygotic embryos of *E. tereticornis* through the callus phase (Prakash and Gurumurthi 2005). However, protocols using juvenile tissue from seeds/seedlings as the explant source do have a number of drawbacks even though juvenile tissues are known to respond easier than tissues taken from plants of selected clones (Liu and Pijut 2008). Shoots differentiated from seedlings are likely to show variations from the mother plant, and such cultures may not be useful for micropropagation and/or being used in genetic improvement programmes of selected elite plants.

Among the different combinations of PGRs tested, shoot organogenesis was observed on media containing combinations with higher concentrations of BA and lower concentrations of auxins (2,4-D or NAA) (Table 4.6). The requirement of BA for the induction of shoot organogenesis has been reported (Ganeshan *et al.* 2006; Prakash and Gurumurthi 2005; Subbaiah and Minocha 1990).

The stage of maturity of the leaf tissue (explants) was also found to influence shoot organogenic potential of these tissues. Middle leaves (third, fourth, fifth) showed a higher potential for shoot organogenesis than younger and mature leaves. Maximum shoot organogenic potential was observed when explants were taken from from the fifth leaf (Table 4.7). A similar finding in terms of the effect of leaf position on shoot organogenesis has been reported for apple and *Anthurium andraeanum* (Martin *et al.* 2003, Yepes and Aldwinckle 1994). The difference in the shoot organogenic potential of the proximal end to distal ends may be due to differences in the maturity of leaves and/or variation in the endogenous levels of PGRs, as reported earlier (James *et al.* 1988, Welander 1988) in case of apple leaves. Many other compounds, in addition to PGRs, are known to influence growth and morphogenesis under both natural and tissue culture conditions (Yu and Wei 2008; Tiwari *et al.* 2006; Teixeira da Silva and Fukai 2001; Nandi *et al.* 1996). The most important of these are antibiotics, which are used in plant tissue culture for different purposes (Yu and Wei 2008; Tiwari *et al.* 2006; Teixeira da Silva and Fukai 2001). In our study, the incorporation of cefotaxime into the medium significantly improved shoot organogenesis from 29.6 to 44.6% explants, whereas the addition of carbenicillin to the medium inhibited shoot organogenesis and that of cephalexin completely inhibited shoot organogenesis (Table 4.8).The beneficial effect of cefotaxime on somatic embryogenesis in wheat has also been reported (Yu and Wei 2008). Li *et al.* (2002) reported the complete inhibition of shoot organogenesis in *Rosa hybrida* cv. 'Carefree Beauty' on medium containing carbenicillin. The growth regulatory activity of these antibiotics has been attributed to their interference with the metabolism of PGRs. Cefotaxime has been shown to interfere with ethylene biosynthesis (Pius *et al.* 1993), whereas carbenicillin may break down into active auxins, such as phenylacetic acid (Holford and Newbury 1992). Therefore, the observed effect of these antibiotics on shoot organogenesis (Table 4.8) could be due to alterations in the endogenous levels of certain PGRs.

The histological studies revealed that the shoot buds differentiated from the surface layers of the explants/callus. Meristematic activity was observed in some of the cells from the surface layer, which subsequently organized into the shoot buds. This is evident from the vascular connection of these shoot-like structures with the parent tissues. Earlier also histological examinations were successfully carried out to study different developmental stages resulting in shoot differentiation were carried out in different plants (Sharma and Millam 2004; Kumar *et al.* 1999).

The three clones of *E. tereticornis* tested in this study showed considerable variation in terms of their shoot organogenic potential (Fig. 4.1). Similar clone specific differences have been reported earlier in *E. camaldulensis* (Mullins *et al.* 1997). Therefore, it is important to develop clone-specific protocols for organogenesis before including any such protocol in an improvement programme based on plant genetic manipulations.

Rooting of microshoots is the first step during hardening of plantlets before preparation for transplanting to the field. Auxins are widely used for induction of roots in microshoots. In this study root induction and root development was observed in all the tested media combinations (Table 4.9), however, maximum rooting of shoots (80.66%) was achieved on 1/4 MS medium supplemented with 5.0 µM IBA. Efficacy of IBA in the induction of rooting in microshoots has also been described in detail by Kato (1985) but the concentration and mode of treatment of IBA varied in different plants (Jha and Sen 1992; Kato 1985). For rooting of microshoots IBA has been reported to be more potent than NAA (Sharma *et al.* 1999b). The reduction of salts in MS was found to be beneficial for rooting of microshoots. Similar results were also reported in many *Eucalypt* species, where lowering of nutrient salt concentration in the medium increased the rooting ability of microshoots (Bennett *et al.* 1994; Sharma and Ramamurthy 2000). PAR light was also found to be more effective for efficient rooting than CFL (Table Table 4.10). Increase in rooting efficiency under PAR light may be due to the involvement of blue light responding cryptochromes and red/far-red light responding phytochromes as reported by Lin (2002). Light quality has been shown to promote rooting efficiency in some plant species (Kumar *et al.* 2003; Rossi *et al.* 1993).

In the present study special emphasis has been given to investigate factors affecting acclimatization of *in vitro* raised plants to increase survival rate and quality of planting material. A major limitation in large scale production of micropropagated plants is higher mortality rate during laboratory to land transfer, mainly due to the extreme differences between *in vitro* and *ex vitro* environmental conditions. In this study, plantlets produced under PAR light showed higher survival rate and growth following transfer to soil and also showed higher chlorophyll content (Table 4.10 and 4.5). Normally, plants produced under *in vitro* conditions develop poor photosynthetic competence and shows low growth potential (Kozai 1991; Brainerd and Fuchigami 1982). Plantlets produced under PAR light showed better survival and growth following transfer to green house conditions; possibly due to higher chlorophyll content. Moreover, modulation of photosynthetic efficiency is thought to be a key property during acclimatization (Jeon *et al.* 2005).

Inoculation of plantlets with bacterial isolates during acclimatization was found to be beneficial for the survival of plants and significant increase in length as well as percentage survival of plants inoculated with these bacterial isolates was observed (Table 4.11). These bacterial isolates namely *Bacillus subtilis* and *Pseudomonas corrugate* were isolated from different regions of the Indian Himalayas and were reported to possess antagonistic properties against various fungi (Trivedi and Pandey 2007). *In vitro* raised plants do not possess sufficient resistance against the soil microbial communities. Such plants succumb to microbial (especially fungal) attack following field transfer (Palni *et al.* 1998). To overcome this problem several antagonistic plant growth-promoting bacteria have been used for biological acclimatzation to increase the survival and augment overall plant growth of micropropagated plants (Ahmad *et al.* 2011; Pandey *et al.* 2002, 2000). These bacterial isolates were earlier used for biological hardening of micropropgated plantlets (Trivedi and Pandey 2007; Pandey *et al.* 2000). Present study confirms the usefulness of these bacterial isolates for biological acclimatization of micropropagated plants of *E. tereticornis.*

The banding profiles of the RAPD and ISSR markers indicate that all of the newly differentiated shoots were similar to each other and to the mother plant, thereby indicating their clonal nature. The utility of RAPD and ISSR markers for testing the clonal fidelity of micropropagated plants has been well documented (Saxena *et al.* 2011**;**

Kumar *et al.* 2010; Chandrika *et al.* 2008; Joshi and Dhawan 2007; Martins *et al.* 2004). RAPD and ISSR markers have also been successfully used for the genetic analysis of *Eucalyptus* (Rani and Raina 1998). In this study, differentiated shoots showed a high degree of genetic uniformity that may be due to the stability of the genome to aseptic manipulations and culture pressures during shoot organogenesis (Kumar *et al.* 2010).

Conclusion

In conclusion, an efficient, reproducible micropropagation and shoot organogenesis protocol has been developed from plants of selected elite clones of *E. tereticornis*. The differentiated shoots/plants were found to be genetically uniform and identical to the mother plants. This protocol can be successfully exploited to undertake the improvement of these clones through trait specific plant genetic manipulations.

Salient findings

- High-frequency shoot multiplication and elongation was achieved on MS medium supplemented with 2.5 µM BA and 0.5 µM NAA and supplemented with 0.1 µM BA and 0.5 µM NAA respectively.
- The initial size of the shoot clump (inoculum) influenced shoot multiplication and elongation, higher number of shoots proliferated per culture vessel with larger shoot clumps (15–20 shoots) as compared to smaller shoot clumps (4–5 shoots).
- Higher the number of elongated shoots and shoot length was achieved in cultures which were inoculated with smaller shoot clumps (4–5 shoots) in comparison to those cultures which were inoculated with larger shoot clumps (15–20 shoots).
- The number of shoots proliferated, elongated, rooting frequency, and subsequent survival of plants after acclimatization were higher in cultures incubated under photosynthetically active radiation (PAR) compared to those incubated under cool fluorescent lights (CFL).

- Osmotic potential of the sap and chlorophyll content of cultures incubated under PAR were also higher than those incubated under CFL.
- High-frequency shoot organogenesis (29.6 %) was achieved on MS medium supplemented with 5.0 µM BA and 1.0 µM 2,4-D.
- Shoot organogenesis response further increased to 40.5 % when fifth leaf from the top was used as explant (14–16 days old).
- Maximum shoot organogenesis occurred in explants cultured on medium containing 500 mg/l cefotaxime (44. 6 %). Carbenicillin and cephalexin inhibited shoot organogenesis from leaf explants.
- Histological studies showed the presence of meristimatic zones that grew into shoot buds which are having vascular connection with the parent tissues
- Amongst the three clones of *E. tereticornis* namely 'T1', 'CE2' and 'Y8' clone 'CE2' performed better than both clone 'T1'and 'Y8' in terms of shoot organogenesis and callus formation.
- Elongated shoots were successfully rooted on one fourth-strength MS medium supplemented with 5.0 µM IBA.
- Following transfer of plants to soil, inoculation with a suspension of *Bacillus subtilis* (plant growth-promoting bacterium) increased the survival rate of plants by 10%, yielding successful transfer of 84 % of plants.
- Clonal natures of the plants were established using different RAPD and ISSR markers.

Development of *Agrobacterium* mediated genetic transformation protocol for *Eucalyptus tereticornis*

Determination of antibiotic sensitivity

Experimental results investigating the sensitivity of the leaf explants showed that the presence of kanamycin in the medium caused considerable toxicity to explant and resulted in drastic decline in shoot regeneration potential compared to those cultured on kanamycin-free medium. On medium lacking kanamycin, about 14 % of explants resulted in shoot organogenesis, whereas incorporation of kanamycin inhibited shoot organogenesis and all the explants died on media containing (50 µg /ml kanamycin (Fig. 5.1). Therefore, the concentration of kanamycin was kept at 50 µg /ml in all experiments (unless otherwise mentioned).

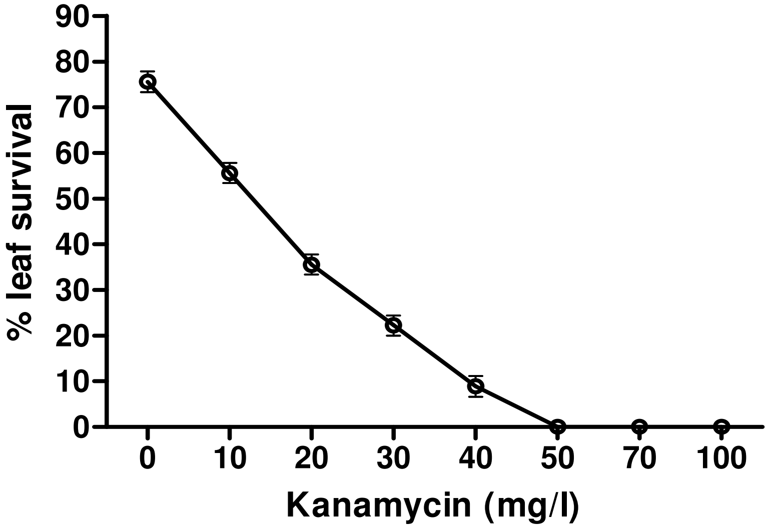


Figure 5.1 The effect of kanamycin on the survival of leaf explants taken from microshoots of *E. tereticornis*. Data were recorded after 6 weeks of culture. Values are the means of three experiments consisting of three replicates each (ten explants in each replicate), values are mean ± standard deviation

Selection of *A. tumefaciens* strain

The efficiency of two strains of *A. tumefaciens* namely, EHA105 and LBA4404 were tested for genetic transformation. Strain EHA105 induced significantly higher transient GUS activity (54.4 % explants) as compared to strain LBA4404 (46.5 % explants). Therefore, strain EHA105 was used in all subsequent experiments (Fig. 5.2).

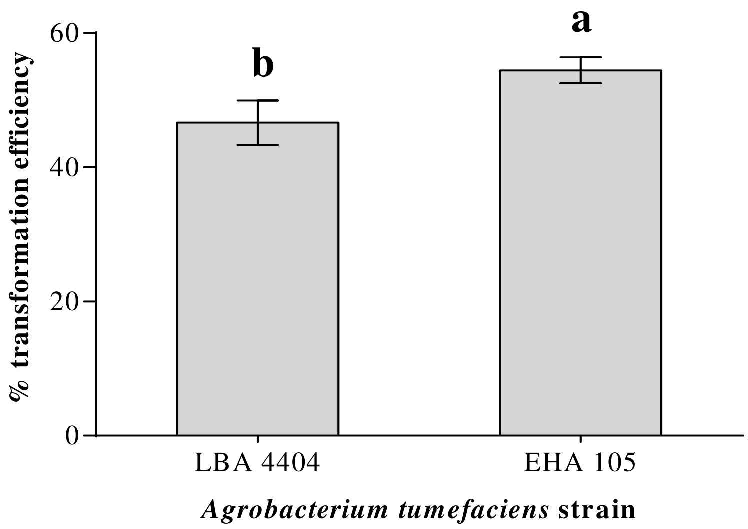


Figure 5.2 Effect of different *Agrobacterium* strains (transformed with binary vector pBI121) on transient gene expression on *E. tereticornis* leaves. Data based on 30 explants per treatment and each experiment repeated thrice. Values sharing a common letter are not significant at $P < 0.05$, values are mean ± standard deviation

Optimization of various conditions for *Agrobacterium*-mediated genetic transformation

The various factors namely pre-culture, bacterial density, mode of injury, incubation conditions, acetosyringone etc., influenced transformation efficiency of leaf explants (Table 5.1). Leaves pre-cultured on PCM medium (Table 3.3) containing 50 µM acetosyringone for 2 days and incubated under 16 h light cycle showed maximum transient GUS activity (59 %; Table 5.1). Incorporation of 100 µM acetosyringone in the co-cultivation medium increased transient GUS activity from 41.1 % (on medium lacking acetosyringone) to 62 % (Table 5.1).

Method of injury to the tissue prior to bacterial infection was also observed to play an important role in T-DNA delivery. Pricking of tissue using hypodermic needle enhanced transient GUS activity from 35.5 % (in intact explants) to 57.7 % explants (Table 5.1).

The pH of medium during co-cultivation also influenced the efficiency of T-DNA delivery. Higher frequency of explants showed transient GUS activity when cultured on medium with pH of 5.2 (54.4 %) as compared to pH of 5.8 (45.5 %), however, these differences were statistically non significant (Table 5.1).

The density of bacterial suspension used for infection of the explant also influenced transient GUS activity (Table 5.1). Maximum transient GUS activity was obtained in explants that were infected with the bacterial suspension having an OD_{590} of 0.8. At higher bacterial density, the decrease in transient GUS activity was observed (Table 5.1).

An approppriate co-cultivation period following *Agrobacterium* infection has significant role in delivery of T-DNA and influenced the expression of transient GUS activity (Table 5.1). A maximum of 56.6 % explants showed transient GUS activity when these were co-cultivated for 2 days. Co-cultivation period of les than two days and more then two days resulted in decrease in transient GUS activity.

In this study, co–cultivation period of more than 3 days caused excessive bacterial growth leading to necrosis of explants. Photoperiod during co-cultivation also had an impact on the transient GUS activity (Table 5.1). A photoperiod of 16-h was found to induce transient GUS activity in maximum explants (55.5%,Fig 5.3 A).

The duration of the exposure interval to *Agrobacterium* cells also influences the transformation frequency of explants. Leaf explants incubated for 10 mins with *Agrobacterium* cells showed a significantly increased frequency of transformation than those transformed for 5 mins, while exposure to *Agrobacterium* for more than 20 mins resulted in decline in transformation frequency as shown in Table 5.1. Maximal number of explants developed GUS positive loci when infection was carried out for 10 mins. However the 30 mins treatment was associated with problems such as elimination of the *Agrobacterium* subsequent to co-cultivation and loss of viability of the explants resulting from the overgrowth of bacteria.

Table 5.1 The effect of different transformation parameters on transient *gus* expression in leaf explants taken from microshoots of *E. tereticornis* co-cultivated with *Agrobacterium tumefaciens* strain EHA105 harboring binary vector pBI121

Factor	**Variable**	**% *GUS* expression**
Infection Time	5 min	44.44 ± 1.92 bc
	10 min	52.22 ± 1.92 a
	15 min	49.99 ± 1.88 ab
	20 min	43.33 ± 2.12 bc
	30 min	38.88 ± 1.89 c
pH of co-cultivation medium	5.2	54.44 ± 2.12 a
	5.5	49.99 ± 2.08 a
	5.8	45.55 ± 1.78 a
Pre-culture	0d	38.88 ± 1.66 d
	1d	47.77 ± 1.99 bc
	2d	58.88 ± 2.15 a
	3d	52.22 ± 1.08 b
	4d	45.55 ± 1.45 c
	5d	39.99 ± 1.92 d
Co-cultivation period	1d	45.55 ± 1.97 bc
	2d	56.66 ± 2.23 a
	3d	48.88 ± 1.89 b
	4d	43.33 ± 1.79 bc
	5d	41.11 ± 1.25 c
O.D Value	0.2	35.55 ± 1.65 d
	0.4	44.44 ± 1.56 c
	0.6	54.34 ± 1.91 ab
	0.8	59.99 ± 1.91 a
	1.0	48.88 ± 1.65 bc
Method of injury	Intact	35.55 ± 1.98 c
	With hypodermic needle	57.77 ± 2.15 a
	With surgical blade	46.66 ± 2.02 b
	With carborundum	42.22 ± 2.02 bc
	With Glass beads	38.88 ± 1.77 c
Acetosyringone	0	41.11 ± 1.66 c
	100	62.22 ± 1.66 a
	200	47.77 ± 1.66 b
Photoperiod	24 h light	43.33 ± 1.76 b
	24 h dark	47.77 ± 1.64 b
	16h light/8 h dark	55.55 ± 1.65 a

Data based on 30 explants per treatment and each experiment repeated thrice. Mean values within column for each factor separately followed by same letter are not significantly different according to Student-Newman-Keuls-Test ($P<0.05$), values are mean ± standard deviation

The transformation efficiency also significantly varied from clone to clone, and amongst the three clones tested namely, ‘T1’, ‘CE2’ and ‘Y8’, the percent explants showing transient GUS expression was 30.0, 41.1 and 24.4 %, respectively (Fig. 5.4).The overall transformation efficiency was also influenced by the regeneration potential of transformants. These transformation and regeneration parameters did not appear to be correlated since the clones showing better regeneration potential did not show better transient expression. Earlier, clone T1 showed higher regeneration potential (Fig. 4.1). It is now observed that the transient expression was higher in clone CE2 (Fig. 5.4). Although, most of the parameters were found to influence T-DNA transfer, yet lower transformation efficiency was observed in this study (Table 5.3). In the present study, it took 35–40 weeks to recover transgenic shoots on selection medium containing kanamycin. Since problems were faced in the regeneration of transgenic shoots following transformation using protocol reported earlier, certain changes were made in the composition of basal medium initially used for selection of transgenic plants (SM-I). The composition of new medium (SM-II) used to achieve shoot organogenesis is mentioned in table 3.3. Potassium nitrate was replaced with 990 mg/l potassium sulphate and ammonium nitrate with 392 mg/l ammonium sulphate. These changes resulted in successful shoot organogenesis from transformed tissues (Fig.5.3B).

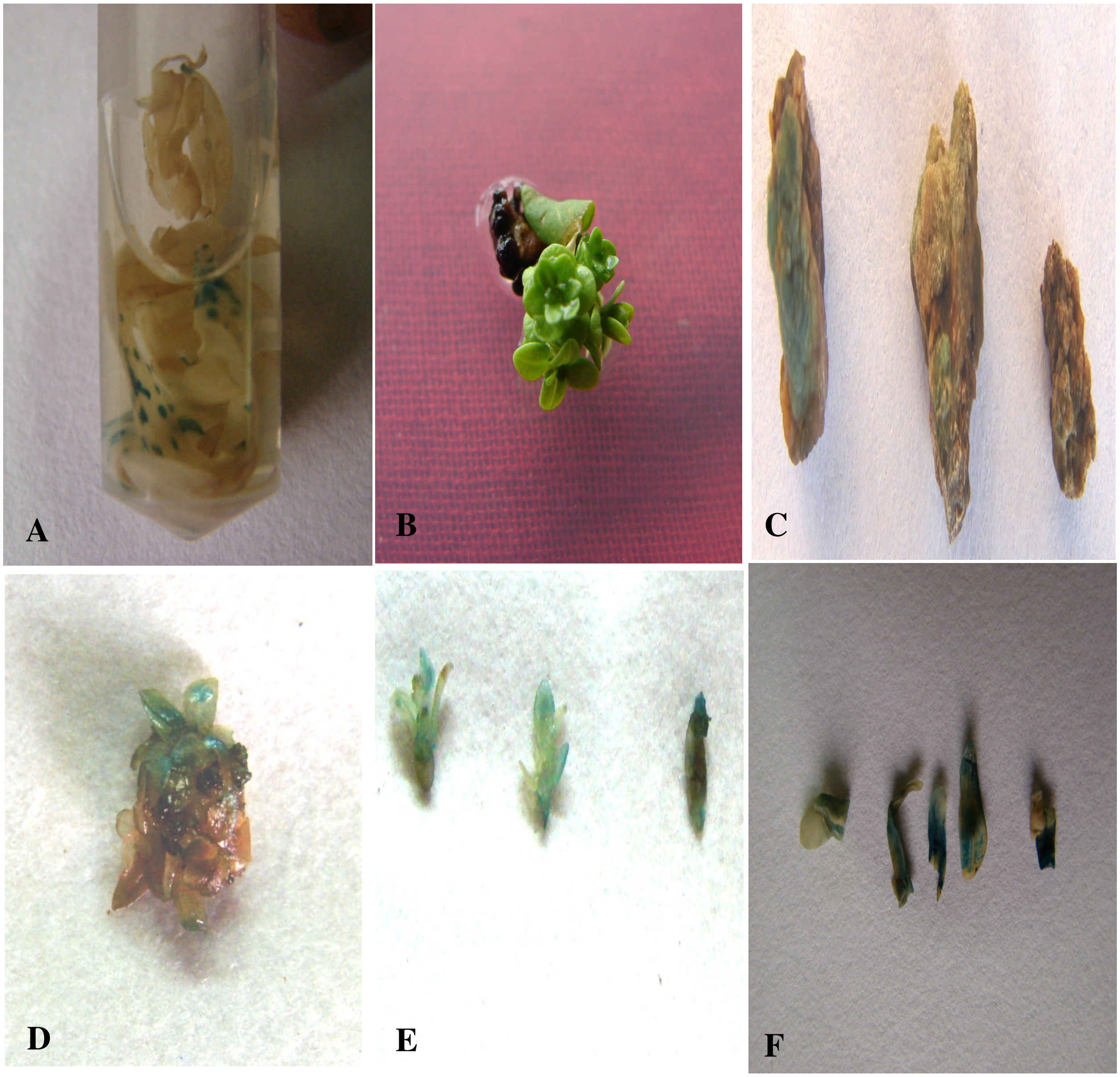


Figure: 5.3 *Agrobacterium* mediated genetic transformation of *E. tereticornis* **A.** *E. tereticornis* leaf explants showing transient GUS activity **B.** Regeneration of putative transformed *E. tereticornis* shoots on selection medium containing kanamycin **C.** Transformed *E. tereticornis* callus showing GUS activity **D-F.** Transformed *E. tereticornis* shoots showing Stable GUS activity after 8 cycles of subculture

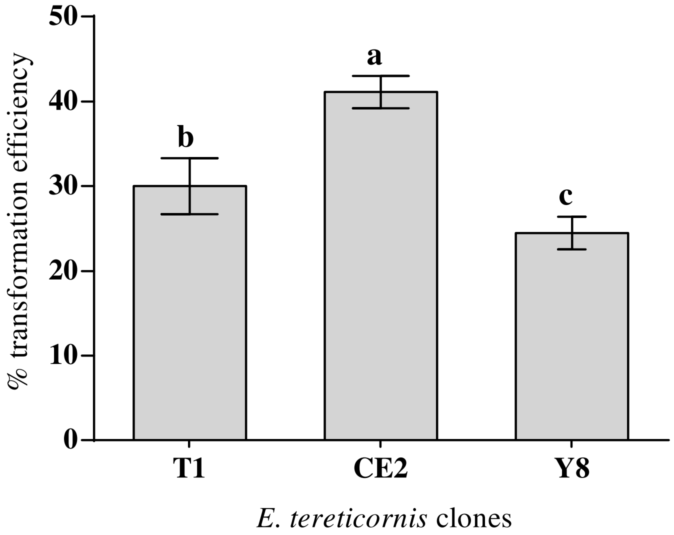


Figure 5.4 Effect of difference in transformation efficiencies of different clones of *E. tereticornis*. Data based on 30 explants per treatment and each experiment repeated thrice. Values sharing a common letter are not significant at $P < 0.05$, values are mean ± standard deviation

Table 5.3 Transformation efficiency of regenerated shoots of *E. tereticornis* on selection medium

Experiment	No. of leaf explants incubated	No. of kanamycin resistant calli	No. of kanamycin resistant shoots	Transformation efficiency (%)
1	600	62	8	1.32 ± 0.89
2	600	58	5	0.83 ± 0.67
3	600	56	9	1.11 ± 0.84

Transformation efficiencies, based on surviving shoots in selective medium expressing the reporter gene and tested positive by PCR, values are mean ± standard deviation

Molecular Analysis

Although the kanamycin resistance shoots was indicative of expression of the *nptII* gene, yet GUS activity was also examined to further confirm the expression of these newly incorporated genes. The kanamycin resistant shoots showed positive GUS activity (Fig. 5.3 D-F). These results were confirmed by PCR amplification of DNA fragments of 720 bp specific to *nptII* gene (Fig. 5.5 B) and 1,500 bp specific to *uidA* gene (Fig. 5.5 C) from transgenic shoots. Amplification of DNA fragment specific to 16S rRNA locus of DNA isolated from transgenic shoots was not observed indicating the complete elimination of bacteria from these tissues (Fig. 5.5 A).

Further, expression of *nptII* gene was also confirmed at transcription level in the transgenic plants by RT-PCR. The cDNA was synthesized from total RNA isolated from control plants and five independent transgenic lines grown under green house conditions were subjected to PCR as mentioned above. Amplification of DNA fragments from five independent transgenic lines yielded a fragments of 1500 bp and 720 bp, specific *uidA nptII* genes respectively, while amplification was not observed in control samples (Fig. 5.5 E-F) indicating that transgenic plants expressed the *nptII* gene. Expression levels of *nptII* gene were studied in different transgenic lines using qRT-PCR (Fig. 5.5F). Different expression levels were observed in different transgenic lines. Line 2 showed maximum expression levels, whereas line 4 showed minimum expression levels of *nptII* gene (Fig. 5.6).

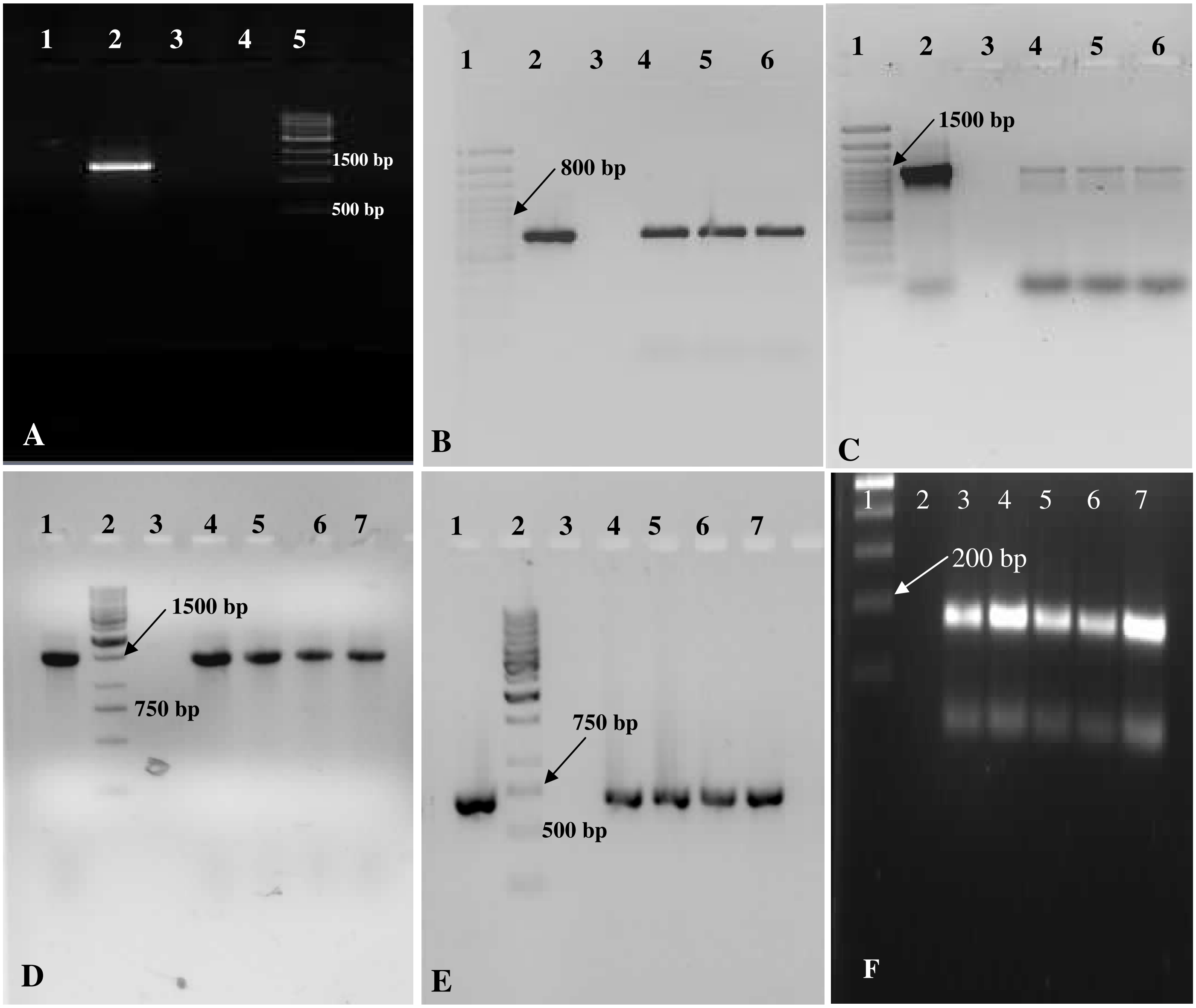


Figure 5.5 Molecular analysis of the putative transgenic shoots of *E. tereticornis* **A:** 16S rRNA analysis of transformed shoots Lane -1: DNA from untransformed plants, Lane-2: DNA from positive control (bacterial genomic DNA, Lane-3-4: DNA from transformed plants, Lane 5: 500 bp ladder **B**: Amplification of *nptll* gene (~720 bp) from genomic DNA of transformed tissue Lane-1:100 bp DNA ladder Lane-2: Positive control (amplification from pBI121) Lane-3: Negative control (Non-transformed tissue) Lane-4-6: Amplification from DNA of transformed Shoot **C**: Amplification of *uidA* gene (~1500 bp) from genomic DNA of transformed tissue Lane-1:100 bp DNA ladder Lane-2: Positive control (amplification from pBI121) Lane-3: Negative control (Non-transformed tissue) Lane-4-6: Amplification from DNA of transformed Shoot **D -E:** RT-PCR analysis of *uidA* (1500 bp) and *nptll gene* (~720 bp) respectively from cDNA of transformed tissue Lane1: Amplification from positive control (amplification from pBI121), Lane-2:1 kb ladder; Lane 4-7: Amplification from cDNA of transformed Shoots **F.** Detection of *nptll* gene from cDNA of transformed and untransformed plants using qRT-PCR, Lane - 1: 100 bp Ladder; Lane -2: Untransformed Plant; Lane 3-7: transformed plants.

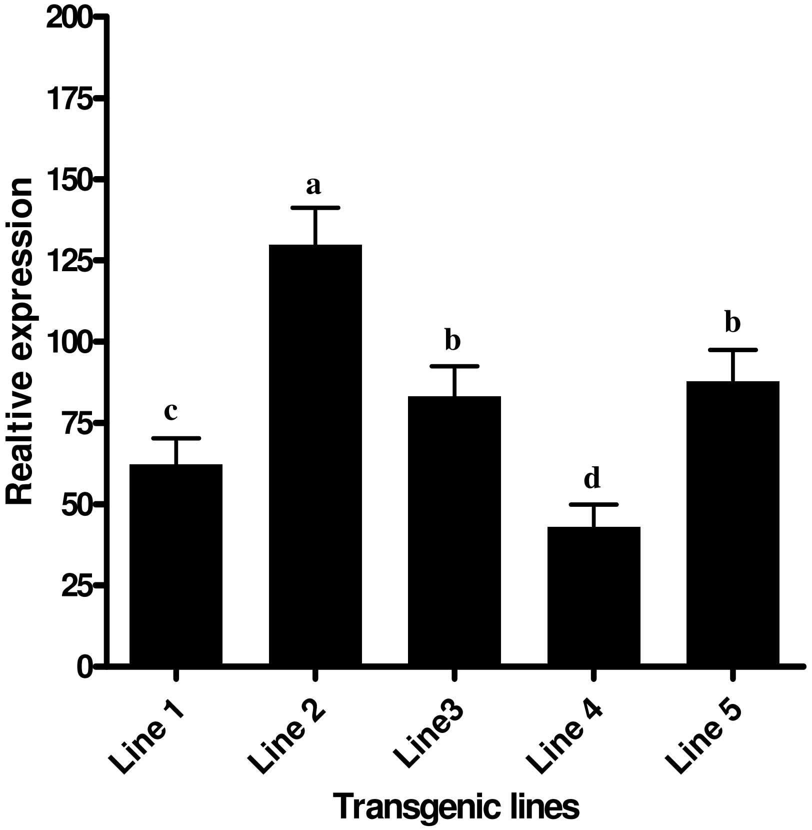


Figure 5.6 Expression levels of *nptll gene* in transgenic *E. terticornis* plant*s* using quantitative real-time PCR analysis. C- Control untransformed plants, T1- T5 different transgenic lines. Error bars standard deviation of the mean, Values sharing a common letter are not significant at $P < 0.05$

Discussion

Despite the clear potential of genetic engineering for improving woody plants due to their vast commercial interest, progress has been slow on these plants especially *Eucalytpus* because of its recalcitrant nature. In particular, regeneration after transformation is often very poor, probably due to the high concentration of phenolic compounds in the cells or due to low endogenous cytokinin content (Azmi 1999). Moreover, organogenesis or embryogenesis capacity is even lower on a selective antibiotic containing medium making it often impossible to recover transgenic shoots even when stable transformation has been achieved (Serrano *et al.* 1996). Nevertheless, transgenic *Eucalyptus* has been recovered in a few species (Tournier *et al.* 2003; Ho *et al.* 1998), mainly from juvenile material that showed better transformation and regeneration capacity than adult clones. However, an efficient improvement programme for the selected clones can only be taken up using mature tissue. Therefore, it is important to develop transformation protocol from the mature plants selected plants of *E. tereticornis.* In this investigation, factors influencing efficiency of T-DNA delivery into the selected elite clones of *E. tereticornis* using *A. tumefaciens* was studied. This is the first report investigating factors influencing the genetic transformation of selected elite clones of *E. tereticornis*.

In order to select the transformed tissue on the selectable marker such as kanamycin (as in present study), the sensitivity of the tissue to this antibiotic is required to be tested. Kanamycin is an aminoglycoside derivative antibiotic and is widely used to select the transformed cells with *nptII* gene. Kanamycin sensitivity appears to be species dependent. A wide range of concentrations has been reported to inhibit organogenesis in various plant species such as, almond (Miguel and Oliveira 1999), apple (Yao *et al.* 1995; Norelli and Aldwinckle 1994), grape (Scorza *et al.* 1996) and citrus fruits (Yao *et al.* 1996). Leaves taken from microshoots of *E. tereticornis* were tested for their sensitivity towards kanamycin. It was found that kanamycin restricted the growth to a greater extent and completely inhibited growth at concentration above 50 mg/l (Fig. 5.1). This sensitivity test of the tissue to kanamycin will be useful in further screening of transformants during genetic transformation studies. These results are in line with the earlier reports on *Eucalyptus* spp. (Prakash and Gurumurthi 2009; Tournier *et al.* 2003; Ho *et al.* 1998), *Leucaena leucocephala* (Jube and Borthakur 2009) and *Quercus suber* (Alvarez and Ordas 2007) where kanamycin was used successfully for selection of transformed plants.

Transformation efficiency often depends on the strain of *A. tumefaciens* used (Kumar and Rajam 2007; Wenck *et al.* 1999; Hood *et al.* 1993). In the present study also two strains of *A. tumefaciens* namely LBA4404 and EHA105 (both transformed with binary vector pBI121) were used and the transient GUS frequencies resulting from the infection of these starins were compared. When EHA105 strain was used to infect the explants 54.4 % explants showed transient GUS activity in comparison with a 46.4% explant showed transient GUS activity with strain LBA4404 (Fig. 5.2). Earlier, several studies have reported that EHA105 is more effective than other strains for transformation in Italian stone pine (Humara *et al.* 1999), sugarcane (Manickavasagam *et al.* 2004) and blueberry (Song and Sink 2004). The different *Agrobacterium* strains are defined by their chromosomal and plasmid genomes. The different strains were reported to be different in virulence because of the lack of the *virF* locus in Ti plasmids of some strains (Melchers *et al.* 1990). The *A. tumefaciens* strain EHA105 is more infectious than LBA4404 because EHA105 is a derivative of the super virulent strain A281 (Hood *et al.* 1993), while LBA4404 is derived from the less virulent strain Ach5 (Hoekema *et al.* 1983). However, the *A. tumefaciens* strain LBA4404 has also been used for many plant transformations because, the elimination of LBA4404 from plant tissues is relatively easy at low concentration of antibiotics (Maheswaran *et al.* 1992). In contrast, it is difficult to eliminate EHA105 from plant tissues. Therefore, in this investigation concentration of cefotaxime was kept 500 µg/ ml in subsequent subculture cycles to completely eliminate the bacteria. Further, it was observed that cefotaxime enhanced shoot organogenic potential of leaf explants of *E. tereticornis* (Table 4.8).

The various factors namely pre-culture, bacterial density, mode of injury, incubation conditions, acetosyringone etc., influenced transformation efficiency of leaf explants (Table 5.1). Culture medium and incubation conditions of the explants, prior to *Agrobacterium* infection have been reported to enhance T-DNA delivery in some plant species (Yevtushenko and Misra 2010; Padmanabhan and Sahi 2009; Ho *et al.* 1998).

Preculture of the explants has also been reported as a significant factor for transformation of several plants (Padmanabhan and Sahi 2009; Ho *et al.* 1998). In present study, leaves pre-cultured on PCM medium (Table 3.3) containing 50 µM acetosyringone for 2 days showed maximum transient GUS activity (59 %; Table 5.1). The higher GUS activity is probably due to the presence of cytokinin resulting in increased cell division (Sangwan *et al.* 1992) and/or presence of acetosyringone that is known to induce *vir* genes and enhance T-DNA transfer (Stachel *et al.* 1985). Earlier, pre-culturing of explants on a particular medium prior to infection with *Agrobacterium* has been reported to enhance transformation efficiency in many plant species (Padmanabhan and Sahi 2009; Yasmeen 2009; Ho *et al.* 1998) including *E. camaldulensis* (Ho *et al.* 1998).

Acetosyringone, a phenolic compound that induces the *vir* genes of *Agrobacterium*, has been shown to be essential for plant transformation (Atkinson and Gardner 1991). *Agrobacterium* recognizes the acetosyringone signal when active growing cells are wounded and is beneficial for plant cell transformation (Stachel *et al.* 1985). In this study incorporation of 100 µM acetosyringone in the co-cultivation medium increased transient GUS activity from 41.1 % (on medium lacking acetosyringone) to 62 % (Table 5.1). Earlier acetosyringone has been shown to enhance *Agrobacterium* mediated genetic transformation efficiency in *Eucalyptus* spp. (Tournier *et al.* 2003) and *Camellia sinensis* (Lopez *et al.* 2004). However, Godwin *et al.* (1991) found that the presence of acetosyringone generally enhanced the virulence of *Agrobacterium tumefaciens*, but its effect on transformation was not always stimulatory and it could also suppress virulence of some *Agrobacterium* strains.

Method of injury to the tissue prior to bacterial infection was also observed to play an important role in T-DNA delivery. Pricking of tissue using hypodermic needle enhanced transient GUS activity from 35.5 % (in intact explants) to 57.7 % explants (Table 5.1).

Wounding of tissue before infection could allow bacterial penetration deep into the tissue facilitating the accessibility of plant cells to *Agrobacterium* on one hand and may stimulate the induction of *vir* genes as a result of phenolics secretion on other (Stachel *et al.* 1986), which could be the main reasons for enhanced bacterial efficiency for T-DNA delivery (Binns and Thomashow 1988). Method of injury to plant tissue before co-cultivation has also been shown to influence transformation frequency in the plants such as *Vitis vinifera* (Dutt *et al.* 2007) and *Vigna radiata* (Sonia *et al.* 2007).

Although *Agrobacterium tumefaciens* was reported to show the best growth at neutral pH (Li *et al.* 2002), previous reports on the *Agrobacterium*-mediated transformation showed that an acidic pH favoured the optimal expression of the *vir* genes (Godwin *et al.* 1991). The transient GUS expression was the strongest when the pH of the co-cultivation medium was adjusted at 5.2 indicating the importance of maintaining the required pH of medium during co-cultivation. Low pH during co-cultivation was reported to be beneficial for *Agrobacterium* mediated transformation across the species by Godwin *et al.* (1991). Thus, we can conclude that the combination of low pH (5.2) and presence of acetosyringone (100 μM) were responsible for the maximum frequency of observed transient GUS expression (Table 5.1).

A critical factor in *Agrobacterium* mediated transformation systems is the density of the *Agrobacterium* inoculum in the inoculation medium. We inoculated leaf explants (as described in materials and methods) with an *Agrobacterium* suspension which was applied at range of cell densities. Maximum transient GUS activity was obtained in explants that were infected with the bacterial suspension having an OD_{590} of 0.8. At higher bacterial density, the decrease in transient GUS activity was observed (Table 5.1). A reduction in the mean transformation frequency was observed following inoculation with higher densities of *Agrobacterium*, possibly due to increased damage, necrosis of plant tissue and increased production of harmful compounds (Saini *et al.* 2003).

The effect of varying length of co-cultivation period was also investigated. Co-cultivation is one of the most important steps for *Agrobacterium*-mediated transformation of plants (James *et al.* 1993). A maximum of 56.6 % explants showed transient GUS activity when these were co-cultivated for 2 days. It is known that co-cultivation of explants with *A. tumefaciens* for an appropriate duration improves the transformation efficiency, but prolonged co-cultivation period was reported to result in death of explants, due to overgrowth of bacteria (James *et al.* 1993). In this study, co–cultivation period of more than 3 days caused excessive bacterial growth leading to necrosis of explants. Although, an increased transformation frequency has been positively correlated with longer co-cultivation periods (2–5 days), yet it has been emphasized that for higher transformation efficiency, an optimum co-cultivation period is required (Niu *et al.* 2000).

Different light conditions during co-cultivation also have a strong affect on the efficiency of transient expression (Zuker *et al.* 1999). A photoperiod of 16-h was found to induce transient GUS activity in maximum explants (55.5 %, Table 5.1). Prakash and Gurumurthi (2009) successfully used 16-h photoperiod for the transformation of hypocotyls segments of *E. tereticornis*. Beneficial effect of light on *Agrobacterium* mediated T-DNA transfer to *Phaseolus acutifolius* and *Arabidopsis thaliana* has been reported (Zambre *et al.* 2003). Photoperiod during co-cultivation was also reported to influence the efficiency of transient expression of reporter gene in *Dianthus caryophyllus* (Zuker *et al.* 1999).

The transformation efficiency also significantly varied from clone to clone, and amongst the three clones tested namely, ‘T1’, ‘CE2’ and ‘Y8’, the percent explants showing transient GUS expression was 30.0, 41.1 and 24.4 %, respectively (Fig. 5.4). Mullins *et* *al.* (1997) also reported clonal variations with respect to *Agrobacterium* mediated genetic transformation in *Eucalyptus* spp. These clonal variations are likely to be due to differential virulence of *Agrobacterium* towards these clones. The overall transformation efficiency was also influenced by the regeneration potential of transformants. Variation in the regeneration potenetial of the different clones of same species has also been observed (Mullins *et al.* 1997). These transformation and regeneration parameters did not appear to be correlated since the clones showing better regeneration potential may not show better transient expression. In presnt also during regeneration, it was observed that the clone T1 showed higher regeneration potential (Fig. 4.1) and now results show that the transient expression is higher in clone CE2 (Fig. 5.4).

Although, most of the parameters were found to influence T-DNA transfer, yet lower transformation efficiency was observed in this study (Table 5.3). This could be due to the use of explants taken from the mature plants of selected elite clones, which is essential in retaining the superior genetic makeup of the existing genotype. It has been reported earlier that tissue taken from selected clones results in lower transformation and organogenesis as compared to juvenile tissues taken from seedlings (Tournier *et al.* 2003). Further, in *E. globulus*, regeneration following transformation was lower, and it was difficult to recover transgenic shoots even if stable transformation was achieved (Serrano *et al.* 1996). In this study, it took 35–40 weeks to recover transgenic shoots on selection medium containing kanamycin.

Further, expression of *nptII* and *uidA* gene was also confirmed at transcription level in the transgenic plants by RT-PCR. The cDNA was synthesized from total RNA isolated from control plants and four independent transgenic lines, were subjected to PCR as mentioned above. Amplification of DNA fragment from four independent transgenic lines yielded a fragments of 720 bp of 1500 bp, specific *nptII* and *uidA* genes , while amplification was not observed in control samples (Fig. 5.5D-E) indicating that transgenic plants expressed the *nptII* gene. Earlier, RT-PCR analysis was successfully utilized for the detection/integration of transgene in the host genome (Bakshi *et al.* 2011; Hazarika and Rajam 2011; Jung *et al.* 2011). Expression levels of *nptII* gene were studied in different transgenic lines using qRT-PCR. Different expression levels were observed in different transgenic lines. Line two showed maximum expression levels, whereas line 4 showed minimum expression levels of *nptII* gene (Fig. 5.6). Differences among transgenic lines in same transformation event are often observed and are likely due to variations in insertion site and events like this can be valuable source for plant genetic improvement (Cai *et al.* 2012; Hong *et al.* 2013).

Since problems were faced in the recovery of transgenic shoots following transformation using protocol reported earlier, certain changes were made in the composition of basal medium initially used for selection of transgenic plants (SM-I). The composition of new medium (SM-II) used to achieve shoot organogenesis is mentioned in table 3.3. Potassium nitrate was replaced with 990 mg/l potassium sulphate and ammonium nitrate with 392 mg/l ammonium sulphate. It has been documented that a proper NO_3^-/NH_4 ratio influences morphogenesis (Ramage and Williams 2002) but the range of optimal ratios depends on the species (Ivanova and Van Staden 2009; Tazawa and Reinert 1969). Recently, Ivanova and Van Staden (2009) also highlighted the role of nitrogen sources and optimal ratio of NO_3^-/NH_4 for successful shoot organogenesis in *Aloe polyphylla*. These changes resulted in successful shoot organogenesis from transformed tissues (Fig. 5.3B). Further increasing meso-inositol concentration improved shoots organogenesis from explants after co-cultivation with *Agrobacterium*. Earlier, the use of higher concentration of mesoinositol has been reported to be beneficial for shoot organogenesis from transformed tissue of hybrid *Eucalyptus* (Tournier *et al.* 2003).

Conclusion

In conclusion, the protocol for delivery of T-DNA using *A. tumefaciens* has been developed and subsequent regeneration of transformed shoots has been achieved in selected clones of *E. tereticornis*. This seems to be first report of successful transformation of elite clones of *E. tereticornis* using *A. tumefaciens*. This protocol has the potential to facilitate work on genetic modification of these clones incorporating genes of important traits.

Salient findings

- Kanamycin was found to be toxic towards *E. tereticornis.* So therefore, kanamycin at concentration of 50 µg /ml was used for the selection of transformed tissue.
- *A. tumefcaiencs* strain than EHA105 was found to be more virulent than strain LBA4404 and used for the development of genetic transformation protocol.
- Pre-culturing of leaves on MS medium supplemented with 5.0 µM BA and 1 µM 2,4-D containing 100 µM Acetosyringone for 2 days prior to infection and incubation under 16 h was found beneficial for enhanced GUS activity.
- Pricking of tissue with hypodermic needle enhanced transient GUS activity from 35.5 % (in intact explants) to 57.7 % explants.
- The pH of medium during co-cultivation was found to influence the efficiency of T-DNA delivery. Lowering of pH of co-cultivation medium was found to be beneficial for T-DNA delivery.
- Infection period of 10 mins with cultures grown at OD_{590}- 0.8 and co-cultivation period of two days resulted in enhanced transient GUS expression.

- Incorporation of 100 µM acetosyringone in the co-cultivation medium further increased the GUS activity.
- Amongst the three clones of *E. tereticornis* namely 'T1', 'CE2' and 'Y8'; clone 'CE2' showed maximum transient expression.
- Transformed shoots showed stable GUS activity.
- Presence of DNA fragments of 720 bp specific to *nptII* gene and 1,500 bp specific to *uidA* gene from transgenic shoots confirms the stable inegrtaion of the genes.
- Amplification of DNA fragment specific to 16S rRNA locus of DNA isolated from transgenic shoots was not observed, indicating the complete elimination of bacteria from these tissues.
- Further, expression of *nptII* gene was confirmed at transcription level in the transgenic plants by RT-PCR and qRT PCR.

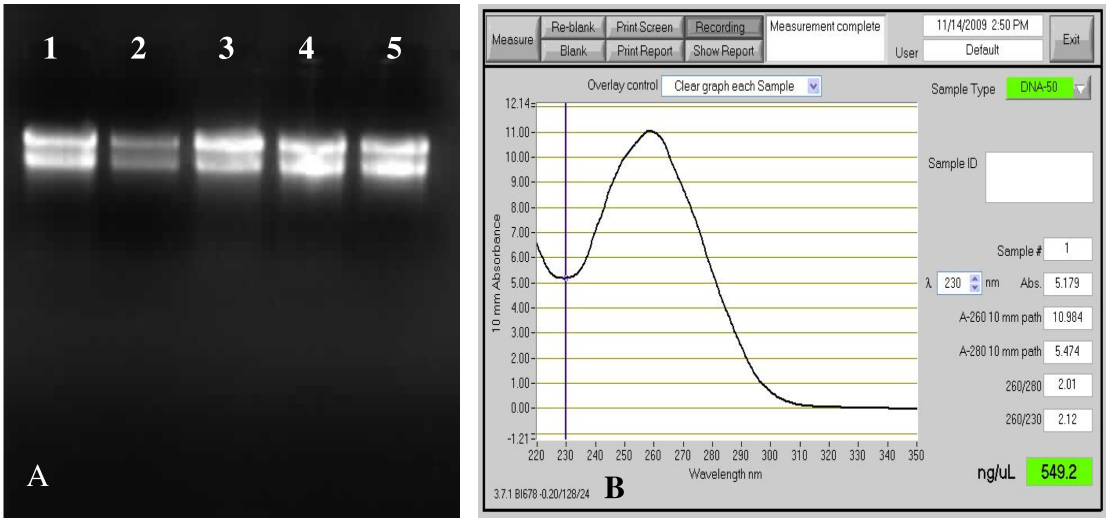
Chapter-6

Molecular cloning of *Korrigan* and *Sucrose Synthase* genes from *Populus deltoides*

Isolation of Total RNA

Protocol for the isolation of high quality RNA from young actively growing shoots of poplar was standardized. Various methods like Trizol reagent method (Simms *et al.* 1993), Iris RNA isolation Kit (CSIR-IHBT, Palampur, India), Guanidine isothiocynate method (Chomczynski and Sacchi 1987) etc. were tried. The best quality RNA was isolated, following CTAB method (Chang *et al.* 1993).

The concentration and purity of RNA was determined using NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Quality of the RNA was assessed using 1.0 % (w/v) agarose gel by loading aliquots of samples. Isolated RNA was intact, high quality and most importantly without any DNA contamination (Fig. 6.1A). The ratio of A_{260}/A_{280} of isolated RNA was 2.01 indicating that the RNA was almost pure (Fig. 6.1B). After checking the concentration of RNA, All extracts were adjusted to 200 ng/µl for subsequent cDNA synthesis.



Measurement complete

11/14/2009 2:50 PM

User Default

Overlay control Clear graph each Sample

Sample Type DNA-50

Sample ID

Sample # 1

λ 230 nm Abs. 5.179

A-260 10 mm path 10.984

A-280 10 mm path 5.474

260/280 2.01

260/230 2.12

ng/uL 549.2

Figure 6.1 A. Lane 1-5: Gel picture showing quality of RNA isolated from *Populus deltoides*. **B**. Spectrophotometric analysis of isolated RNA sample using Nano drop

Isolation and cloning of partial cDNA clones of *korrigan (KOR)* and *sucrose synthase (SUS)* from *P. deltoides*

The reported sequences of *KOR* and *SUS* were retrieved from the NCBI database and aligned using ClustalW. In the first instance, primers were designed from the conserved region from aligned sequences to amplify partial fragments (1800 bp fragment in case of *KOR* and 1200 bp in case of *SUS,* Table 3.5). Using the designed primers, cloning of partial segments of *KOR* and *SUS* genes was carried out from *P. deltoides* cDNA synthesized from RNA by reverse transcription (RT). Amplification of fragments of above genes was carried out using PCR and lead to the successful amplification of desired fragments of 1800 bp in case of *KOR* (Fig. 6.2A) and 1200 bp in case of *SUS* (6.2B).

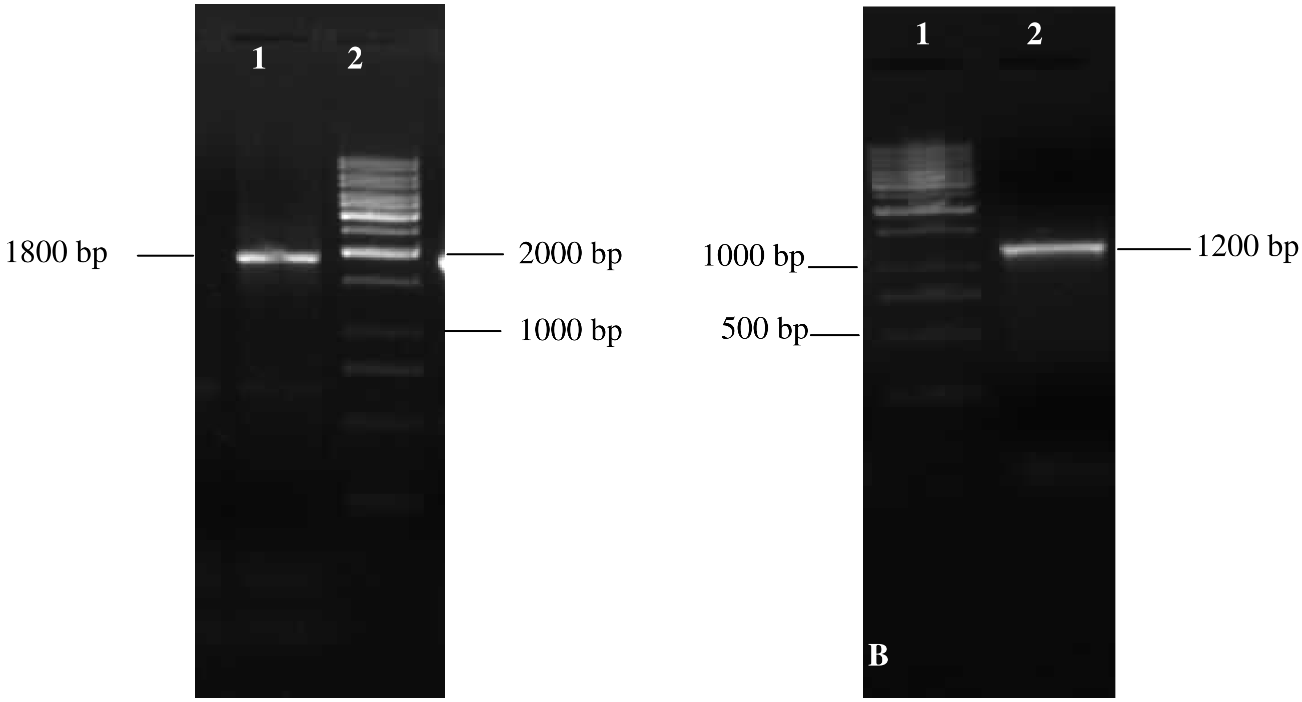


Figure 6.2 Amplification of partial fragments *KOR* and *SUS* from *P. deltoides* cDNA
A. Lane 1: Amplification of partial *KOR* fragment; Lane 2: 1kb DNA Ladder
B. Lane 1: 1 kb DNA ladder; Lane 2: Amplification of partial *SUS* fragment

The PCR products amplified from *P. deltoides* cDNA were excised and purified from agarose gel electrophoresis using a Qiagen gel extraction kit (Qiagen, Valencia, CA, USA) following the manufactures instructions and cloned into pGEM®-T easy vector (Promega Corporation, USA). Competent *E. coli* DH5α cells were transformed with these ligated products by a heat shock method. The positive clones were screened by α-complementation and the plasmid DNA of the recombinant cells was isolated using the alkaline lysis method (Fig. 6.3). Plasmid containing partial fragments of *KOR* and *SUS* were identified by PCR amplification of corresponding fragments using gene specific primers (Fig. 6.4 and 6.5). The inserts in the plasmids were sequenced by chain termination method (Sanger *et al.* 1977) using an Applied Biosystems automatic sequencer (DNA sequencing facility, Department of Biochemistry, South campus, Delhi university, New Delhi, India). Sequence analysis was performed with BLASTN program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence analysis of both the fragments showed 99 % similarity with reported sequences of other *Populus* species.

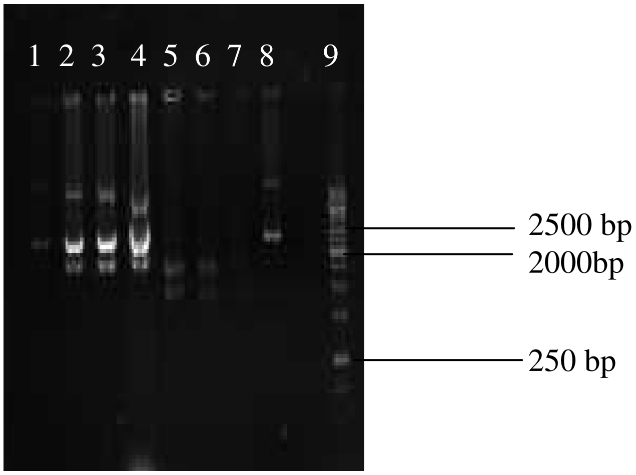


Figure 6.3 Plasmid DNA isolated from *E. coli* DH5α cells using alkaline lysis method. Lane 1-8: Plasmid (pGEM-T Easy vector) with fragments of interest (*KOR* and *SUS*); Lane 9: 1 kb DNA ladder

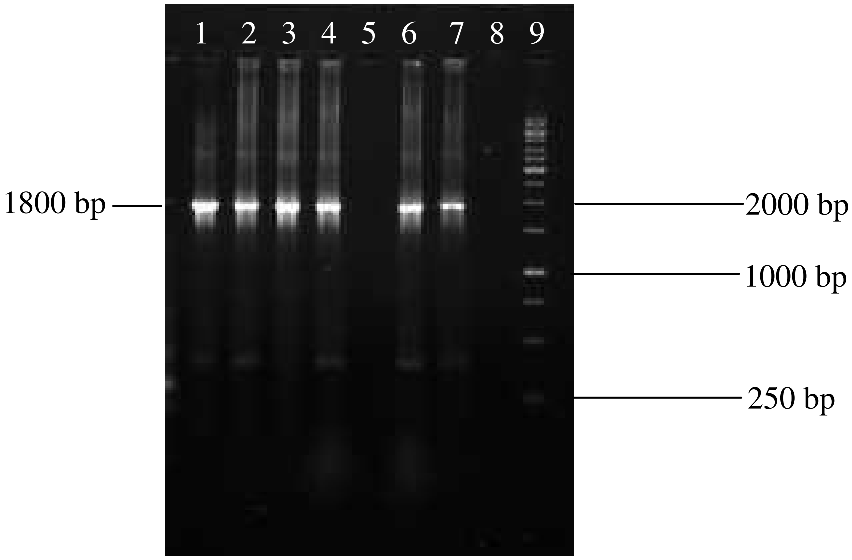


Figure 6.4 Screening of *KOR* from the isolated plasmid of positive clones. Lane 1: Positive control amplification from *P. deltoides* cDNA; Lane 2-8: Amplification from positive clones using gene specific primers; Lane 9: 1 kb DNA Ladder

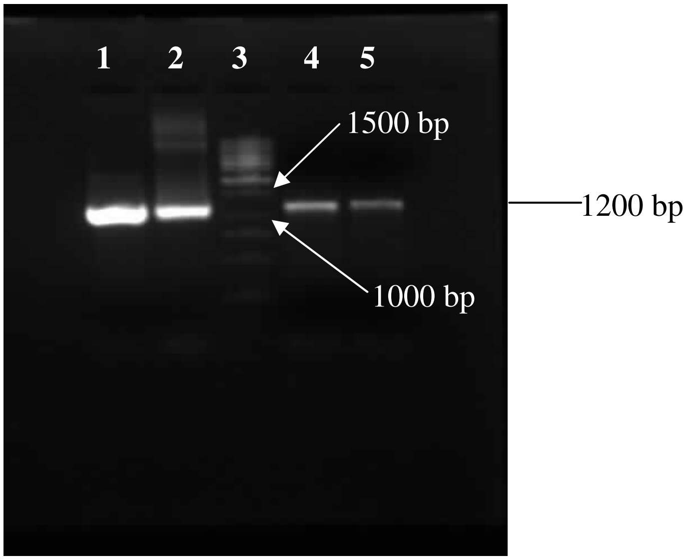


Figure 6.5 Screening of *SUS* from isolated plasmid of positive clones. Lane 1: Positive control, amplification from *P. deltoides* cDNA; Lane 2, 4 & 5: Amplification from positive clones using gene specific primers; Lane 9: 1 kb DNA Ladder

Isolation and cloning of full length cDNA clones of *korrigan* and *sucrose synthase* from *P. deltoides*

Upon knowing the sequences of these partially cloned fragments of both *KOR* and *SUS,* these were analyzed using BLAST N. As these sequences showed 99 % similarity with reported sequences (*P. tremuloides* endo-1,4-beta–glucanase mRNA GenBank accession: AY535003 in case of *KOR* and *P. tremuloides* sucrose synthase mRNA GenBank accession AY341026 in case of *SUS*). Primers for cloning of full length fragments of these genes were designed by taking above mentioned sequences as template. Overhangs of restriction enzymes BamH1 and Sac1 in case of *KOR* and BamH1 and Sma1 in case of *SUS* were added to the respective primers (Table 3.6) for the sub cloning of these genes in transformation vector pBI121 (Fig. 3.2). Using a set of these designed primers (Table 6.2), PCR reactions lead to the successful amplification of desired DNA fragments corresponding to the full length coding sequences of *KOR* and *SUS* (Fig. 6.6).

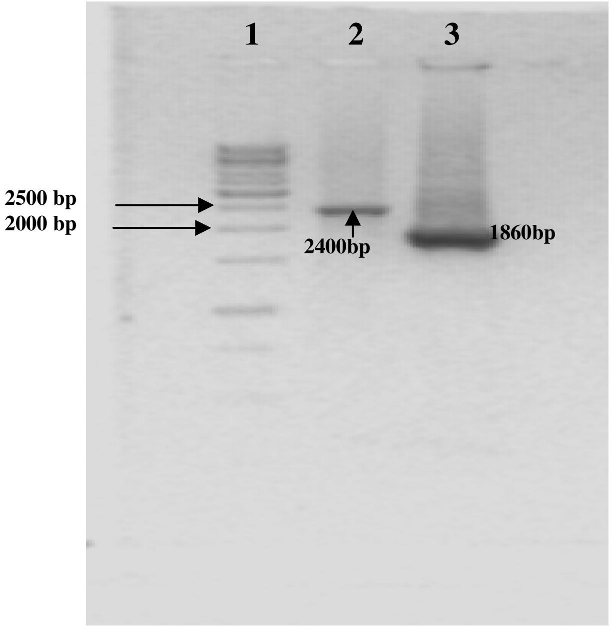


Figure 6.6 Amplification of full length genes of *korrigan* and *sucrose synthase* from *P. deltoides* cDNA. Lane-1:1kb DNA ladder; Lane-2 *sucrose synthase* gene*;* Lane-3: *korrigan* gene

The PCR amplified products from *P. deltoides* cDNA were excised and purified from agarose gel after electrophoresis using a Qiagen gel extraction kit (Qiagen, Valencia, CA, USA) following manufactures instructions and cloned into pGEM®-T easy vector (Promega Corporation, USA) used for PCR cloning. Competent *E. coli* DH5α cells were transformed with the ligated products following heat shock method. The positive clones were screened by α-complementation and the plasmid DNA from various colonies of recombinant cells was isolated using the alkaline lysis method (Fig. 6.7). Presence of these genes in plasmids was confirmed by PCR amplification of corresponding fragments using gene specific primers (Fig. 6.8A and 6.8B). The inserts in the plasmids were sequenced by chain termination method (Sanger *et al.* 1977) using an Applied Biosystems automatic sequencer (DNA sequencing facility, Department of Biochemistry, South campus, Delhi university, New Delhi, India). The DNA sequences (Annexure 1) thus obtained was used to characterize these genes using various bioinformatics tools.

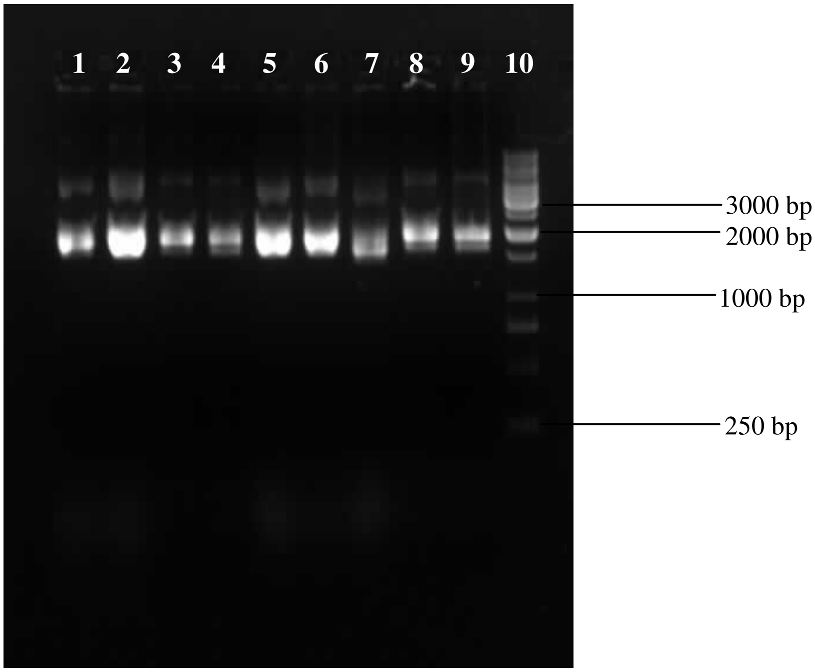


Figure 6.7 Plasmid DNA isolation from *E. coli* DH5α cells using alkaline lysis method. Lane 1-9: Plasmid (pGEM-T Easy vector) with fragments of interest (*KOR* and *SUS*) ; Lane 9: 1 kb DNA ladder

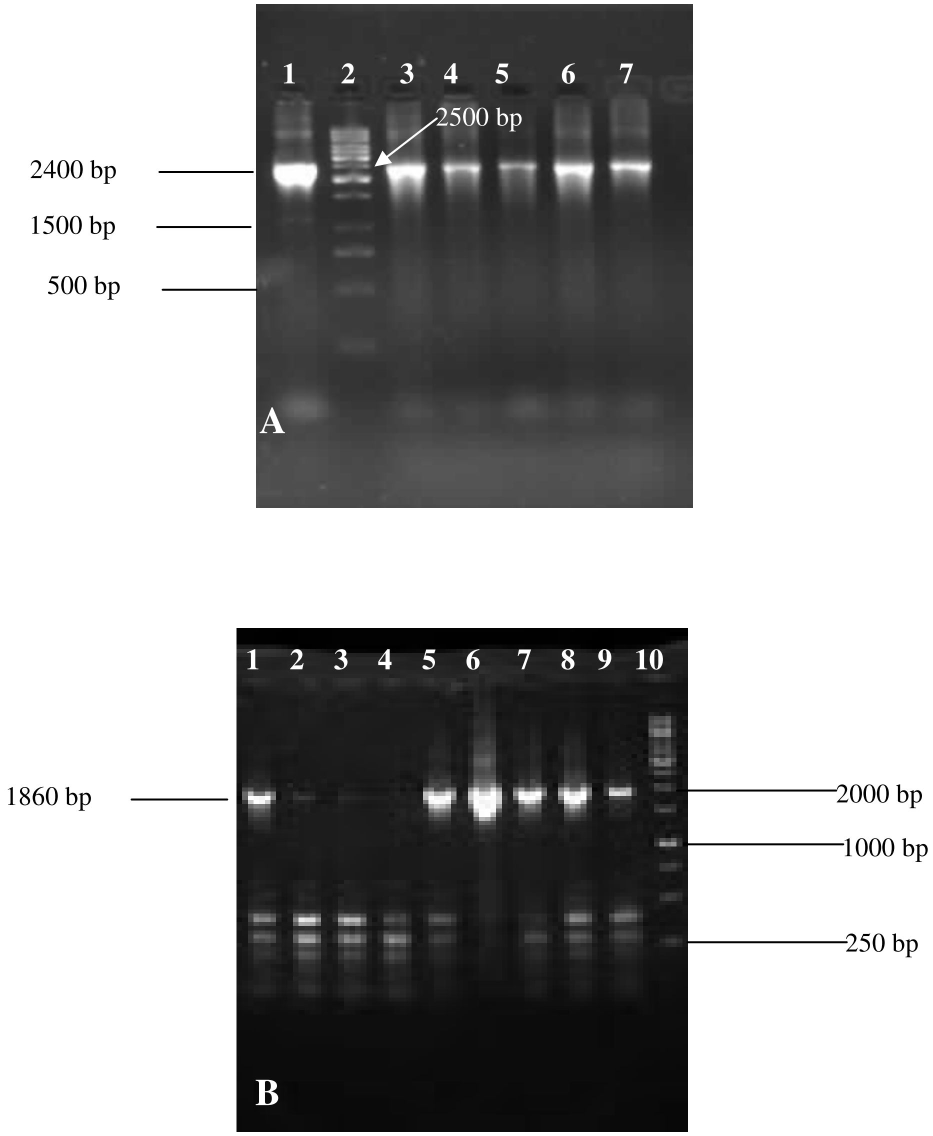


Figure 6.8 A Screening of *SUS* from plasmid isolated from positive clones Lane 1: Positive control amplification from P. *deltoides* cDNA; Lane 2: 1 kb DNA Ladder; Lane 3-7: Amplification from positive clones using gene specific primers. **B.** Screening of *KOR* from plasmid isolated from positive clones. Lane 1: Positive control amplification from *P. deltoides* cDNA; Lane 2-9: Amplification from positive clones using gene specific primers; Lane 10: 1 kb DNA Ladder

Characterization of *korrigan*

Sequence analysis of the cloned *KOR* full length gene fragment was performed with BLASTN programmme (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Isolated KOR gene sequence showed 99 % similarity with reported sequences of other *Populus* species like *P. trichocarpa* and *P. tremuloides* (Table. 6.1).

Table 6.1 BLASTN analysis of full length *korrigan* from *P. deltoides (1860 bp)* with homologus sequences of other species

Accession	Description	Max score	Total score	Query coverage	E value	Max identity
XM_002332660.1	*Populus trichocarpa* hypothetical protein (POPTRKOR2), mRNA	3358	3358	100%	0.0	99%
AY660967.1	*Populus tremula x Populus tremuloides* Cel9A mRNA, complete cds	3247	3247	100%	0.0	98%
AY535003.1	*Populus tremuloides* endo-1,4-beta-glucanase (Kor1) mRNA, complete cds	3236	3236	100%	0.0	98%
GU324115.1	*Populus alba x populus grandidentata* membrane bound endo-beta 1, 4-zlucanase mRNA, complete cds	3219	3219	100%	0.0	98%

The identification of the open reading frame (ORF) and the amino acid sequences encoded by *KOR* nucleic acid sequence was deduced using the ORF finder programme from NCBI. The predicted ORF of *KOR* gene was found to encode a protein of 619 amino acids (Fig. 6.9) with a calculated molecular mass of 68.45 kDa and an isoelectric point of 8.92.

Homologues amino acid sequences corresponding to *KOR* were retrieved from databases using BLAST P. These sequences were aligned using MAFFT and edited using BIOEDIT programme. A comparison of these protein sequences with that of other reported KOR proteins from different plant species showed the strong overall similarity. (Fig. 6.10).

Figure 6.9 cDNA and deduced amino acid (ORF) sequences of the *KOR* gene in *P. deltoides*

```
   1  ATGTACGGAAGAGATCCATGGGGAGGACCCCTGGAGATAAATGCAGCAGATTCAGCAACAGATGAT
       M  Y  G  R  D  P  W  G  G  P  L  E  I  N  A  A  D  S  A  T  D  D
  67  GACAGGAGCAGGAACTTGAATGACTTAGACAGGGCAGCTCTGTCAAGGCCATTGGATGAGACTCAG
       D  R  S  R  N  L  N  D  L  D  R  A  A  L  S  R  P  L  D  E  T  Q
 133  CAGAGCTGGTTGCTGGGTCCAGCCGAGCAAAAGAAGAAGAAGAAATATGTGGATCTGGGCTGTGTC
       Q  S  W  L  L  G  P  A  E  Q  K  K  K  K  K  Y  V  D  L  G  C  V
 199  ATTGTTAGTCGCAAGATCTTTGTGTGGACTGTTGGGAGTATCGTTGCTGCTGGTCTATTGGTTGGT
       I  V  S  R  K  I  F  V  W  T  V  G  S  I  V  A  A  G  L  L  V  G
 331  CTTATTACTCTTATTGTTAAAACTGTGCCTCGCCATCATCACTCTCATGCTCCAGCTGATAACTAC
       L  I  T  L  I  V  K  T  V  P  R  H  H  H  S  H  A  P  A  D  N  Y
 397  ACTCTTGCTCTACATAAGGCACTCATGTTCTTCAATGCTCAAAGATCGGGAAAGCTTCCGAAGCAT
       T  L  A  L  H  K  A  L  M  F  F  N  A  Q  R  S  G  K  L  P  K  H
 463  AACAACGTGTCATGGAGGGGAAGCTCGTGTTTGGGCGATGGGAAAGGTAAACAAGGTAGTTTTTAC
       N  N  V  S  W  R  G  S  S  C  L  G  D  G  K  G  K  Q  G  S  F  Y
 529  AAAGATCTGGTGGGTGGATATTATGATGCTGGGGATGCAATAAAGTTCCACTTCCCTGCTTCTTTT
       K  D  L  V  G  G  Y  Y  D  A  G  D  A  I  K  F  H  F  P  A  S  F
 595  TCCATGACAATGTTGAGCTGGAGTGTTATTGAATATAGTGCGAAATATGAAGCTTCTGGTGAGCTT
       S  M  T  M  L  S  W  S  V  I  E  Y  S  A  K  Y  E  A  S  G  E  L
 661  AACCATGTCAAAGAACTTATAAAGTGGGGAGCTGACTACTTTCTGAAGACATTTAATAGTTCTGCT
       N  H  V  K  E  L  I  K  W  G  A  D  Y  F  L  K  T  F  N  S  S  A
 727  GATACCATCGACAGGATAGTTGCACAGGTTGGCTCGGGGGATACTTCTGGTGGGAGTACCACTCAA
       D  T  I  D  R  I  V  A  Q  V  G  S  G  D  T  S  G  G  S  T  T  Q
 793  AACGATCATTATTGCTGGATGCGTCCTGAGGACATTGATTACGACAGACCTGTAACTGAATGCAGC
       N  D  H  Y  C  W  M  R  P  E  D  I  D  Y  D  R  P  V  T  E  C  S
 854  TCCTGCTCTGATCTTGCTGCAGAAATGGCTGCTGCCTTAGCTTCTGCATCTATCGTTTTCAAAGAT
       S  C  S  D  L  A  A  E  M  A  A  A  L  A  S  A  S  I  V  F  K  D
 923  AACAAGGCCTACTCTCAGAAGCTTGTTCATGGTGCCAAAACTCTCTTTAAATTTGCAAGGGACCAG
       N  K  A  Y  S  Q  K  L  V  H  G  A  K  T  L  F  K  F  A  R  D  Q
 991  CGAGGTAGATACAGTGCAAGTGGTTCGGAAGCCGCCATTTTCTATAATTCAACTAGTTACTGGGAT
       R  G  R  Y  S  A  S  G  S  E  A  A  I  F  Y  N  S  T  S  Y  W  D
1057  GAATTTATTTGGGGTGGAGCTTGGCTATATTATGCAACTGGAAATAATTCCTATCTTCAGCTTGCT
       E  F  I  W  G  G  A  W  L  Y  Y  A  T  G  N  N  S  Y  L  Q  L  A
1123  ACAATGCCTGGCTTAGCCAAGCATGCCGGTGCATTTTGGGGAGGGCCAGACTATGGTGTGCTTAGC
       T  M  P  G  L  A  K  H  A  G  A  F  W  G  G  P  D  Y  G  V  L  S
1189  TGGGACAACAAGCTTGCTGGTGCTCAGCTACTTCTGAGCCGCCTAAGATTGTTCTTGAGCCCTGGT
       W  D  N  K  L  A  G  A  Q  L  L  L  S  R  L  R  L  F  L  S  P  G
1255  TATCCATATGAAGAAATACTGAGTACATTTCACAACCAGACCAGTATAATCATGTGCTCATATCTG
       Y  P  Y  E  E  I  L  S  T  F  H  N  Q  T  S  I  I  M  C  S  Y  L
1321  CCAATTTTCACAAAGTTTAATAGAACAAAAGGAGGCTTGATCGAGTTAAACCATGGAAGGCCTCAG
       P  I  F  T  K  F  N  R  T  K  G  G  L  I  E  L  N  H  G  R  P  Q
1387  CCCCTTCAGTATGTCGTCAACGCTGCCTTCCTAGCAACCCTATTTAGTGGTTATCTTGAAGCTGCT
       P  L  Q  Y  V  V  N  A  A  F  L  A  T  L  F  S  G  Y  L  E  A  A
1453  GATACACCTGGATGGTATTGTGGACCAAATTTCTACTCTACTGATGTGTTGCGTGATTTCGCCAAG
       D  T  P  G  W  Y  C  G  P  N  F  Y  S  T  D  V  L  R  D  F  A  K
1519  ACTCAAATTGATTACATCCTGGGCAAAAATCCTCGTAAAATGAGCTATATTGTGGGTTTTGGTAAT
       T  Q  I  D  Y  I  L  G  K  N  P  R  K  M  S  Y  I  V  G  F  G  N
1585  CATTACCCAAAACATCTCCACCATAGAGGTGCATCTATCCCTAAGAACAAGATCAGGTATAACTGT

       K  G  G  W  K  W  R  D  T  S  K  P  N  P  N  T  L  V  G  A  M  V
1651  AAAGGAGGATGGAAATGGAGGGACACCTCGAAGCCAAACCCAAATACACTTGTTGGAGCCATGGTT
       A  G  P  D  R  H  D  G  F  H  D  V  R  T  N  Y  N  Y  T  E  P  T
1717  GCGGGCCCTGACCGGCATGATGGTTTCCATGATGTTCGCACCAACTATAATTATACAGAGCCAACG
       I  A  G  N  A  G  L  V  A  A  L  V  A  L  S  G  D  K  T  T  G  I
1783  ATTGCTGGAAATGCAGGATTAGTTGCAGCACTTGTGGCCTTGTCTGGTGACAAAACTACTGGGATT
       D  K  N  T  I  F  S  A  V  P  P  M  F  P  T  P  P  P  P  P  A  P
      TGGAAACCATGA
       W  K  P  *
```

Initiation and stop code are shown in bold. Asterisk indicates the termination codon

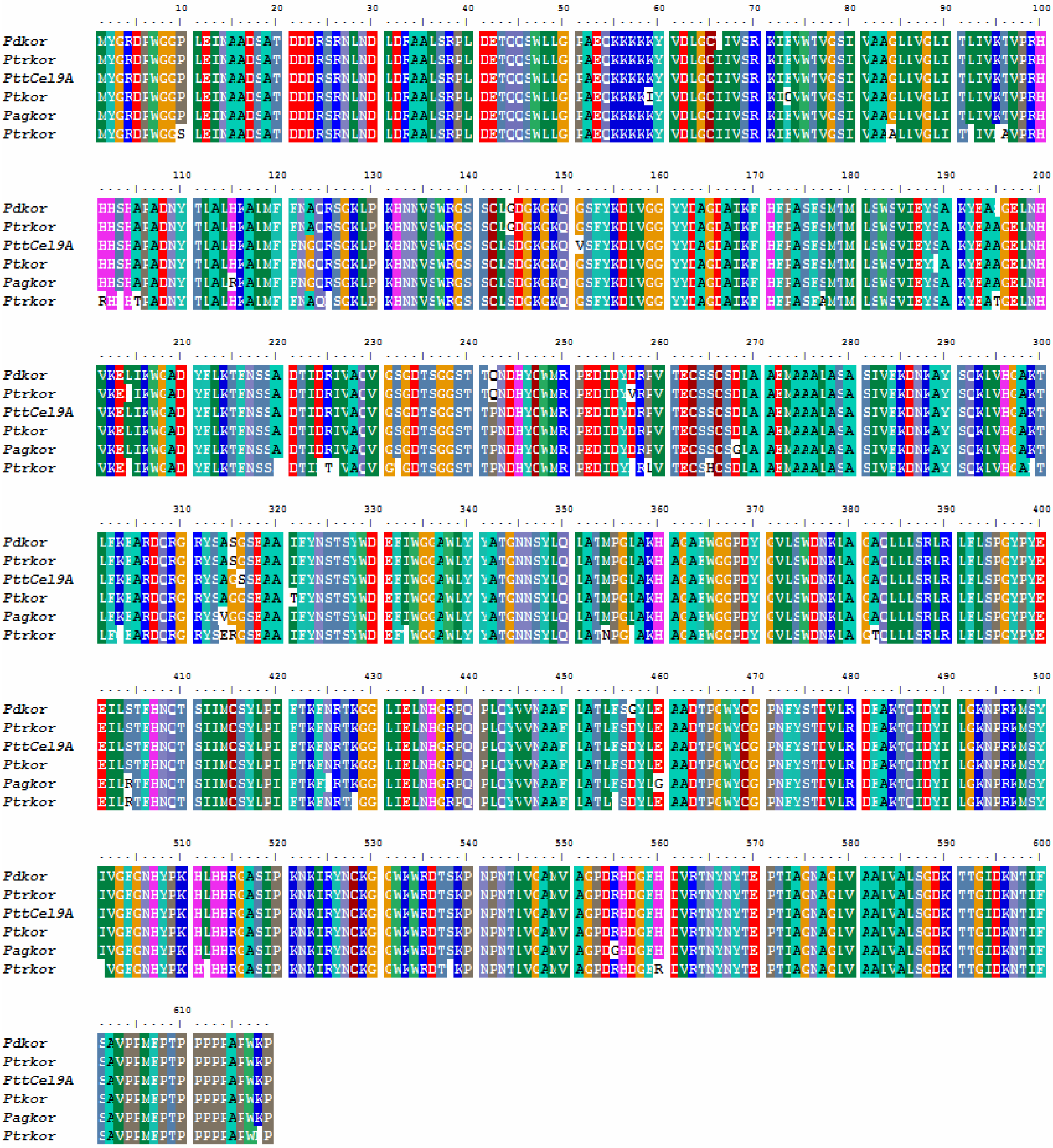


Figure 6.10 Alignment of *P. deltoides* amino acid sequence of *KOR* with different endo-1,4-b-glucanase protein sequences. *Pdkor- Populus deltoides* (Query); *Ptrkor- Populus trichocarpa*; *Pagkor- Populus alba x grandidentata*; *Ptkor- Populus tremuloides ,Pttkor- Populus tomestosa*

Potential transmembrane domains were identified using TMHMM-2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0) programme. In case of KOR protein, a single transmembrane domain was detected (Fig. 6.11) between amino acids 72–94, a feature that is similar to other known KOR proteins. The glycosylation and phophorylation sites in deduced amino acid sequence were identified using NetNGlyc 1.0 and NetPhos 2.0 Server of technical University of Denmark. In KOR protein, 8 glycosylated sites (Fig. 6.12) and total 40 phosphorylation sites (out of which 22 are at serine residue, 9 each at threonine and tyrosine residues) were detected (Fig. 6.13), indicating that *KOR* might be undergoing post translational modifications for proper functioning. InterProScan programme of European Bioinformatics institute, predicted that the isolated KOR from *P. deltoides* belongs to glycosyl hydrolase family 9 (GH9) of proteins (Fig. 6.14).

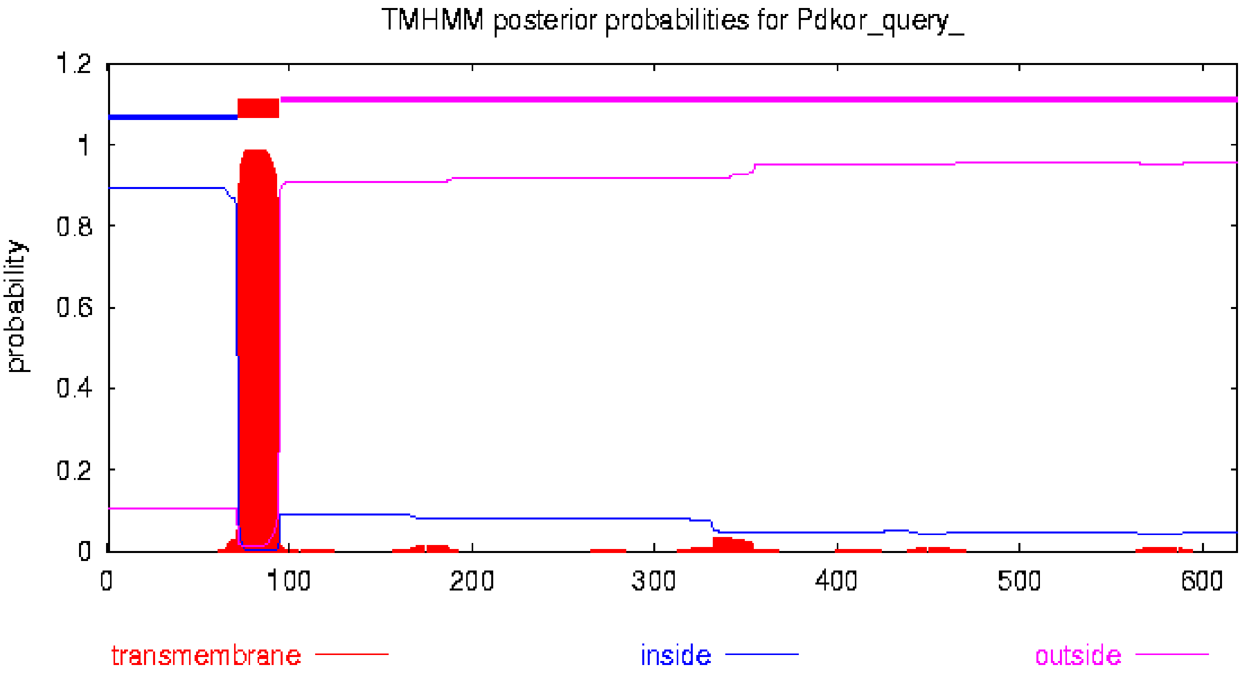
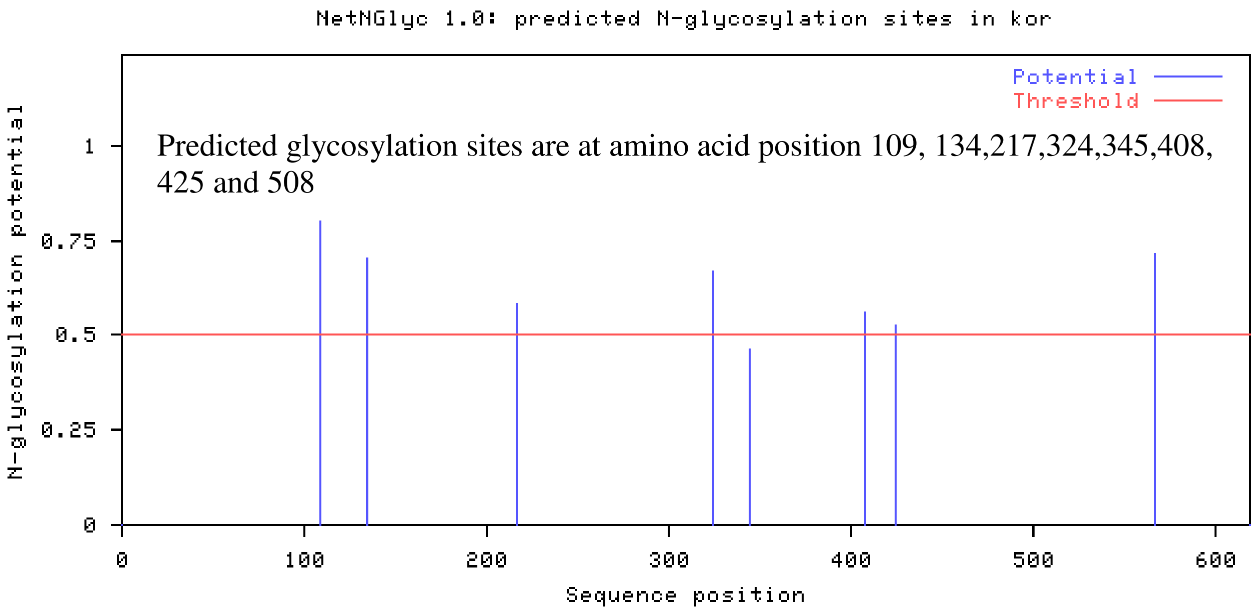
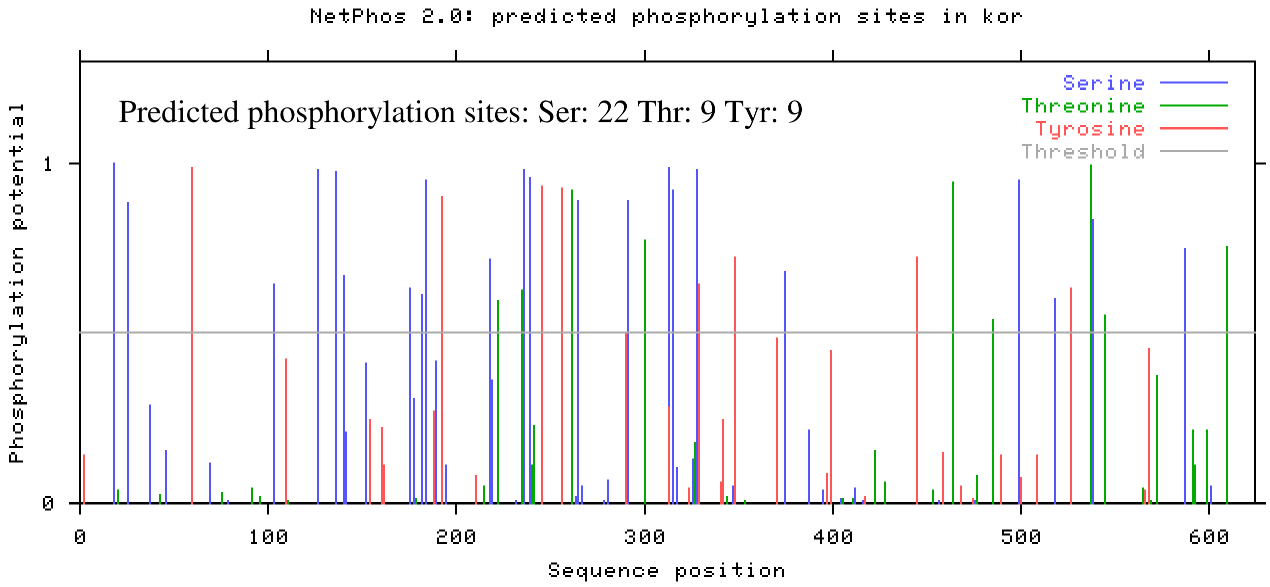


Figure 6.11 Prediction of transmembrane domain in cloned KOR protein (*P. deltoides*) using TMHMM-2.0 http://www.cbs.dtu.dk/services/TMHMM-2.0) programme.



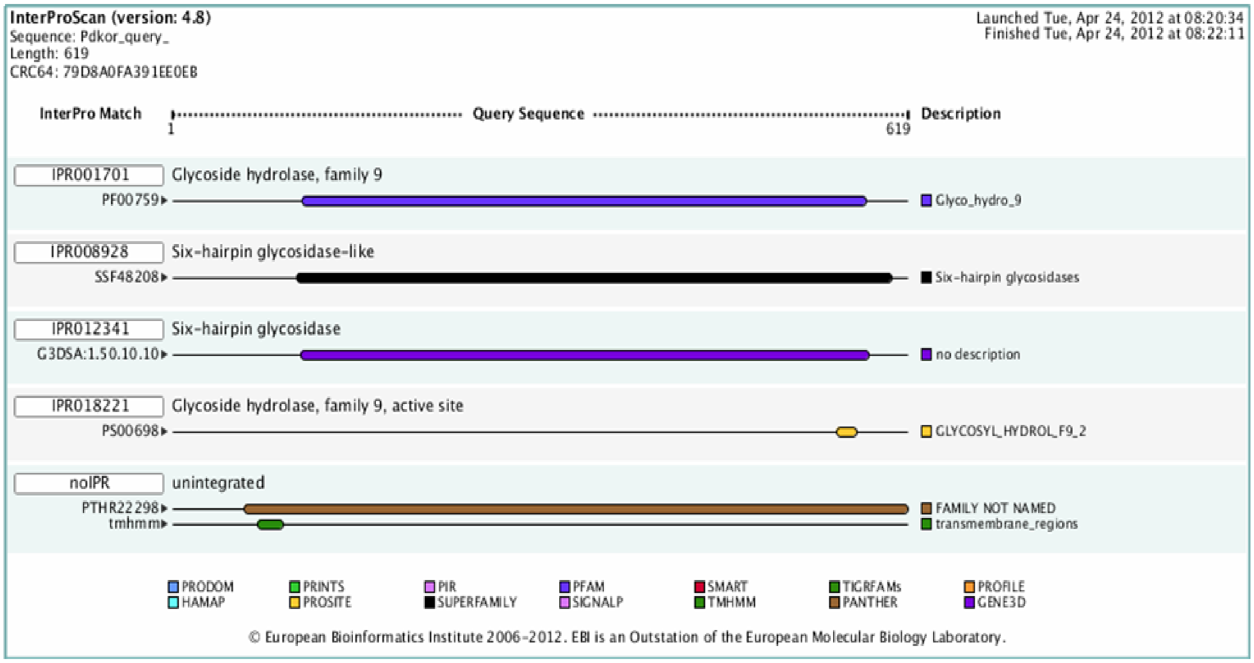
Predicted glycosylation sites are at amino acid position 109, 134,217,324,345,408, 425 and 508

Figure 6.12 Prediction of glycosylation sites in KOR protein *(P. deltoides*) using NetNGlyc 1.0 Server (http://genome.cbs.dtu.dk//cgi-bin/webface?jobid=netNglyc)



Predicted phosphorylation sites: Ser: 22 Thr: 9 Tyr: 9

Figure 6.13 Prediction of phosphorylations sites in KOR protein (*P. deltoides*) using NetPhos 2.0 Server (http://genome.cbs.dtu.dk//cgi-bin/webface?jobid=netphos)



InterProScan (version: 4.8)
Sequence: Pdkor_query_
Length: 619
CRC64: 79D8A0FA391EE0EB

Launched Tue, Apr 24, 2012 at 08:20:34
Finished Tue, Apr 24, 2012 at 08:22:11

Figure 6.14 Pictorial representation of InterProScan analysis of KOR protein (*P. deltoides*)

Characterization of *sucrose synthase*

Sequence analysis of the cloned *SUS* full length gene fragment was performed with BLASTN programmme (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Isolated *SUS* gene sequence showed 99 % similarity with reported sequences of other *Populus* species like *P. trichocarpa* and *P. tremuloides* (Table. 6.2).

Table 6.2 BLASTN analysis of full length *sucrose synathse* from *P. deltoides* (2400 bp) with homologus sequences of other species

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
GU559729.1	*Populus trichocarpa* sucrose synthase 1 (SuS1) mRNA, complete cds	4316	4316	99%	0.0	99%
XM_002324100.1	*Populus trichocarpa* hypothetical protein (PtrSuSY1), mRNA	4316	4316	99%	0.0	99%
GU559727.1	*Populus tomentosa* sucrose synthase 1 (SuS1) mRNA, complete cds	4235	4235	99%	0.0	98%
AY341026.1	*Populus tremuloides* sucrose synthase mRNA, complete cds	3635	3635	99%	0.0	94%
GU559730.1	*Populus trichocarpa* sucrose synthase 2 (SuS2) mRNA, complete cds	3463	3463	98%	0.0	93%

The predicted ORF of *SUS* gene was found to encode a protein of 805 anino acids (Fig. 6.15) with a calculated molecular mass of 92.29 kDa and an isoelectric point of 6.12. Homologues amino acid sequences corresponding to *SUS* gene were retrieved from databases using BLAST P. These sequences were aligned using MAFFT and edited using BIOEDIT programme. A comparison of these protein sequences with that of other reported SUS cproteins from different plant species showed the strong overall similarity. (Fig. 6.16).

No transmembrane domain was detected in case of SUS protein (Fig. 6.17)**.** **S**US protein has also shown the presence of glycosylations as well as phosphorylation sites in his sequence. 4 glycosylation sites (Fig. 618) and 38 phosphorylation sites, out of which 15 are at serine residue, 14 are at threonine residue and 9 are at tyrosine residue were detected in SUS protein (Fig. 6.19). InterProScan programme from European Bioinformatics institute, predicted that isolated KOR from *P. deltoides* belongs to glycosyl hydrolase family 9 (GH9) of proteins and SUS belongs to sucrose synthase family of proteins, a member of UDP-glycosyltransferase (GT1) super family (Fig. 6.20).

Figure 6.15 cDNA and deduced amino acid (ORF) sequences of the *SUS* gene in *P. deltoides*

```
  1  ATGACTGAACGTGCTCTTACTCGTGTCCATAGCATTCGTGAACGTGTAGATGAGACTCTCAAGGCT
      M  T  E  R  A  L  T  R  V  H  S  I  R  E  R  V  D  E  T  L  K  A
 66  CATCGCAATGAAATTGTTGCGTTACTCACAAGGATCGAAGGTAAGGGAAAAGGAATTCTCCAACAC
      H  Q  I  V  A  E  F  E  A  I  P  E  D  T  R  K  T  L  A  G  G  A
132  CACCAGATTGTTGCTGAGTTTGAAGCAATTCCTGAAGACACCAGGAAAACATTGGCAGGCGGTGCT
      F  A  E  V  L  R  S  T  Q  E  A  I  V  V  P  P  W  I  A  L  A  L
198  TTTGCTGAAGTTCTCAGATCCACACAGGAAGCAATTGTTGTGCCTCCATGGATTGCTCTTGCTCTG
      R  P  R  P  G  V  W  E  Y  I  R  L  N  V  Q  A  L  V  V  E  E  L
330  CGCCCGAGGCCTGGTGTCTGGGAGTACATTAGACTGAATGTCCAAGCACTTGTTGTTGAGGAGCTG
      R  V  A  E  Y  L  H  F  K  E  E  L  V  D  G  G  S  N  G  N  F  V
396  CGTGTTGCTGAGTATCTTCATTTCAAGGAGGAGCTTGTTGATGGAGGCTCGAACGGCAACTTTGTG
      L  E  L  D  F  E  P  F  N  A  S  L  P  R  P  T  L  S  K  Y  I  G
462  CTTGAATTGGACTTCGAACCATTCAATGCATCTCTCCCTCGCCCAACTCTTTCAAAGTATATTGGT
      N  G  V  E  F  L  N  R  H  L  S  A  K  L  F  H  D  K  E  S  L  H
528  AATGGTGTTGAGTTCCTTAATCGCCACCTTTCGGCTAAGTTGTTCCATGACAAGGAAAGCCTGCAT
      P  L  L  A  F  L  K  V  H  C  H  K  G  K  N  M  M  L  N  D  R  I
594  CCCCTGCTTGCATTTCTCAAAGTGCACTGTCACAAGGGGAAGAATATGATGCTGAATGACAGAATT
      R  N  L  D  S  L  Q  Y  V  L  R  K  A  E  E  F  L  S  T  L  E  P
660  CGTAACCTAGACTCTCTGCAATATGTTCTGAGAAAGGCTGAGGAGTTTCTGTCTACTCTGGAACCT
      D  T  P  Y  S  Q  F  E  H  K  F  Q  E  I  G  L  E  R  G  W  G  D
726  ACTGCCGAGCGTGTTCTTGAGACGATTCGACTTCTTTTGGATCTTCTTGAGGCACCAGATCCCTGC
```

```
        T  A  E  R  V  L  E  T  I  R  L  L  L  D  L  L  E  A  P  D  P  C
 792  ACTCTCGAAACATTCCTAGGCAGAATTCCTATGGTCTTCAATGTTGTGATTATGTCCCCTCATGGA
        T  L  E  T  F  L  G  R  I  P  M  V  F  N  V  V  I  M  S  P  H  G
 852  TACTTTGCCCAAGACAATGTTTTGGGGTATCCTGATACTGGAGGCCAGGTTGTTTACATTTTGGAT
       Y  F  A  Q  D  N  V  L  G  Y  P  D  T  G  G  Q  V  V  Y  I  L  D
 924  CAAGTCCGTGCCTTGGAGAATGAAATGCTTCAGCGTATCAAGAAGCAAGGACTTGATATTACCTCC
        Q  V  R  A  L  E  N  E  M  L  Q  R  I  K  K  Q  G  L  D  I  T  S
 990  CGAATTCTCATTATTACTCGACTACTCCCTGATGCAGTAGGAACCACTTGTGGTCAACGTTTGGAG
        R  I  L  I  I  T  R  L  L  P  D  A  V  G  T  T  C  G  Q  R  L  E
1056  AAAGTGTATGGATCTGAGCATTGTGATATTCTTCGAGTTCCCTTCAGAGATGGAAAGGGTATGGTC
        K  V  Y  G  S  E  H  C  D  I  L  R  V  P  F  R  D  G  K  G  M  V
1122  CGCAAATGGATCTCTCGCTTTGAAGTGTGGCCATACCTAGAAACTTTCGCCGAGGACGTTGCTGCT
        R  K  W  I  S  R  F  E  V  W  P  Y  L  E  T  F  A  E  D  V  A  A
1188  GAAATTGCTAAGGAGTTGCAGGGCAAGCCTGATCTTATCATTGGAAATTACAGTGATGGAAACATC
        E  I  A  K  E  L  Q  G  K  P  D  L  I  I   G  N  Y  S  D  G  N I
1254  GTTGCCTCCTTGTTAGCACACAAATTAGGTGTTACAGAGTGCACCATTGCGCATGCTCTAGAAAAA
        V  A  S  L  L  A  H  K  L  G  V  T  E  C  T  I  A  H  A  L  E  K
1320  ACAAAGTATCCAGACTCAGATATATACTGGAAGAAGTTTGATGAAAAGTACCACTTCTCATGCCAG
       T  K  Y  P  D  S  D  I  Y  W  K  K  F  D  E  K  Y  H  F  S  C  Q
1386  TTTACAGCTGATCTTTTTGCAATGAACCATACAGATTTCATTATCACCAGCACATTCCAAGAGATT
        F  T  A  D  L  F  A  M  N  H  T  D  F  I  I  T  S  T  F  Q  E  I
1452  GCTGGAAGCAAGGATACTGTTGGACAGTATGAAAGCCACACTGCTTTCACTCTCCCTGGCCTCTAT
        A  G  S  K  D  T  V  G  Q  Y  E  S  H  T  A  F  T  L  P  G  L  Y
1518  AGAGTTGTTCATGGTATTGATGTCTTTGATCCCAAACTCAACATTGTATCCCCTGGCGCCGATGAG
        R  V  V  H  G  I  D  V  F  D  P  K  L  N  I  V  S  P  G  A  D  E
1584  AGCATATACTTCCCCTACACTGACGAGAAACGTAGGTTGACTTCTTTCCATCCAGAAATTGACGAG
        S  I  Y  F  P  Y  T  D  E  K  R  R  L  T  S  F  H  P  E  I  D  E
1650  CTTCTTTACAGCCCTGTTGAGAATGAAGAGCACTTATGTGTTCTAAAAGACCGAAACAAACCAATT
        L  L  Y  S  P  V  E  N  E  E  H  L  C  V  L  K  D  R  N  K  P  I
1716  CTATTTACCATGGCAAGGCTGGACAGAGTTAAGAATTTATCTGGTCTTGTAGAATGGTATGGAAAG
        L  F  T  M  A  R  L  D  R  V  K  N  L  S  G  L  V  E  W  Y  G  K
1782  AACACCAAGCTACGTGAATTAGTTAATCTTGTTGTAGTTGGTGGAGATAGAAGAAAGCAGTCTAAA
        N  T  K  L  R  E  L  V  N  L  V  V  V  G  G  D  R  R  K  Q  S  K
1848  GATCTAGAAGAGCAAGCTGAGATGAAGAAAATGTACAGTCATATAGAGAAATACAACTTGAATGGC
        D  L  E  E  Q  A  E  M  K  K  M  Y  S  H  I  E  K  Y  N  L  N  G
1914  CAGTTCAGATGGATTTCTTCCCAGATGAACCGTGTGAGGAATGGAGAGCTTTACCGTTACATTTGT
        Q  F  R  W  I  S  S  Q  M  N  R  V  R  N  G  E  L  Y  R  Y  I  C
1980  GATACCAAGGGAGCTTTCGTGCAGCCTGCTTTGTATGAGGCTTTTGGATTGACAGTTGTTGAGGCC
       D  T  K  G  A  F  V  Q  P  A  L  Y  E  A  F  G  L  T  V  V  E  A
2046  ATGACTTGTGGTTTGCCAACTTTTGCTACCTGCAATGGTGGTCCTGCTGAGATCATTGTTAATGGA
        M  T  C  G  L  P  T  F  A  T  C  N  G  G  P  A  E  I  I  V  N  G
2112  AAATCCGGATTCCACATCGATCCTTATCATGGTGAGCAGGCTGCTGAGCTCCTTGTTGACTTCTTT
        K  S  G  F  H  I  D  P  Y  H  G  E  Q  A  A  E  L  L  V  D  F  F
2178  GAGAAGTGCAAGGTTGATCCCGCTCACTGGGACAAAATCTCCCACGGAGGTCTGCAACGAATCCAA
       E  K  C  K  V  D  P  A  H  W  D  K  I  S  H  G  G  L  Q  R  I  Q
2244  GAGAAGTATACCTGGCAAATTTACTCTCAACGGCTCCTGACGCTCACAGGAGTTTATGGATTCTGG
        E  K  Y  T  W  Q  I  Y  S  Q  R  L  L  T  L  T  G  V  Y  G  F  W
2310  AAGCACGTTTCGAACCTTGATCGTCTTGAGAGCCGTCGGTATATGGAAATGTTCTATGCACTCAAA
        K  H  V  S  N  L  D  R  L  E  S  R  R  Y  M  E  M  F  Y  A  L  K
2376  TACCGCAAATTGGCTGAGTCTGTTCCTTTGA
       Y  R  K  L  A  E  S  V  P  *
```

Initiation and stop code are shown in bold. Asterisk indicates the termination codon

Figure 6.16 Alignment of *P. deltoides* amino acid sequence of *SUS* with different SUS proteins of other plants. *PdeSUS- Populus deltoides* (Query); *PtriSUS- Populus trichocarpa*; *PtomSUS- Populus tomestosa; EgSUS- Eucalyptus grandis; AhySUS: Arachis hypogea; CaSUS- Cicer ariterium*

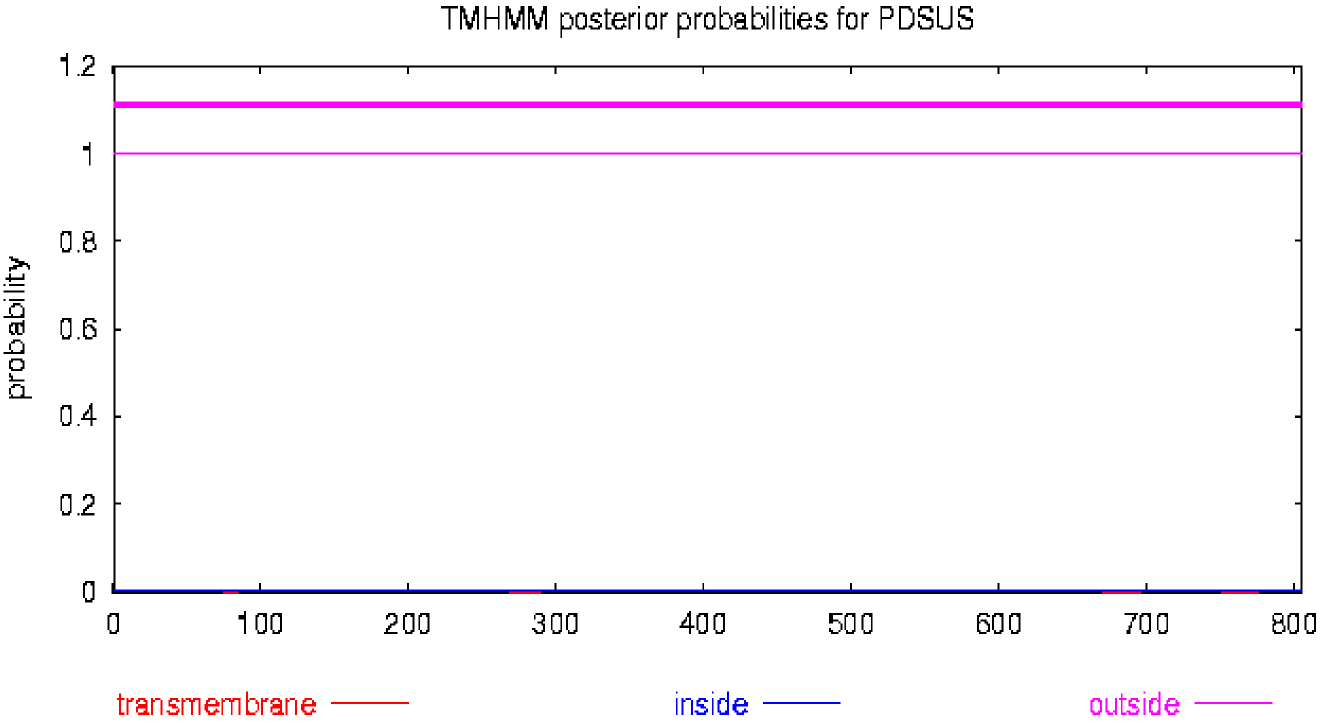
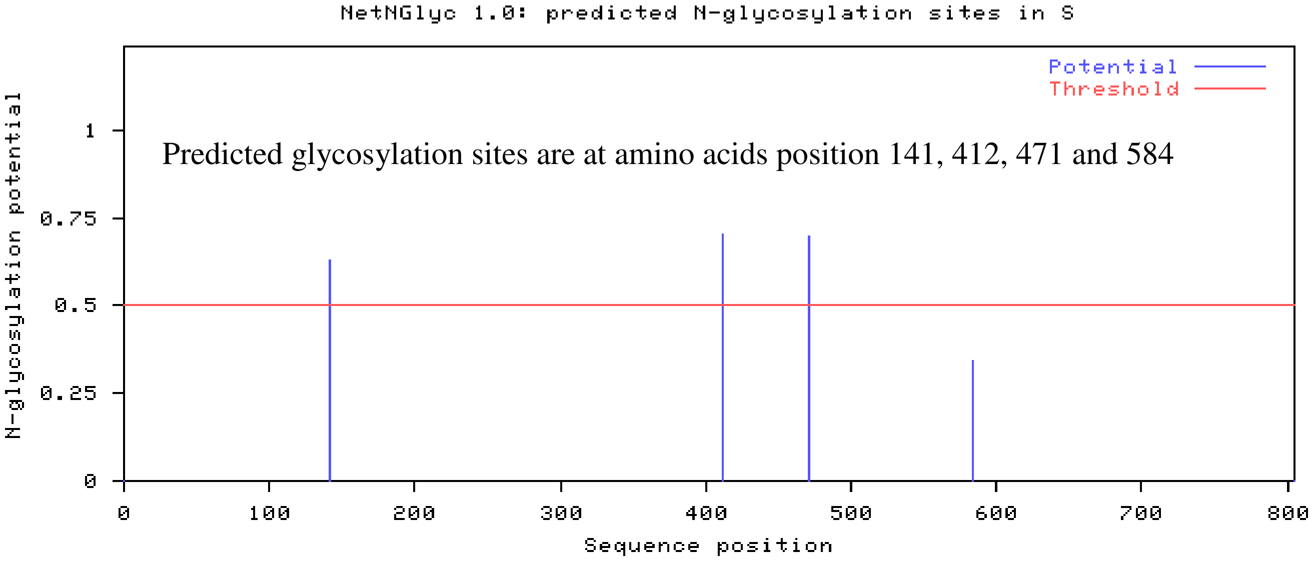


Figure 6.17 Prediction of transmembrane domain in SUS protein using TMHMM-2.0 http://www.cbs.dtu.dk/services/TMHMM-2.0) programme (please note that no transmembrane was detechted)



Predicted glycosylation sites are at amino acids position 141, 412, 471 and 584

Figure 6.18 Prediction of glycosylation sites in SUS protein using NetNGlyc 1.0 Server (http://genome.cbs.dtu.dk//cgi-bin/webface?jobid=netNglyc)

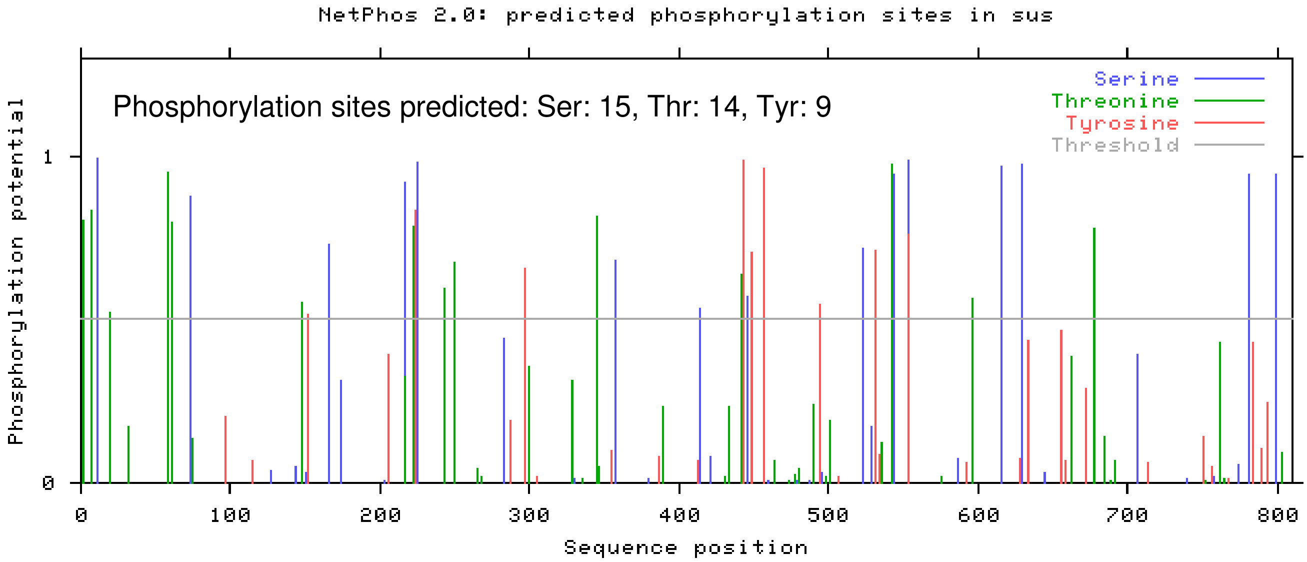
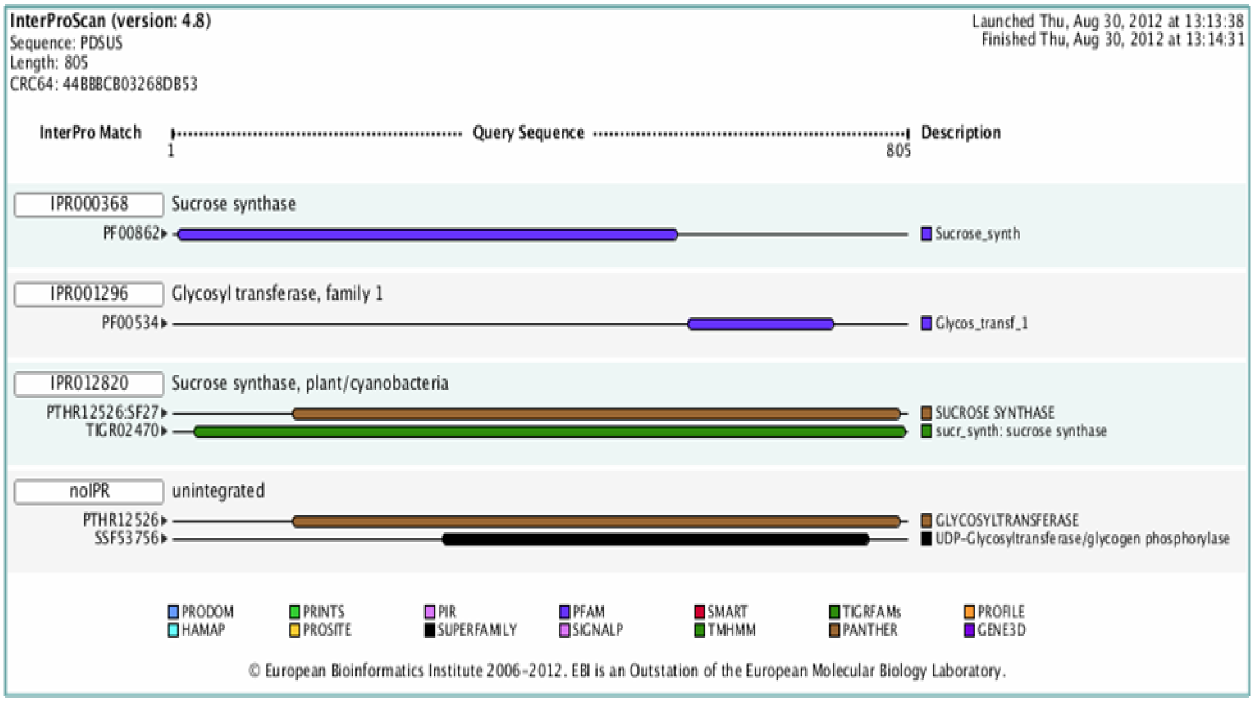


Figure 6.19 Prediction of phosphorylations sites in SUS protein using NetPhos 2.0 Server (http://genome.cbs.dtu.dk//cgi-bin/webface?jobid=netphos)



InterProScan (version: 4.8)
Sequence: PDSUS
Length: 805
CRC64: 44BBBCB03268DB53

Launched Thu, Aug 30, 2012 at 13:13:38
Finished Thu, Aug 30, 2012 at 13:14:31

InterPro Match	Query Sequence (1–805)	Description
IPR000368	Sucrose synthase	
PF00862		■ Sucrose_synth
IPR001296	Glycosyl transferase, family 1	
PF00534		■ Glycos_transf_1
IPR012820	Sucrose synthase, plant/cyanobacteria	
PTHR12526:SF27		■ SUCROSE SYNTHASE
TIGR02470		■ sucr_synth: sucrose synthase
noIPR	unintegrated	
PTHR12526		■ GLYCOSYLTRANSFERASE
SSF53756		■ UDP-Glycosyltransferase/glycogen phosphorylase

© European Bioinformatics Institute 2006-2012. EBI is an Outstation of the European Molecular Biology Laboratory.

Figure 6.20 Pictorial representation of InterProScan analysis of SUS protein

Discussion

Cellulose the most abundant, renewable polymer resource available worldwide is mostly used as raw material for two general purposes, first as a construction material, mainly in the form of intact wood and secondly as fibers such as cotton, flax, or in the form of paper and board. Cellulose consists of crystalline assemblies of parallel 1,4-β–linked glucan chains, which show a tensile strength comparable to that of steel and most commonly found in plant cell walls (Crawford 1981). Cellulose is synthesized at the plasma membrane by the coordinated action of multiple protein complex arranged in a hexagonal rosette like structure called terminal complex (Somerville 2006; Delmer 1999). These plasma membrane rosettes contain the cellulose synthase catalytic subunit (CESA) proteins that are encoded by the *CESA* genes (Somerville 2006).

In addition to CESA proteins, cellulose biosynthesis requires the action of other proteins (Matthysse *et al.* 1995). Two such important genes are, plasma membrane bound endo -1, 4- β- D-glucanase *Korrigan (KOR)*, which is important for normal cell wall assembly and cell elongation (Szyjanowicz 2004; Lane *et al.* 2001; Nicol *et al.* 1998) and *Sucrose synthase* (*SUS*) involved in providing precursor UDP-glucose for cellulose biosynthesis (Delmer 1999). As cellulose is an important component of plant cell walls, genetic modulation of cellulose synthesis can have a direct impact on several aspects of plant growth and development (Somerville *et al.* 2004). A thorough understanding of the intricacies of the cellulose biosynthetic processes is pivotal for genetic improvement of cellulose production in economically important plants (Joshi *et al.* 2004; Doblin *et al.* 2002; Williamson *et al.* 2002; Delmer 1999). Therefore present study was taken up for the cloning and characterization of *KOR* and *SUS*, two key genes involved in cellulose biosynthesis from *P. deltoides.*

A clean and intact RNA is important for any functional genomic study. Unfortunately, extracting intact RNA is difficult due presence of polyphenolic and polysaccharide compounds upon cell disruption (Rubio-Pina and Vazquez-Flota 2008). Furthermore, samples from different species and tissues show differences in the composition of these interfering compounds which lead to differences in quality of isolated RNA (Wu *et al.* 2008; Hibino *et al.* 2001). The difficulties in isolating high quality RNA have also been reported in other studies (Gonzalez-Mendoza *et al.* 2008; Vasanthaiah *et al.* 2008; Yang *et al.* 2008; Zeng *et al.* 2006). Most of these studies followed different RNA isolation protocols like guanidinium thiocynate, Cetyl trimethyl ammonium bromide (CTAB) or SDS/Phenol, suggesting that various plants requires different methods for isolation of quality RNA. Two of the most used methods for RNA extraction of woody plants are based on CTAB and SDS extractions (Gonzalez-Mendoza *et al.* 2008; Miyama and Tada 2008; Yang *et al.* 2008; Rodrigues *et al.* 2007; Chang *et al.* 1993) which are modified for plant tissues rich in polysaccharides and polyphenols. In the present study also various methods were tried and CTAB method (Chang *et al.* 1993) was found to be best for isolation of high quality RNA from *P. deltoides* (Fig. 6.1A). Isolated RNA was successfully used for the synthesis of cDNA and amplification of desired fragments from cDNA. The utility of the CTAB method for isolation of high quality RNA from woody species was reported by number of workers (Gonzalez-Mendoza *et al.* 2008; Miyama and Tada 2008; Yang *et al.* 2008; Rodrigues *et al.* 2007)

In the first attempt partial *KOR and SUS* fragments were amplified from cDNA using specific oligonucleotide primers specific to corresponding fragments (Table 3.5), PCR lead to the successful amplification of partial fragments of 1800 bp specific to *KOR* (Fig. 6.2) and 1200 bp specific to *SUS* (Fig. 6.3). BLAST N analysis confirmed the similarity of these fragments with already reported sequences in databases. *KOR* was found to share maximum similarity of 99 % with *P. tremultoides* (AY535003.1), whereas *SUS* also shares maximum similarity again with *P. tremultoides* (AY341026.1). Based on this information and sequence similarity of partial cDNA clones, primers for full length amplification of these genes (*KOR* and *SUS)* were designed from *P. tremuloides* endo-1,4-beta–glucanase mRNA (GenBank accession: AY535003) in case of *KOR* and *P. tremuloides* sucrose synthase mRNA (GenBank accession AY341026) in case of *SUS*. The amplification of DNA fragments of 1860 bp corresponding to full length coding sequence of *KOR* gene (Fig. 6.6) with overhangs of *Bam*H1 at 5' position and *Sac*1 at 3' position and 2400 bp corresponding to full length coding sequence of *SUS* gene (Fig. 6.6) with overhangs of *Bam*H1 at 5' position and *Sma*1 at 3' position was achieved from cDNA of *P. deltoides*. The amplified full length fragments of *KOR* and *SUS* shared identity of up to 99 % with other *KOR* and *SUS* from *P. trichocarpa* (Table 6.1 and 6.2). This suggests that these proteins are highly conserved in plant species and play an important function in the growth and development (Zheng 2011; Lin *et al.* 2009).

The predicted open reading frame (ORF) of *KOR* encodes a protein of 619 amino acids (Fig. 6.9) with a calculated molecular mass of 68.45 kDa and an isoelectric point of 8.92, whereas ORF of *SUS* encodes a protein of 805 amino acids (Fig. 6.15) with a calculated molecular mass of 92.29 kDa and an isoelectric point of 6.12. The ORFs of KOR and SUS began with an ATG start codon and ended with a TGA stop codon. The predicted protein of KOR and SUS shared a high sequence identity with other closely related homologs in the database. The predicted ORF of both KOR and SUS shares maximum similarity of 82 % and 85 % with *Arabidopsis thaliana* amino acid sequence. The *Arabidopsis KOR* gene was found to be involved in cytokinesis, pectin metabolism in the primary cell wall and cell elongation (His *et al.* 2001; Lane *et al.* 2001; Nicol *et al.* 1998). This indicates that KOR from *P. detoides* might be playing a similar role during plant development. cDNA sequences of both *KOR* and *SUS* from *P. deltoides* were deposited with GenBank under accession numbers KF 471020 and KF 471021 respectively (Annexure 1).

In case of *KOR*, G+C content in the sequence was 45.10 %, while in case of *SUS* GC content was found to be 44.66 %. Genomic nucleotide landscapes strongly vary among organisms. In eukaryotes, mean GC content ranges from about 20 % to 60 % and from 30 % to 50 % in animals and land plants (Lynch 2007). The GC content of both sequences was found to be in the reported range. For the predicted protein of SUS, the instability index (II) was computed as 33.06, which classify this protein as stable. The amino acid composition data also revealed that some of the amino acids such as Val (7.0 %), Phe (4.7 %), Glu (8.6 %), Tyr (4.0 %), Val (7.0 %) and Leu (11.2 %) occurred more frequently as compared to their average occurrence, whereas, the amino acids, namely Gln (3.4 %), Asp (3.6 %), Gly (6.6 %), Ala (6.0 %), Met (1.9 %), Ser (4.5 %) occurred less frequently (Doolittle 1989). Similarly the amino acid composition of KOR protein showed the amino acids that occurred in more frequency then average are Gly (8.9 %), Ala (9.4 %), Phe (4.4 %), Tyr (5.2 %), Asp (6.1 %), His (3.1 %), Ser (7.8 %) and amino acids which occurred less frequently are then average are Glu (2.4 %), Val (4.7 %), Leu (8.6 %), Met (1.8 %) (Doolittle 1989). Average amino acid composition passively reflects random permutations of the genetic code and reported to has evolutionary significance (Dyer 1971; King and Jukes 1969). Further, amino acid composition in protein structure may have an effect on overall quality of a protein. Recently, similar studies were carried out for sequence and gene expression analysis of vacuolar invertase (β -fructofuranosidase) from potato (Kumari and Das 2013).

One of the major post-translational protein modifications is glycosylation, an enzymatic process that links saccharides to specific amino acids of the polypeptide chain.

Glycosylation can be classified into four types based on the nature of chemical linkage between specific acceptor residues in the protein and sugar: N-linked and O-linked glycosylation, C-mannosylation, and glycosyl phosphatidyl inositol (GPI) anchors (Drickamer and Taylor 2006). N-glycosylation is a major posttranslational modification of proteins in all eukaryotic cells (Strasser 2007). Knowledge of these modifications is extremely important because these alter physical and chemical properties of the protein and thereby affect their folding, distribution, stability and thus influencing their biological activities (Haltiwanger and Lowe 2004; Rudd and Dwek 1997).

Similarly protein phosphorylation is also one of the most widespread and best understood posttranslational modifications. Phosphorylation turns many protein enzymes on and off, thereby altering their function and activity (Ciesla *et al.* 2011). Within a protein, phosphorylation can occur on several amino acids. Phosphorylation on serine is the most common, followed by threonine (Thomason and Kay 2000). Keeping in view these modifications, presence of phosphorylation sites were analyzed in both the proteins using NetPhos 2.0 Server. Both the proteins have shown the presence of glycosylation and phosphorylation sites (Fig. 6.12, 6.13, 6.18, 6.19), suggesting that these protein might be highly modified after translation. Earlier, both glycosylation and phosphorylation sites were reported in KOR proteins from other plants like *Picea glauca, Pinus taeda, Arabidopsis thaliana, Brassica napus* and *Populus trichocarpa* (Maloney and Mansfield 2010; Nairn *et al.* 2008; Bhandari *et al.* 2006; Molhoj *et al.* 2002; Nicol *et al.* 1998). Similarly both phosphorylation and glycosylation sites were also reported in other SUS protein homolog from different plant species (Jaysree *et al.* 2008; Carlson *et al.* 2002; Huber *et al.* 1996; Geigenberger and Sttit 1993; Sanchez de la Hoz *et al.* 1992). *SUS* has been reported to undergo post-translational modifications by reverse protein phosphorylation (Hardin *et al.* 2003; Winter and Huber 2000), thought to be important for determining its cellular distribution between the cytoplasm, actin cytoskeleton and plasma membrane. The phosphorylation site on maize sucrose synthase protein was identified as Ser15 (Huber *et al.* 1996) and in soybean at Ser11 (Zhang and Chollet 1997). Deduced sequences from SUS cDNAs cloned from monocotyledonous and dicotyledonous species show that this phosphorylation sites are conserved. Moreover, phosphorylation is reported to activate SUS protein and reported to have a regulatory significance (Huber *et al.* 1996). By prediction of glycosylation and phosphorylation sites from protein sequences, we can obtain much valuable information that can be useful for further research.

In case of KOR protein, a single transmembrane domain was detected between amino acids 72–94**,** a feature that is similar to other known KOR proteins (Molhoj *et al.* 2002), This motif has often been found in other membrane-anchored Endoglucanases (EGases) (Brummell *et al.* 1997). This suggests that *KOR* is a membrane-anchored enzyme involved in cellulose biosynthesis during plant cell wall assembly (Libertini *et al.* 2004). The specific functions (s) of membrane-anchored EGases are currently unknown, but they are located at the plasma membrane and participate in cellulose metabolism at the inner layers of the cell wall (Molhoj *et al.* 2001; del Campillo 1999; Nicol *et al.* 1998; Brummell *et al.* 1997). The signatures of Glycosyl hydrolase family 9 (Pfam Acc. No. PF00759) (Fig. 6.16) and "GLYCOSYL_HYDROL_F9_2" were found in *P. deltoides* KOR protein (Fig. 6.16), indicating that the KOR is a member of the glycoside hydrolase family 9 (GH9), which is reported to possess the activity of endoglucanase or cellobiohydrolase (Henrissat and Bairoch 1993, 1996). A six-hairpin glycosidase signature (Pfam Acc. No. SSF48208) was also located in KOR protein which was proposed to be the catalytic domain of EGase (Itoh *et al.* 2006).

In a report on the classification of glycosyl hydrolases, Henrissat (1991) found that most of the endoglucanases present in plants belong to glycoside hydrolase family 9 (GH9).

The reaction mechanism of the GH9 family is distinctive and upon hydrolysis the hydroxide on the anomeric carbon is inverted to the α-position. Generally, plant endoglucanases lack a catalytic binding domains (CBD), posses an N terminal endoplasmic reticulum import sequence, and are secreted into the apoplast (Molhoj *et al.* 2002). With the exception of a few putative CBD containing endoglucanases of unknown function (Trainotti *et al.* 1999), most plant endoglucanases are believed to depolymerize non-crystalline glucans, although these proposed catalytic mechanisms have yet to be fully understand (Libertini *et al.* 2004; Master *et al.* 2004; Ohmiya *et al.* 2003; Molhoj *et al.* 2001; Ohmiya *et al.* 2000). Enzymes within the GH9 family those are located at the plasma membrane act at the innermost layers of the cell wall and those that are secreted out of this cell act anywhere within the cell wall or at the outer layer (Rose *et al.* 2004; del Campillo 1999). Previous reports suggest that plasma-membrane anchored endoglucanases function in the biosynthesis of the cell wall either by editing the cellulose microfibril or during the assembly of the cellulose-hemicellulose network (Molhoj *et al.* 2001; Sato *et al.* 2001; Zuo *et al.* 2000; Nicol *et al.* 1998; Brummell *et al.* 1997).

No transmembrane domain was detected in case of SUS protein as sucrose metabolism proteins are generally believed to be cytosolic proteins. Plasma membrane association of SUS was first described by Amor *et al.* (1995), where the enzyme was found as the most abundant Uridine diposphate glucose (UDPG) binding protein on the plasma membrane in developing cotton fiber cells. This suggested a potential role for SUS in channelling UDPG into glucan synthesis directly from cytosolic sucrose. Membrane association of SUS is relatively strong and can only be released by strong detergents such as digitonin, CHAPS or SDS (Amor *et al.* 1995) or by phosphorylation of membrane vesicles (Winter *et al.* 1997). The mechanism of SUS association with membrane is not well understood but it has been shown that protein phosphorylation could be involved (Winter *et al.* 1997). *In vitro* studies have shown that dephosphorylation of SUS causes increased association with the membrane fraction whereas phosphorylation of membrane proteins caused the release of SUS from the membrane (Winter *et al.* 1997).

SUS belongs to sucrose synthase family of proteins, a member of UDP-glycosyltransferase (GT1) super family. The signatures of Sucrose synathse (Pfam Acc. No. PF00862) (Fig. 6.23) and "Glycos_Transf_1" (Pfam Acc. No. PF00534) were found in predicted *P. deltoides* SUS protein. Members of the SUS family are divergent and differentially expressed and act as the limiting factor in cellulose biosynthesis during plant growth and development. A comprehensive study of the structure, expression, and evolution of the SUS family in tree species is currently lacking (Zhang *et al.* 2011). Consequently, the identification of new cDNAs and studies involving structure, expression profiling, and phylogeny of the SUS family from tree species seems essential for improving wood fiber traits).

Moreover the glycosylation process is catalyzed by glycosyltransferases (GTs) enzymes (Mackenzie *et al.* 1997). GTs from diverse species have been classified into 92 families based on the amino acid sequence similarities, catalytic mechanisms and the presence of conserved sequence motifs (http://www.cazy.org/GlycosylTransferases.html). Among these, the glycosyltransferase family 1 is the largest family, the enzymes of which generally catalyze transfer of the glycosyl group from nucleoside diphosphate-activated sugars (e.g., UDP-sugars) to a diverse array of substrates (Jones and Vogt 2001, Ross *et al.* 2001). Consequently, a thorough knowledge of the expression patterns of *SUS* gene (SUS) families during tree development is required to understand the role of *SUS* in regulating wood fiber properties and effectively manipulating cellulose biosynthesis.

Conclusion

In conclusion, two important genes namely *Korrigan* (*KOR)* and *Sucrose synathse* (*SUS*) involved cellulose biosynthesis were cloned and characterized from *P. deltoides.* Both the cloned genes showed sequence similarity of 99 % with other homologues from the databases. Further, characteristics features to these genes were found in cloned genes.

Salient findings

High quality RNA was isolated using CTAB method from *P. deltoides*. Isolated RNA was successfully used for the synthesis of cDNA.

- In the first attempt partial *KOR* (1800 bp) *and SUS* (1200 bp) fragments were amplified from cDNA using specific oligonucleotide primers. BLAST N analysis confirmed the similarity of these fragments with already reported sequences in databases.
- The amplification of full length fragments corresponding to *KOR* gene and *SUS* gene was achieved from cDNA of *P. deltoides*. The amplified full length fragments of *KOR* and *SUS* shared identity of up to 99 % with other *KOR* and *SUS* genes from databases.
- The predicted open reading frame (ORF) of *KOR* encodes a protein of 619 amino acids, whereas ORF of *SUS* encodes a protein of 805 amino acids.
- cDNA sequences of both KOR and SUS from *P. deltoides* were deposited with GenBank under accession numbers KF 471020 and KF 471021 respectively.
- Both the genes have shown the presence of glycosylation and phosphyrtaion sites in their respective sequences.
- In case of KOR protein, a single transmembrane domain was detected between amino acids 72–94**,** whereas no transmembrane domain was detected in case of SUS protein.

- The predicted KOR belongs to glycosyl hydrolase family 9 (GH9) of proteins and SUS belongs to sucrose synthase family of proteins, a member of UDP-glycosyltransferase (GT1) super family.

Chapter 7

Transformation of *Eucalyptus* with *korrigan* and *sucrose synthase*

Cloning of *KOR* and *SUS* in binary vector pBI121

After characterization of *KOR* and *SUS* genes, these were directionally cloned into binary vector (pBI121). The fragments of both the genes were amplified using XT-20 Taq DNA Polymerase (Taq with proof reading activity, Banglore Genei, India) from the cloning vector isolated from *E. Coli* DH5α positive clones. As the fragments of both the genes are amplified with primers having overhangs for respective restriction enzymes, the fragments were digested with respective restriction enzymes, excised and purified from agarose gel electrophoresis using a Qiagen gel extraction kit (Qiagen, Valencia, CA, USA). Similarly pBI121 was also digested with respective restriction enzymes after digestion it was also excised and purified from agarose gel electrophoresis using a Qiagen gel extraction kit (Qiagen, Valencia, CA, USA). After purification of digested fragments and pBI121, fragments were directionally cloned into binary vector pBI121 at respective restriction sites (Fig. 7.1 and 7.2). Competent cells of *E. Coli* DH5α were transformed with ligated products. The positive clones were screened by growing the cells on LB medium containing kanamycin as selection marker and the plasmid DNAs of the recombinant cells were isolated using the alkaline lysis method**.** Plasmid containing full length *KOR* and *SUS* inserts were confirmed by PCR amplifications using gene specific primers. The plasmids with gene of interest were selected and transformed into competent cells of *A. tumefaciens* strain EHA105 using freeze thaw method (Holsters *et al.* 1978). *A. tumefaciens* strain EHA105 was selected for transformation as this strain was found more virulent then *A. tumefaciens* strain LBA4404 for transformation *E. tereticornis* (Fig. 5.2). Positive clones of strain EHA105 were selected on LB medium containing kanamycin (50 mg/l) and rifampicilin (15 mg/l). Plasmid DNAs of the recombinant cells were isolated using the alkaline lysis method (Fig. 7.3) and the presence of genes in positive clones was again confirmed with the help of PCR using gene specific primers (Fig. 7.4a and 7.4b).

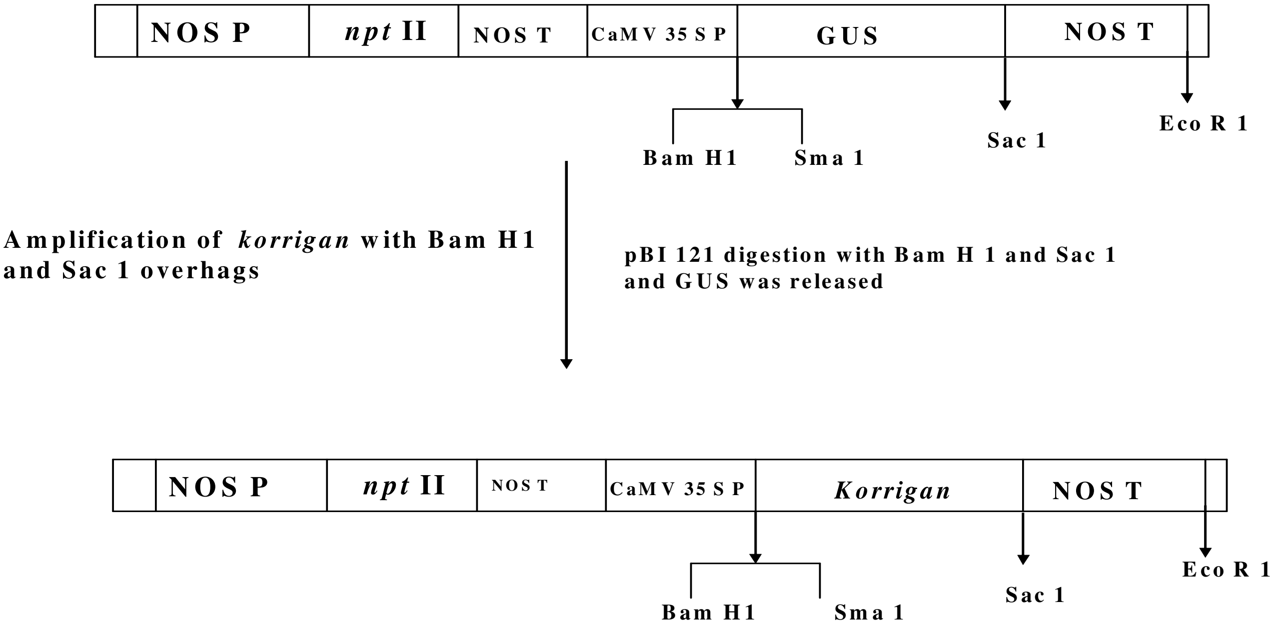


Figure 7.1 Schematic representation of ligation of full length gene fragment *KOR* in pBI121 (*KOR* was ligated between Bam H1 and Sac1 sites in place of GUS down stream of CaMV 35S promoter)

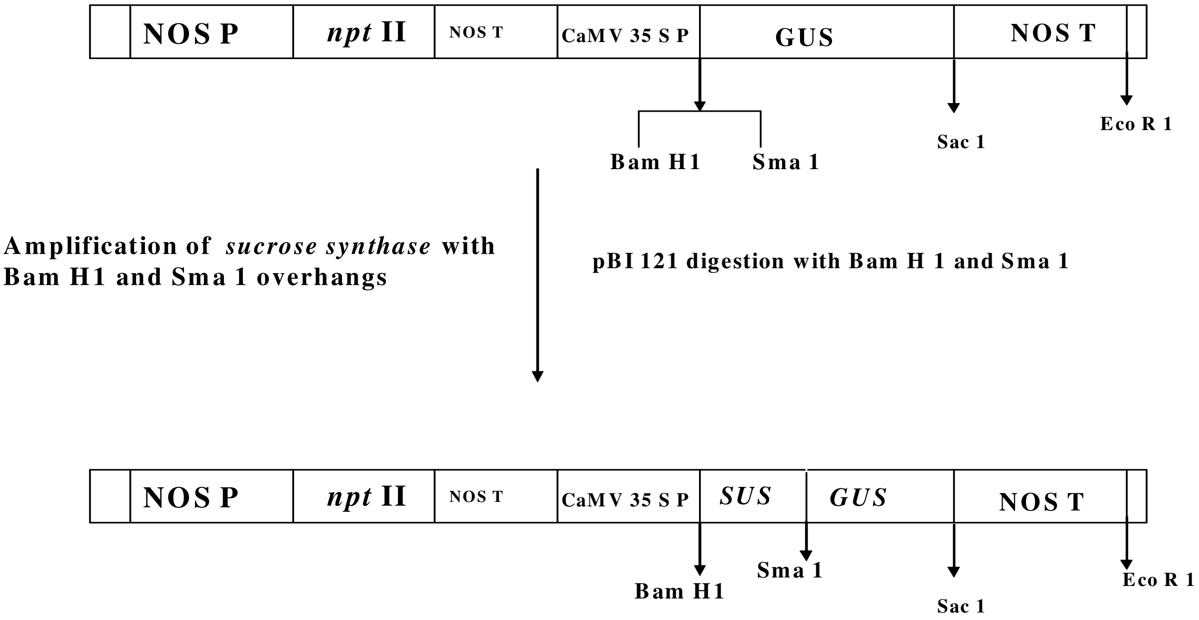


Figure 7.2 Schematic representation of ligation of full length gene fragment of *SUS* in pBI121 (*SUS* was ligated at Bam H1 and Sma 1sites, before GUS down stream of CaMV 35S promoter)

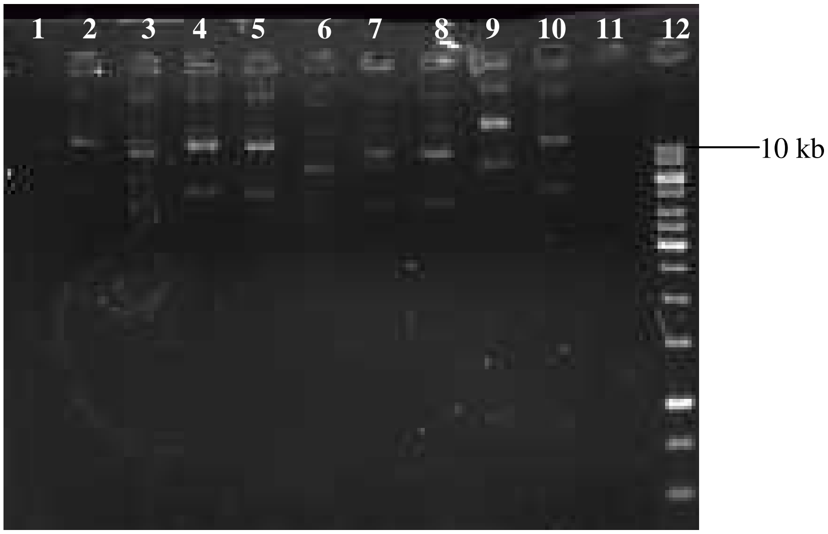


Figure 7.3 Plasmid DNA isolation from *A. tumefaciens* strain EHA105 cells using alkaline lysis method. Lane 1-11: Plasmid (pBI121) with fragments of interest (*KOR* and *SUS*) ; Lane 12: 1 Kb DNA ladder

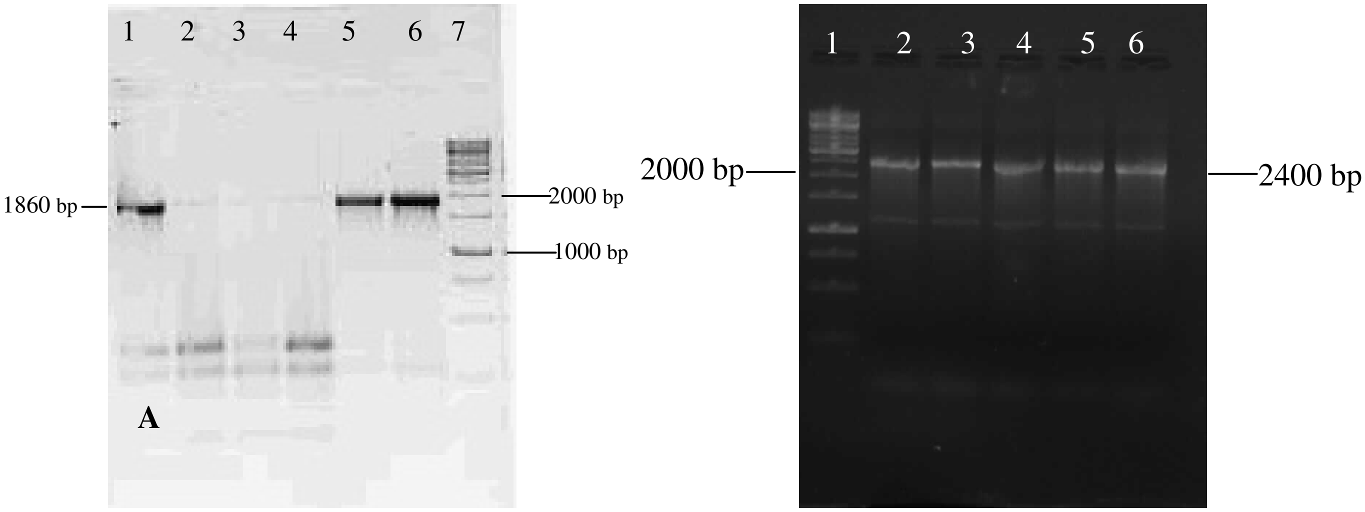


Fig: **A** Screening of *KOR* from plasmid isolated from positive clones of *A. tumefaciens* strain EHA 105; Lane 1-6 Screening of *KOR* from plasmid isolated from positive clones; Lane 7 – 1kb DNA ladder. **B** Screening of *SUS* from plasmid isolated from positive clones of *A. tumefaciens* strain EHA 105; Lane -1: 1 Kb DNA Ladder; Lane 2-6: Screening of *SUS* from plasmid isolated from positive clones

Transformation of *Eucalyptus* with *korrigan* and *sucrose synthase*

After confirmation of positive clones of *A. tumefaciens* strain EHA105 with gene of interests, these were used for the genetic transformation of *E. tereticornis* clone CE-2 following the already standardized protocol. Cells of *A. tumefaciens* strain EHA105 harbouring binary vector pBI121 with gene of interest were grown on LB medium containing 50 mg/l of kanamycin and 15 mg/l of rifampicilin, supplemented with 100 µM acetosyringone up to OD_{590} value of 0.8. Precultured leaf explants were infected with these cells (10 mins) and kept for co-cultivation of 2 days. After co cultivation, leaves were washed for removal of extra bacteria, blotted dried and transferred to selection medium for regeneration of transformed shoots. After 35-40 weeks of transformation, shoot regeneration (Fig.7.5A and B) was obtained on selection medium 'SM-ll' (Table 3.3) and these shoots were successfully multiplied on MS medium containing 2.5 µM BA, 0.5 µM NAA and supplemented with 50 mg/l of kanamycin (Fig.7.5C). Multiplied shoots were elongated on MS medium supplemented with 0.1 µM BA and 0.5 µM NAA and 50 mg/l of kanamycin (Fig.7.5D). Elongated shoots were excised from clumps just below the node, leaves were removed from lower nodes and microshoots were cultured on 1/4 strength MS medium supplemented with 5 µM IBA for rooting. Root induction was not observed on this medium (Fig.7.5E), whereas same medium was used for successful rooting of untransformed microshoots. For induction of roots in transformed microshoots, these were inoculated on MS medium supplemented with 50.0 µM IBA for 36 hrs and transferred to PGR-free MS medium. Roots were observed in transformed shoots after 10 days of culture on PGR free medium following pulse treatment of IBA (Fig.7.5F).

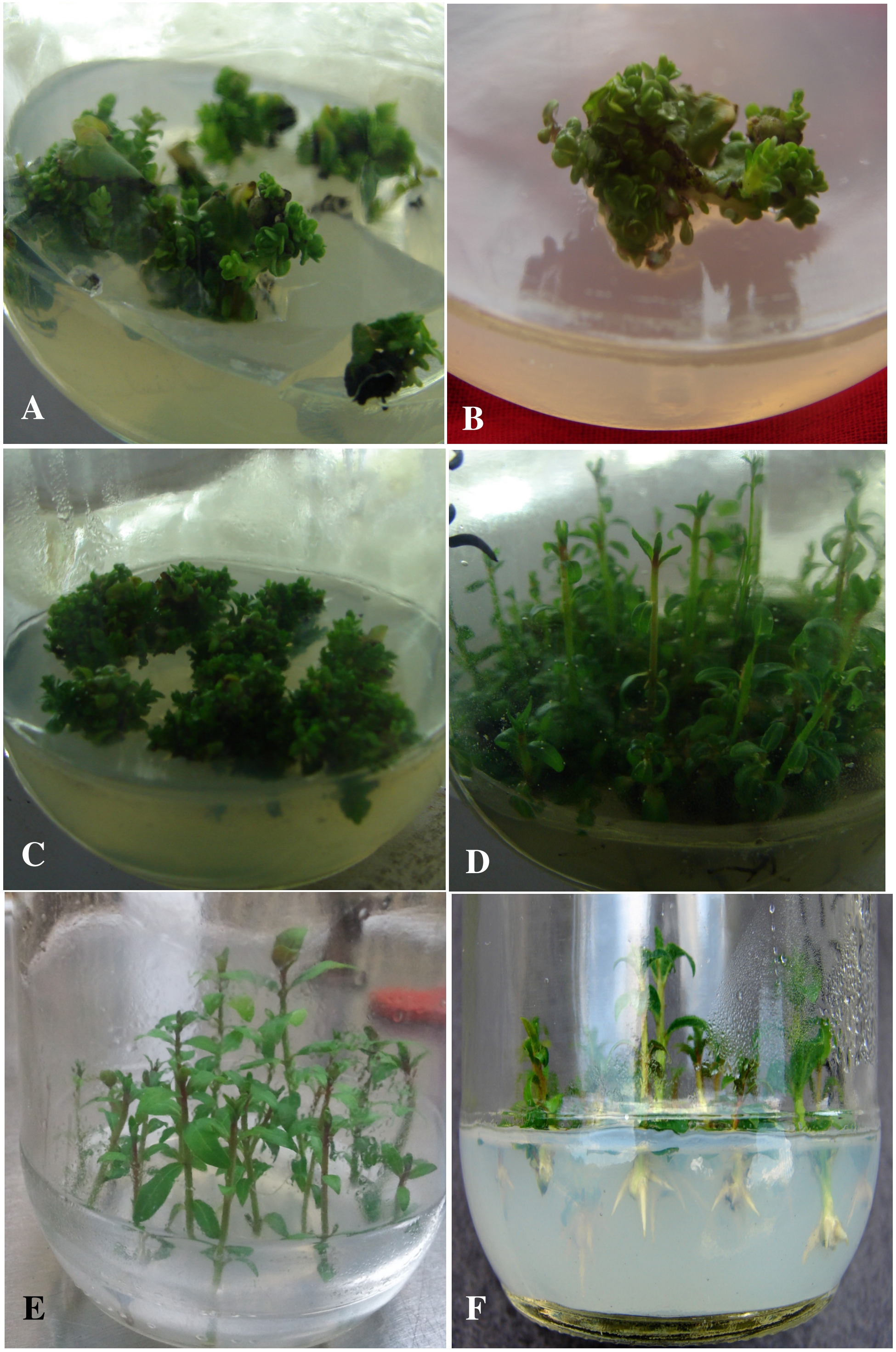


Figure 7.5 A and B. Regenerated transformed shoots of *E.tereticronis* (clone CE2) on selection medium (SM-ll) after infection with *A. tumefaciens* strain EHA105 containing pBI121 with KOR, **C.** Multiplication of transformed shoots on MS medium supplemented with 2.5 µM of BA and 0.5 µM of NAA and 50mg/l of kanamycin, **D.** Elonagtion of transformed shoots on MS medium supplemented with 0.1 µM of BA and 0.5 µM of NAA and 50mg/l of kanamycin, **E.** Microshoots fail to root on ¼ strength MS medium supplemented with 5 µM of IBA and 50mg/l of kanamycin, **F.** Rooting of microshoots on PGR free MS medium after treatment on MS medium supplemented with 50.0 µM of IBA for 36 hrs

Analysis of transformed shoots

The putative transformed shoots were analyzed for the presence of transgene using PCR amplification of fragments. Genomic DNA isolated from both transformed lines and untransformed tissues was subjected to PCR amplification using gene specific as well as *nptll* primers for the presence of these genes. Amplification of fragment corresponding to original cloned gene and *nptll gene* confirmed the integration of transgene into the host genome (Fig. 7.6A and 7.6B). To rule out the possibility of bacterial contamination in transformed shoots 16S rRNA locus was amplified from transformed shoots taking bacterial genomic DNA as positive control. No amplification was observed in DNA isolated form transformed shoots. (Fig. 7.6 C)

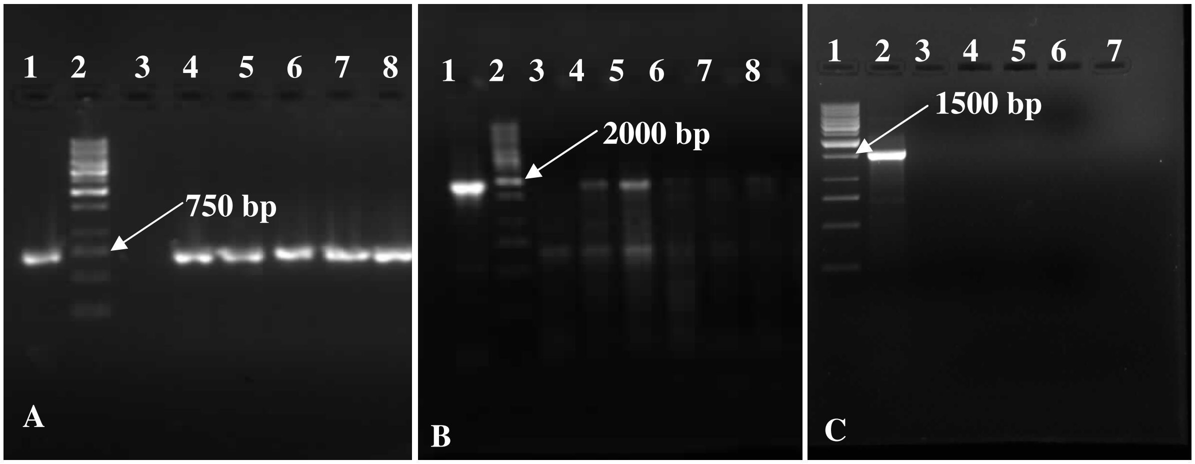


Figure 7.6 Analysis of transgenic *Eucalyptus* plants. **A.** Detection of *nptll* gene (~ 720 bp) from genomic DNA of transformed and untransformed Plants, Lane 1: Amplification from positive control (pBI121); Lane 2- 1kb DNA Ladder; Lane -3: Untransformed plant; Lane 4-8: Transformed plants. **B.** Detection of *KOR* gene (1860bp) from genomic DNA of transformed and untransformed Plants, Lane 1: Amplification from positive control (Plasmid); Lane 2- 1kb DNA Ladder; Lane -3: Untransformed plant; Lane 4-8: Transformed plants. **C.** 16S rRNA analysis of transformed plants, Lane- 1kb ladder; Lane-2: Amplification from positive control (bacterial genomic DNA), Lane 3-7: Amplification from DNA isolated from transformed plants

Quantitative real-time polymerase chain reaction (*qRT-PCR*) analysis of *KOR* gene was conducted using the Real Master Mix SYBR ROX Master Mix on Realplex 2.2 real-time PCR system to determine critical thresholds (Ct), using gene-specific primers (forward primer 5'-CTCTGTCAAGGCCATTGGAT-3' and reverse primer 5'-TCGTTGCTGCTGGTCTATTG -3'). For quantification of gene expression in transgenic lines, actin-9 (forward primer 5'-CTATTCTCCGCTTTGGACTTGGCA and reverse primer 5- AGGACCTCAGGACAACGGAAACG -3') as described previously by Volkov *et al.* (2003) was used as an endogenous control. cDNA synthesiszed form isolated RNA of transformed and untransformed plants was used as template for PCR amplifications. Conditions for the RT-PCR reactions were as follows: 95^{o}C for 2 min, followed by 40 cycles of 95 ^{o}C for 15 s, 55 ^{o}C for 15 s, and 68 ^{o}C for 20 s. Gene expression was quantified using the comparative method Ct: $2^{-\Delta\Delta Ct}$ method. Results showed the different levels of expression of transgene (*KOR* and *nptll*) in the various lines of transformed shoots (Fig. 7.7A and 7.7B). Expression level was found to vary in different transgenic lines and the highest level of expression of *KOR* was detected in transgenic line 2 (Fig. 7.8).

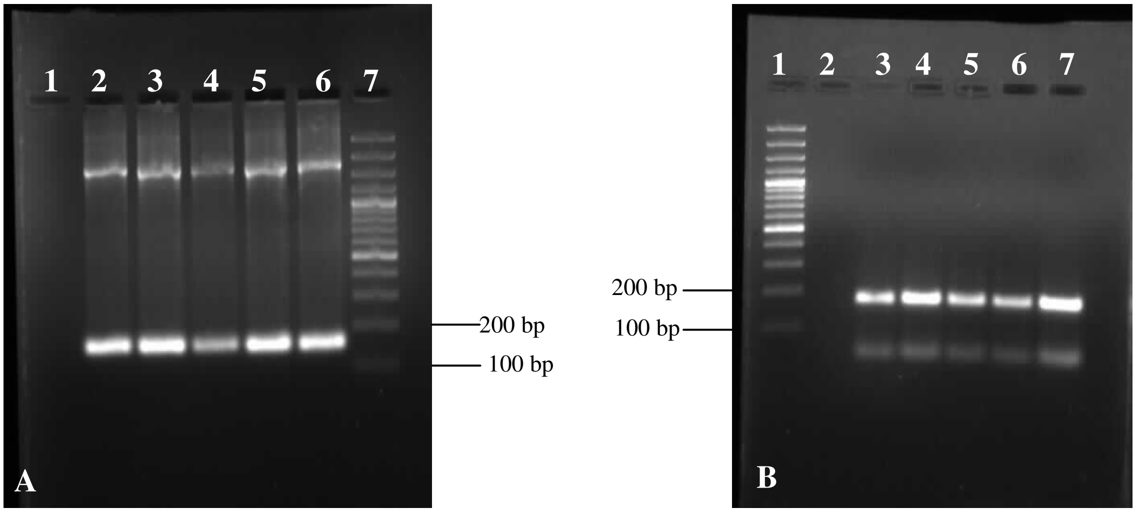


Figure 7.7 Analysis of transgenic *Eucalyptus* plants using real time PCR. **A.** Detection of *KOR* gene from cDNA of transformed and untransformed plants Lane 1: Untransformed plant; Lane 2-6: Transformed plants; Lane 7: 100 bp Ladder. **B.** Detection of *nptll* gene from cDNA of transformed and untransformed plants, Lane - 1: 100 bp Ladder; Lane -2: Untransformed Plant; Lane 3-7: transformed plants.

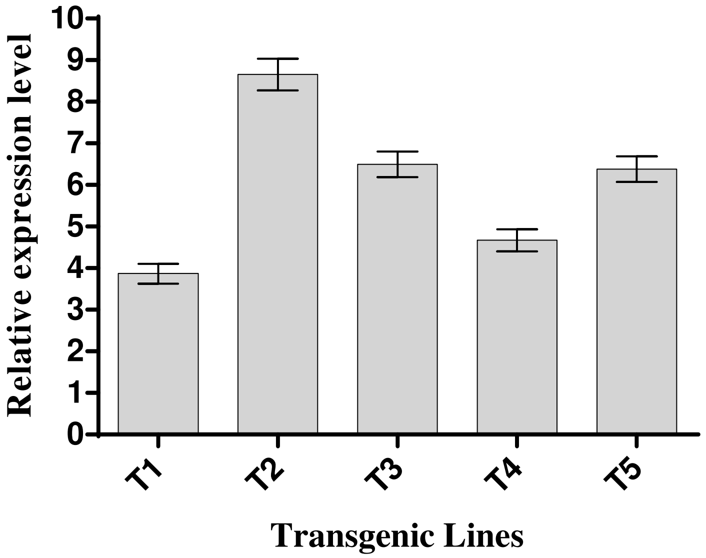


Figure 7.8 Expression levels of *KOR gene* in transgenic *E. terticornis* plants using quantitative real-time PCR analysis. C- Control untransformed plants, T1- T5 different transgenic lines. Error bars standard error of the mean

After the confirming the presence of transgene in transformed plants, cellulose content of the transgenic plants growing in culture was analyzed following the method of Upendgaff *et al.* (1969). Although quantitative real-time PCR analysis confirms the over expression of *KOR* gene in all the transgenic lines, but no significant differences were noticed in cellulose content of transgenic line in culture as compared to untransformed plants.

Discussion

Despite the importance of *Eucalyptus* for pulp and paper industry, very little work have been done to modify wood quality (by altering the chemical composition of cell walls), or to increase growth, which may be due to the recalcitrant nature of *Eucalyptus* plants for regeneration following transformation (Tournier *et al.* 2003). Further, there is an urgent need for improvement of *Eucalyptus* with respect to reduced lignin content and/or higher cellulose content through plant genetic manipulation to meet the ever increasing demand of eucalypt pulp. Wood quality and biomass enhancement are of major concern for wood based industries. Changes in the cell wall components qualitatively and quantitatively can lead to changes in cell wall properties and are responsible for variations in wood properties (Spokevicius *et al.* 2007). As endo-glucanases are reported to be involved in cell elongation through the hydrolysis of cellulose-xyloglucan links allowing the cellulose chains to move freely (Molhoj *et al.* 2002). Over expression of these genes in plants can induce elongation of cells more rapidly which in turn may result in faster growth and development of transgenic *Eucalyptus* trees (Shani *et al.* 2003, 2004). So therefore, the present study an effort was made to transform an elite plant of *E. tereticornis* (an important source of raw material for pulp and paper industry in tropical and subtropical part of the world) with *Korrigan*, an important enzyme involved in cellulose biosynthesis.

After cloning and characterization of KOR protein from *P. deltoides*, it was ligated in binary vector pBI121 in place of GUS under the control of CaMV 35S promoter (Fig. 7.1). The efficient expression of transgene in the host system, dictates the success of any genetic transformation study. Selection of a suitable promoter that initiates high levels of expression is, therefore, important. The class of non-constitutive promoters includes tissue specific, cell type specific and inducible promoters that direct transcription initiation in specific tissue such as leaves, stem, roots, seeds, cell types, organs and stress inducible, respectively (Girijashaker 2011). Whereas a constitutive promoter is active under most environmental conditions and in almost all plant parts. The frequently used promoter for wood transformation studies is CaMV 35S. It has been found to produce larger phenotypic changes when compared to wood-specific promoters (Yahiaoui *et al.* 1998). This constitutive promoter is also the most extensively used regulatory sequence in genetic transformation studies (Macrae and Van Staden 1999). Further, binary vector pBI121 has been reported to be widely used in plant transformation studies (Xu and Joshi 2010; Rohini and Sankara Rao 2001; Zheng *et al.* 2001; Sharma and Anjaiah 2000) and considered as valuable vector for plant transformation (Hellens *et al.* 2000). The T-DNA region, which integrates into plant genomes is considered as the most important part of any plant transformation vector, carries the features like *nptll* as selection marker (kanamycin resistance) and *uidA* as reporter gene (GUS activity) both under separate promoters giving more freedom for cloning (Fig. 3.1). After cloning of *KOR* in pBI121, plasmid was transformed in competent cells of *A. tumefaciens* strain EHA105 and positive clones were selected, which were subsequently used for transformation.

Eucalypts are known for there low regeneration ability due to the high concentration of phenolic compounds and low endogenous cytokinin content, which eventually restricts the genetic transformation procedures to few species and genotypes (Teulieres and Marque 2007; Tournierr *et al.* 2003). Moreover, to recover transgenic shoots on selective antibiotic medium is reported to be difficult even when stable transformation is achieved (Tournier *et al.* 2003; Serrano *et al.* 1996). Therefore, in the present study special efforts were made to develop an efficient and reproducible shoot organogenesis as well as *Agrobacterium* mediated genetic transformation protocol for recovery of transformed lines. Factors influencing shoot organogenesis and genetic transformation were studied in details (chapter 4 and 5). Transformation efficiency of nearly 1.5 % from leaves taken from microshoots of established culture is the major advantage of the present study. An efficient gene transfer method is developed for *E. tereticornis* which can be suitable for recovering economically important transgenic lines. By using the standardized protocol, five lines of transformed *Eucalyptus* plants carrying the *KOR* were established which were confirmed by PCR amplification with gene-specific primers (Fig. 7.6A and 7.6B). Review of literature did not reveal any report of *Eucalyptus* transformation with this gene.

The presence of bacteria is a serious problem in plant tissue cultures as they may affect culture growth under *in vitro* conditions (Lata *et al.* 2006) Sometimes presence of bacteria makes regeneration difficult or even impossible (Leifert and Cassells 2001). So, therefore elimination of residual bacteria is important step during *Agrobacterium* mediated genetic transformation. Amplification of DNA fragment specific to 16S rRNA locus of DNA isolated from transgenic shoots was not observed indicating the complete elimination of bacteria from these tissues (Fig. 7.6C). Earlier, such analysis has been successfully used for the detection of bacterial contamination in the plant tissues (Luna *et al.* 2008; Thomas *et al.* 2007).

Elongated transformed shoots were excised and transferred to the rooting medium which was used successfully for the induction of untransformed micro shoots (Table 4.9). However, no rooting was induced in transformed micro shoots. It has been reported that after being inoculated with *A. tumefaciens* and maintained on media containing kanamycin, putative transgenic shoots became rather difficult to root (Teulieres *et al.* 1994; Ho *et al.* 1998). So therefore, for induction of roots in these transformed micro shoots, these were inoculated on MS medium supplemented with 50 µM of IBA for 36 h. after 36 hrs of pulse treatment with high concentration of IBA, micro shoots were transferred to PGR free MS medium. After 10 days of culturing on this medium rooting was induced in transformed shoots with an average rooting frequency of 80 %. It has been suggested that exposing shoots to an excessive amount of cytokinin during the regeneration process could result in poor rhizogenesis (Debergh and Read 1991). Therefore, exposing shoots to high amount of auxin possibly diminishes the adverse rooting effect of residual cytokinin carried over through the shoot induction stage, hence resulting in the rooting of transformed shoots (Ho *et al.* 1998).

Reserve transcription combined with the polymerase chain reaction (RT-PCR) has proven to be a powerful method to quantify gene expression (Noonan *et al.* 1990; Murphy *et al.* 1990). Real time PCR technology has been adapted to perform quantitative RT-PCR (qRT-PCR) (Heid *et al.* 1996). Two different methods of analyzing data from real-time, quantitative PCR experiments exist: absolute quantification and relative quantification (Livak and Schmittgen 2001). Absolute quantification determines the input copy number of the transcript of interest, usually by relating the PCR signal to a standard curve. Relative quantification describes the change in expression of the target gene relative to some reference group such as an untreated control or a sample at time zero in a time-course study (Livak and Schmittgen 2001). So, therefore in the present study qRT-PCR was used to study the over expression of transgene. Results obtained confirm the presence (Fig.7.7) as well as over expression of transgene in transgenic lines. Transgenic line 2 showed the highest expression level among the different transgenic lines (Fig. 7.8). Differences among transgenic lines in the same transformation event are often observed and likely due to variations in insertion site and events like this can provide valuable variation for plant genetic improvement (Cai *et al.* 2012; Hong *et al.* 2013).

The shoots obtained are being analyzed for cellulose content, but no significant change in amount of cellulose content was observed. Since cellulose is an important component of plant cell walls, genetic modifications of related to cellulose biosynthesis can have a direct impact on several aspects of plant growth and development (Somerville 2006) including, cell division and expansion, plant morphogenesis, and plant responses to the environment. Moreover, earlier investigations on KOR protein indicated that it is important for cell wall development (Robert *et al.* 2004). Therefore, transgenic shoots obtained are required to be grown in field to analyze for cellulose quality, quantity and wood characteristics for pulping. With the long-term aim of obtaining plants with modified cellulose content the current work can provide new insight to advance overall knowledge about the gene transfer in economical important plants like *Eucalyptus*.

Moreover, cloning and characterization of genes involved in cellulose biosynthesis and their expression patterns from trees is a prerequisite for pursuing such experiments.

Conclusion

In conclusion, an elite clone *E. tereticornis* was transformed with *KOR* gene and transgenic shoots showing overexpression of *KOR* was obtained. Over expression of *KOR* gene in an elite clone of *Eucalyptus* seems to be the first report.

Salient findings

- After cloning and characterization of KOR protein from *P. deltoides*, it was ligated in binary vector pBI121 and transformed in to A. *tumefaciens* strain EHA105.
- Five lines of transformed *Eucalyptus* plants carrying the *KOR* were established which were confirmed by PCR amplification with gene-specific primers.
- 16S rRNA analysis of transgenic shoots ruled the possibility of bacterial presence in transgenic lines.
- Transformed shoots were rooted on PGR free MS medium after pulse treatment of 36 hrs on MS medium supplemented with 50 µM of IBA.
- Stable integration as well as over expression of transgene in transgenic lines was carried out using RT-PCR and qRT-PCR. Transgenic line 2 showed the highest expression level among the different transgenic lines
- The shoots obtained were analyzed for cellulose content, but no significant change in amount of cellulose content was observed.
- These plants are being acclimatized and transferred in the containement facility for further analysis.

Chapter-8

Summary

Eucalyptus (Family: Myrtaceace) is among the fastest growing woody plant in the world and comprises of nearly 700 species distributed throughout the world. It is grown world wide due to its wide adaptability, extremely fast growing nature and most importantly excellent wood and fiber properties which make it an important source of raw material for pulp and paper industry. Cellulose, the most abundant biopolymer on earth, consists of crystalline assemblies of parallel 1,4-β–linked glucan chains. Current models envision plant cellulose biosynthesis to be a three step process: (1) plasma membrane-associated sucrose synthase (*SUS*) which directly channel the UDP-glucose substrate to cellulose synthesizing machinery (2) coordinately expressed multiple cellulose synthase genes, organized in the form of hexagonal rosettes, polymerize glucose monomers into glucan chains while recycling liberated UDP back to *SUS* and (3) a membrane-associated cellulase (β-1,4–glucanase; *korrigan (KOR)*, acts as an editor of newly produced glucan chains. These two genes (namely *KOR* and *SUS*) could be important target for up regulation of the cellulose content in wood. Thus, there is a possibility for improvement of selected clones of *Eucalyptus* for up-regulation of cellulose content through plant genetic manipulation using these genes.

Plants of elite clone (self pruning and higher biomass productivity) of *E. tereticornis* growing at Thapar University Campus were selected for the present study. Cultures were established from nodal explants taken from freshly coppiced shoots of elite plant of *E. tereticornis* (10 years old). Cultures were initiated on MS medium supplemented with 2.5 µM BA and 0.5 µM NAA. Maximum numbers of shoots per culture vessel (342) were obtained on MS supplemented with 2.5 µM BA in combination with 0.5 µM NAA, whereas shoot elongation was achieved on MS medium supplemented with 0.1 µM BA and 0.5 µM NAA. Out of the three tested cytokinins i.e BA, KIN and TDZ, BA was found to be better for both shoot proliferation and elongation followed by KIN and TDZ. Average number of elongated shoots per culture vessel was significantly higher (65) when smaller shoot clump (4-5 shoots/culture) were inoculated on medium containing 0.1 µM BA along with 0.5 µM NAA as compared to larger shoot clumps (15-20 shoots per clump, 54). However, shoots proliferated per culture vessel were significantly lower (245 shoots per culture vessel) when smaller shoots clumps were cultured as compared to larger shoot clumps (342 shoots per culture vessel). Therefore, it was concluded that larger shoot clumps are better for shoot multiplication whereas, for shoot elongation smaller shoot smaller shoot were required.

The effect of two light sources i.e. PAR and CFL was examined on shoot proliferation and elongation. Both, shoot proliferation rates and number of shoots elongated per culture vessel were significantly higher in cultures incubated under PAR light as compared to those inoculated under CFL. Both, chlorophyll content and osmotic potential of sap were also higher in cultures incubated under PAR light.

Development of an efficient shoot regeneration protocol (through shoot organogenesis and/or somatic embryogenesis) is prerequisite for the development of an efficient genetic transformation protocol. In the present study, effect of BA along with 2,4 – D or NAA was examined on shoot regeneration from leaf segments taken from microshoots. Explants showed callus formation in all combinations. However, shoot regeneration was observed in some of the combinations only. Higher percent of explants showed shoot regeneration on medium containing 2,4–D as compared to those containing NAA. On medium supplemented with 5.0 µM BA and 1.0 µM 2,4–D, a maximum of 29.8 percent explants showed shoot regeneration with an average of 14.6 shoots per explant .

Leaf maturity was found to influence shoot regeneration potential of leaf segments (3rd to 7th leaf from top of microshoot). Maximum percent of explants showed shoot regeneration (40.6) with 16.0 shoots per explant, when explants were taken from 5th leaf from top. This response was found to decrease from mature and younger leaves. Addition of Cefotaxime (antibiotic generally used for the elimination of *Agrobacterium* from cultures after co-cultivation) increased shoot regeneration potential of explants. Both percent explants showing shoot regeneration and number of shoots regenerated per explants increased with increasing concentrations of cefotaxime. Others antibiotics like carbenicillin and cephalexin inhibited shoot regeneration potential of leaf explant.

The shoot regeneration potential also varied amongst different clones of *E. tereticornis.* Amongst the three clones tested higher number of explants showed shoots regeneration as well as callus differentiation in case of clone 'T1'.

Histological studies indicate that some of the cells in the surface layer of the callus showed intense cell division and get organized into the shoot apical meristematic zone. These meristematic zones were later organized into the shoot bud like structures, which have vascular connection with the parent tissues. These shoot buds were further observed to grow into individual shoots. Thus direct shoot regeneration was observed from these explants.

Clonal uniformity of *in vitro* plants with that of mother plant was established using RAPD and ISSR markers. Out of 40 primers used (20 each in RAPD and ISSR), 16 RAPD and 12 ISSR primers resulted in the amplification of scorable bands. These primers gave maximum of seven and minimum of two bands. Out of the total 133 markers obtained with RAPD and ISSR, 58 markers were scored with ISSR and 75 markers were scored with RAPD primers. Size of amplified markers ranged from 200 bp to 2500 bp. Similarity in banding profile of RAPD and ISSR markers in *in vitro* propagated plants to that of mother plant indicates the clonal nature of these palnts.

The effect of different auxins i.e. NAA, IAA and IBA and strength of MS medium (Full strength, 1/2 strength and 1/4 strength) was examined on rooting of microshoots. Maximum number of shoots rooted (80.66 %) on ¼ MS medium containing 5.0 µM IBA. Maximum numbers of roots per rooted shoot (4.25) were also observed on same medium, whereas maximum root length (2.90 cm) was recorded on full strength MS medium supplemented with 5.0 µM IBA. The effect of two light sources was also examined on rooting of microshoots. Percent shoots rooted, average root length and average numbers of roots per rooted shoot were also significantly higher in cultures incubated under PAR light.

In the present study, special emphasis was given to investigate the factors affecting acclimatization of plants to increase survival rate and quality of planting material. The effect of different plant growth promoting bacterial isolates namely *Bacillus subtilis* and *Pseudomonas corrugate* was tested on growth and survival of micropropagated plants. Higher survival percentage (74.6 %) was recorded for plantlets which were incubated under PAR light as compared to plantlets incubated under CFL (70.4 %). Plant survival further increased to 84 % when plants were inoculated with *Bacillus subtilis* followed by plantlets inoculated with *Pseudomonas corrugata* (80.8 %) as compared to control plants (74.6 %) where no bacteria were inoculated.

For the development of genetic transformation protocol, *Agrobacterium tumefaciens* mediated approach was adopted. *A. tumefaciens* strain EHA105 harboring binary vector pBI121 was used for the development of genetic transformation protocol. Tolerance limits (sensitivity) of the leaf segments for kanamycin was determine by culturing these on shoot induction medium supplemented with different concentrations of kanamycin

(0, 10, 20, 30, 40,50, 70 and 100 mg/l). It was observed that the presence of kanamycin in the regeneration medium caused a drastic decline in shoot regeneration potential as compared to control (cultured on medium lacking kanamycin).

The effect of various factors namely pre-culture, bacterial density, mode of injury, incubation conditions. pH, phenolics etc. on transformation of leaf explants was evaluated. In the present study; leaves, pre-cultured on medium containing 50 µM acetosyringone for 2 days and incubated under 16-h light cycle showed maximum transient GUS activity (59 %). Addition of acetosyringone (100 µM) to the co-cultivation medium recorded higher transient GUS activity (62 %) in the explants. Injury of explants with the help of hypodermic needle enhanced transient GUS activity. Lower pH was found to be beneficial for T-DNA delivery. Density of bacterial suspension used for explant infection also influenced transient GUS activity. A co cultivation period of 2 days was found to be optimum. An increase in the number of explants showing GUS activity was observed, when explants were co-cultivated in 16-h light regime.

Following the genetic transformation, difficulties were encountered in achieving shoot organogenesis. Therefore certain changes were made in the basal MS medium to achieve shoot organogenesis. Potassium nitrate in MS medium was completely replaced with 990 mg/l of potassium sulphate, ammonium nitrate was reduced to 391.8 mg/l and 323 mg/l of ammonium sulphate was also added to the medium. Furthermore, mesoinositol was increased to 200 mg/l. Despite optimizing most of the parameters influencing transformation were optimized, lower transformation efficiency was observed. In the present study, it took 35-40 weeks to recover transgenic shoots.

The transformation efficiency varied from clone to clone, and amongst the three clones tested namely, ‘T1’, ‘CE2’ and ‘Y8’, maximum genetic transformation efficiency was recorded in case of clone ‘CE2’.

The kanamycin resistant shoots showed positive GUS activity. These results were confirmed by PCR amplification of DNA fragments of 750 bp specific to *nptII* gene and 1500 bp specific to *uidA* gene from transgenic shoots. Amplification specific to 16s rRNA from DNA samples isolated from transgenic shoots was not observed indicating the complete elimination of bacteria from these tissues.

Isolation and cloning of *korrigan (KOR)* and *sucrose synthase (SUS)* genes was carried out using poplar (*Populus deltoides*) cDNA. High quality RNA from young actively growing shoots of poplar was using CTAB method.

The reported sequences of *KOR* and *SUS* were retrieved from the NCBI and primers were designed to amplify partial fragments (1800 bp fragment in case of *KOR* and 1200 bp in case of *SUS*). After successful amplification, these fragments were cloned in *E. coli* DH5α cells and sequenced. Sequences were analyzed using BLAST and sequence analysis of both the fragments showed 99 % similarity with reported sequences from other *Populus* species.

Subsequently, on the basis of this information, primers for full length amplification of these genes (*KOR* and *SUS)* along with overhangs of restriction enzymes *BamH1* and *Sac1* in case of *KOR* and *BamH1* and *Sma1* in case of *SUS* were designed for the directional cloning of these genes in the transformation vector pBI121. Using these primers; fragments of 1860 bp for *KOR* and 2400 bp for *SUS* were amplified. After successful amplification, these fragments were cloned in *E. coli* DH5α cells and sequenced. Homology of these fragments to their respective genes was confirmed by NCBI search tool BLAST.

The identification of the ORF and amino acid sequences coded by these fragments was deduced using the ORF finder program. The predicted open reading frame (ORF) of *KOR* gene was found to encode a protein of 619 amino acids with a calculated molecular mass of 68.45 kDa and an isoelectric point of 8.92, whereas *SUS* gene was found to encode a protein of 805 amino acids with a molecular mass of 92.29 kDa and an isoelectric point of 6.12. In case of KOR protein, a single transmembrane domain was detected between amino acids 72–94**,** a feature that is similar to other known KOR proteins, whereas no transmembrane domain was detected in case of SUS protein. Both the proteins have shown the presence of glycosylations and phosphorylation sites in their sequences which indicate that both proteins undergo post translational modifications for proper functioning. The predicted KOR belongs to glycosyl hydrolase family 9 (GH9) of proteins and SUS belongs to sucrose synthase family of proteins, a member of UDP-glycosyltransferase (GT1) super family.

After theses analyses, these genes were directionally cloned into binary vector (pBI121). The ligated products were transformed into competent cells of *A. tumefaciens* strain EHA105 for genetic transformation of *E. tereticornis* clone 'CE2'.

Utilizing the protocol developed for the genetic transformation, *E. tereticornis* clone 'CE2' was transformed with 5 different transgenic lines were selected for further analysis. The putative transformed shoots were analyzed for the presence of *KOR* using PCR. Amplification of fragment corresponding to original cloned gene confirmed the integration of *KOR* gene in the host genome. qRT- PCR analysis showed over expression of mRNA specific to *KOR* in transformed shoots of *E. tereticornis*. Expression level was found to vary in different transgenic lines.

Chapter-9

References

Ahmad I, Khan MSA, Aqil F and Singh M (2011)**:** Microbial applications in agriculture and the environment: a broad perspective. In: Iqbal Ahmad, F. Ahmad and J. Pichtel. (Eds). Microbes and Microbial Technology: Agriculture and Environmental Applications; Springer New York. pp 1-27

Albrecht G and Mustroph A (2003): Localization of sucrose synthase in wheat roots: increased in situ activity of sucrose synthase correlates with cell wall thickening by cellulose deposition under hypoxia. *Planta* 217: 252-260

Almehdli AA and Parfitt DE (1986): *In vitro*, propagation of peach: Propagation of 'Lovell' and 'Nemguard' peach rootstocks. *Fruit Varieties Journal* 40: 12–17

Altman A and Loberant B (1998): Micropropagation: Clonal plant propagation *in vitro*. In: A. Altman, ed. (1998). Agricultural Biotechnology. Marcel Dekker, Inc., New York, pp19-42

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W and Lipman DJ (1997): Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research* 25: 3389-3402

Alvarez R, Ordas RJ (2007): Improved genetic transformation protocol for cork oak (*Quercus suber* L.). *Plant Cell Tissue and Organ Culture* 91:45-52

Amor Y, Haigler CH, Johnson S, Wainscott M and Delmer DP (1995): A membrane-associated form of sucrose synthase and its potential role in synthesis of cellulose and callose in plants. *Proceedings of National Academy of Science of USA 92*: 9353-9357

Anonymous (1994): Annual report (1993–1994), Department of Biotechnology, Ministry of Science and Technology, India

Appenroth KJ, Mec*x*o R, Jourdan V and Lillo C (2000): Phytochrome and post-translational regulation of nitrate reductase in higher plants. *Plant Sci*ence 159:51–56

Arezki O, Boxus P, Kevers C and Gaspar T (2001): Changes in peroxidase activity and level of phenolic compounds during light-induced plantlet regeneration from

Eucalyptus camaldulensis Dehn. nodes in *vitro. Plant Growth Regulation* 33: 215–219

Arnon DI (1949): Copper enzymes in isolated chloroplasts - polyphenol oxidases in *Beta cularis*. *Plant Physiology* 24:*1*-15

Atkinson RG and Gardner R (1991): *Agrobacterium* mediated transformation of pepino and regeneration of transgenic plants. *Plant Cell Reports* 10:208–212

Azmi A (1999): Bases cellulaires et physiologiques de la régénération et de la transformation génétique chez Eucalyptus globules Labill. PhD Thesis, Pierre et Marie Curie University, Paris, France

Azmi A, Noin M, Landré P, Proteau M, Boudet AM and Chriqui D (1997): High frequency plant regeneration from *Eucalyptus globulus* Labill. Hypocotyls: ontogenesis and ploidy level of the regenerants. *Plant Cell Tissue and Organ Culture* 51: 9-16

Bajaj YPS (1989): Biotechnology in agriculture and forestry 5: Trees II. Springer-Verlag, Berlin, Germany

Bakshi S, Sadhukhan A, Mishra S and Sahoo L (2011): Improved *Agrobacterium* mediated transformation of cowpea via sonication and vacuum infiltration. *Plant Cell Reports* 30:2281–2292

Barratt DH, Derbyshire P, Findlay K, Pike M, Wellner N, Lunn J, Feil R, Simpson C, Maule AJ and Smith AM (2009): Normal growth of *Arabidopsis* requires cytosolic invertase but not sucrose synthase. *Proceedings of the National Academy of Sciences of USA* 106: 13124–13129

Barrueto Cid LP, Machado ACM, Carvalheira SRC and Brasileiro ACM (1999): Plant regeneration from seedling explants of *Eucalyptus grandis X E.urophylla*. *Plant Cell Tissue and Organ Culture* 56: 17–23

Beck SL and Dunlop RW (2001): Micropropagation of the *Acacia* species – a review. *In Vitro Cellular and Developmental Biology- Plant* 37:531–538

Bennett IJ, McDavid D and McComb JA (1994): The influence of ammonium nitrate, pH and indole butyric acid on root induction and survival in soil of micropropagated *Eucalyptus globules*. *Biologia Plantarum* 47:355–360

Bhandari S, Fujino T, Thammanagowda S, Zhang D, Xu Fand Joshi CP (2006): Xylem-specific and tension stress-responsive coexpression of KORRIGAN endoglucanase and three secondary wall-associated cellulose synthase genes in aspen trees. *Planta* 224: 828 – 837

Bimboim HC and Doly J (1979): A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* 7: 1513-1523

Binns AN and Thomashow MF (1988): Cell biology of *Agrobacterium* infection and transformation of plants. *Annual Review of Microbiology* 42: 575– 606

Bisht MS, Vyas P, Bag N and Palni LMS (1998): Plant Tissue Culture and Molecular Biology-Application and prospects, Narosa Publishing House, New Delhi, pp 126-170

Bjellqvist B, Hughes GJ, Pasquali CH, Paquet N, Ravier F, Sanchez J Ch, Frutiger S and Hochstrasser DF (1993): The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences. *Electrophoresis* 14: 1023-1031

Bonga JM and DJ Durzan (1987): Cell and tissue culture in Forestry General Principles and Biotechnology 1: pp422, Specific principles and methods 2: pp447, Case histories 3:pp416 Martinus Nijhoff Publishers, Dordrecht, Netherlands. ISBN 90-247-3433-9 (set).

Boyle JR (1999): Planted forests: views and viewpoints. *New Forest* 17: 5–9

Brainerd KE and Fuchigami LH (1982): Stomatal functioning of *in vitro* and greenhouse apple leaves in darkness, mannitol, ABA and CO_2. *Journal of Experimental Botany* 33: 388–392

Brooker MIH (2000): A new classification of the genus *Eucalyptus* L'Her. (Myrtaceae). *Australian Systematic Botany* 13: 79– 148

Brown Jr RM, Saxena IM and Kudlicka K (1996): Cellulose biosynthesis in higher plants. *Trends in Plant Science* 1:149- 156

Brown Jr RM (1996): The biosynthesis of cellulose. *Journal of Macromolecular Science: Pure and Applied Chemistry* 33: 1345—1373

Brummell DA, Catala C, Lashbrook CC and Bennett AB (1997): A membrane-anchored E-type endo-1,4-beta -glucanase is localized on Golgi and plasma membranes of higher plants. *Proceedings of National Academy of Science of USA* 94: 4794-4799

Brummell DA, Lashbrook CC and Bennett AB (1994): Plant endo-1,4-β-glucanases: Structure, properties and physiological function. In Enzymatic Conversion of Biomass for Fuels Production,ME, Himmel JO, Baker and Overend RP (eds) American Chemical Society, Washington, DC pp100–129

Brunner AM and Li J et al. (2007): Genetic containment of forest plantations. *Tree Genetics and Genomes* 3(2):75–100

Burger DW (1987): *In vitro* micropropagation of *Eucalyptus sideroxylon*. *HortScience* 22:496- 497

Burton RA, Shirley NJ, King BJ, Harvey AJ and Fincher GB (2004): The CesA gene family of barley (*Hordeum vulgare*): quantitative analysis of transcripts reveals two groups of co-expressed genes. *Plant Physiology* 134: 224–236

Cai P, Long H, Deng GB, Pan ZF, Peng ZS and Yu MQ (2012) Molecular cloning, characterization, and expression analysis of genes encoding gibberellin 20-oxidase in *Dasypyrum villosum* dwarf mutant. *Plant Molecular Biology Reporter* 30:1110–1116

Carlson SJ and Chourey PS (1996): Evidence for plasma membrane-associated forms of sucrose synthase in maize. *Molecular and General Genetics* 252: 303-10

Carlson SJ, Chourey PS, Helentjaris T and Datta R (2002): Gene expression studies on developing kernels of maize sucrose synthase (SuSy) mutants show evidence for a third SuSy gene. *Plant Molecular Biology* 49: 15-29.

Carvalho LC, Goulao L, Oliveira C, Goncalves JC and Amancio S (2004): RAPD assessment for identification of clonal identity and genetic stability of *in vitro* propagated chestnut hybrids. *Plant Cell Tissue and Organ Culture* 77:23 –27

Cass LG, Kirven KA and Christoffersen RE (1990): Isolation and characterization of a cellulase gene family member expressed during avocado fruit ripening. *Molecular Genetics* and *Genomics* 223: 76-86

Chandrika, M, Thoyajaksha, Ravishankar RV and Ramachandra KK (2008): Assessment of genetic stability of *in vitro* grown *Dictyospermum ovalifolium*. *Biologia Plantarum* 52: 735 – 739

Chang S, Puryear J and Cairney J (1993): A simple and efficient method for isolating RNA from pine trees. *Plant Molecular Biology Reporter* 11:113-116

Chang SH, Donald DGM and Jacobs G (1992): Micropropagation of *Eucalyptus radiata* ssp. *radiata* using explants from mature and coppice material. *South African Forestry Journal* 162:43–47

Chen Z, Chang S, Ho C, Chen Y, Tsai J and Chiang V (2001): Plant production of transgenic *Eucalyptus camaldulensis* carrying the *Populus tremuloides* cinnammate 4-hydroxylase gene. *Taiwan Journal of Forest Science* 16:249–258

Chezhian P, Yasodha R and Ghosh M (2010): Genetic diversity analysis in a seed orchard of *Eucalyptus tereticornis. New Forests* 40:85–99

Ching A, Dhugga KS, Appenzeller L, Meeley B, Bourret TM, Howard RJ and Rafalski A (2006): Brittle stalk 2 encodes a putative glycosylphosphatidylinositol-anchored protein that affects mechanical strength of maize tissues by altering the composition and structure of secondary cell walls. *Planta* 224 1174–1184.

Chomczynski P and Sacchi N (1987): Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* 162:156-159

Chriqui D, Adam S, Caissard JC, Noin M and Azimi A (1992): Shoot regeneration and *Agrobacterium*-mediated transformation of *E. globulus* and *E. gunni*. In Schonau APG (ed) Proceedings of IUFRO symposium on intensive forestry: the role of *Eucalyptus.* Pretoria, South Africa: South African Institute of Forestry 70–80

Ciesla J, Fraczyk T and Rode W (2011): Phosphorylation of basic amino acid residues in proteins: important but easily missed. *Acta Biochimica Polonica* 58: 137–47

Coleman HD, Ellis DD, Gilbert M and Mansfield SD (2006): Up-regulation of sucrose synthase and UDP-glucose pyrophosphorylase impacts plant growth and metabolism. *Plant Biotechnol Journal* 4: 87-101

Coleman HD, Yan J and Mansfield SD (2009): Sucrose synthase affects carbon partitioning to increase cellulose production and altered cell wall ultra structure. *Proceedings of National Academy of Sciences of USA* 106: 13118-13123

Contin A, Van der Heijden R, Ten Hoopen HJG and Verpoorte R (1998): The inoculum size triggers tryptamine or secologanin biosynthesis in a *Catharanthus roseus* cell culture. *Plant Science* 139: 205-211

Crawford RL (1981): Lignin biodegradation and transformation. John Wiley and Sons, New York. ISBN 0471057436

Das T & Mitra GC (1990): Micropropagation of *Eucalyptus tereticornis* Smith. *Plant Cell Tissue and Organ Culture* 22: 95–103

De Fossard RA, Barker PA and Boume RA (1977): The organ culture of nodes of four species of *Eucalyptus*. *Acta Horticulturae* 78: 157-l 65.

Debergh PC and Read PE (1991): Micropropagation. In: Debergh PC, Zimmerman RH (eds) Micropropagation: technology and application, Kluwer, Dordrecht, pp 1–13

del Campillo E (1999): Multiple endo-1,4- beta- D -glucanase (cellulase) genes in *Arabidopsis* . *Current Topics in Developmental Biology* 46: 39 – 61

del Campillo E and Bennett AB (1996): Pedicel breakstrength and cellulase gene expression during tomato flower abscission. *Plant Physiology* 111: 813-82

Delmer DP (1999): Cellulose biosynthesis: exciting times for a difficult field of study. *Annual Review of Plant Physiology and Plant Molecular Biology 50*: 245 – 276

Dhiman B and Singh M (2003): Molecular detection of Cashew husk (*Anacardium occidentale*) adulteration in market samples of dry tea (*Camellia sinensis*). *Planta Medica* 69: 882-884

Dibax R, Eisfeld CL, Cuquel FL, Koehler H and Quoirin M (2005): Plant regeneration from cotyledonary explants of *Eucalyptus camaldulensis*. Scientia Agricola (Piracicaba, Brazil) 62: 406-412

Djerbi S, Lindskog M, Arvestad L, Sterky F and Teeri T (2005): The genome sequence of black cottonwood *Populus trichocarpa* reveals 18 conserved cellulose synthase (CesA) genes. *Planta* 221:739-746

Doblin MS, Kurek I, Jacob-Wilk D, Delmer DP (2002): Cellulose biosynthesis in plants: from genes to rosettes. *Plant and Cell Physiology* 43:1407-1420

Doolittle RF (1989): Redundancies in protein sequences. In prediction of protein structure and the principles of protein conformation (Fasman GD ed.), Plenum Press, New York. pp. 599–623. ISBN 0-306-43131-9

Doughty RW (2000): The *Eucalyptus*- A natural and commercial history of the gum tree. The Johns Hopkins University Press, Baltimore and London. ISBN 0-8018-6231-0

Doyle JJ, and Doyle JL (1990): Isolation of plant DNA from fresh tissue. *Focus* 12:13-15

Drickamer K and Taylor ME (2006): Introduction to Glycobiology (2nd ed.). Oxford University Press, USA. ISBN 978-0-19-928278-4

Duncan DB (1955): Multiple range and multiple F tests. *Biometrics* 11:1–42

Dutt M, Li ZT, Dhekney SA and Gray DJ (2007): Transgenic plants from shoot apical meristems of *Vitis vinifera* L. "Thompson Seedless" via *Agrobacterium*-mediated transformation. *Plant Cell Reports* 26:2101-2110

Dyer KF (1971): The quiet revolution: A new synthesis of biological knowledge. *Journal of Biological Education* 5:15-24

Eldridge K, Davidson J, Harwood C and Van WG (1993): Eucalypt domestication and breeding. Clarendon Press, Oxford.

Etxeberria E and Gonzalez P (2003): Evidence for a tonoplast-associated form of sucrose synthase and its potential involvement in sucrose mobilization from the vacuole. *Journal of Experimental Botany* 54: 1407-1414.

Evans J (1992): Plantation forestry in the tropics. Second edition. Oxford Science Publication, USA. p403

FAO (2000): The global outlook for future wood supply from forest plantations by C. Brown. Working paper GFPOS/WP/O3, Rome. p129

Fenning TM and Gershon J (2002): Where will the wood come from? Plantation forests and the role of biotechnonology. *Trends in Biotechnology* 20: 291-296

Figueiredo SFL, Simoes C, Albarello N, and Viana VRC (2000): *Rollinia mucosa* cell suspension cultures: establishment and growth conditions. *Plant Cell Tissue and Organ Culture* 63: 85-92

Franclet A and Boulay M (1982): Micropropagation of frost resistant eucalypt clones. *Australian Journal of Forest Research* 13:83-89

Gamborg OL, Miller RA and Ojma K (1968): Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Reserach* 50:151–158

Ganeshan S, Weir BJ, Baga M, Rossnagel BG and Chibbar RN (2006): Evaluation of the enhanced regeneration system for *in vitro* regeneration in Barley. *Canadian Journal Plant Science* 86:63-69

Geigenberger P and Stitt M (1993): Sucrose synthase catalyzes a readily reversible reaction in vivo in developing potato tubers and other plant tissues. *Planta* 189: 329-339

Gelvin SB (2003): Improving plant genetic engineering by manipulating the host. *Trends in Biotechnology* 21: 95-98

George EF (1996): Plant Growth Regulators. *In:* Plant propagation by tissue culture. Part 1 The Technology, 2nd edition. Exegetics Ltd, England, pp 420-476

Girijashankar V (2011): Genetic transformation of *Eucalyptus*. *Physiology and Molecular Biology* of *Plants* 17: 9-23

Girijashankar V and Swathisree V (2009): Genetic Transformation of *Sorghum bicolor*. *Physiology and Molecular Biology* of *Plants* 15:1–16

GIT Forestry (2008) Cultivated eucalyptus global map. http:// www.git-forestry.com

Glocke P, Delaporte K, Collins G and Sedgley M (2006): Micropropagation of juvenile tissue of *Eucalyptus erythronema x Eucalyptus Stricklandii* cv. ‘urrbrae gem’. *In Vitro Cellular and Developmental Biology- Plant* 42:139–143

Godwin I, Todd G, Ford-Lloyd B and Newbury H J (1991): The effect of acetosyringone and pH on *Agrobacterium*-mediated transformation vary according to plant species. *Plant Cell Reports* 9:671–675

Goins GD, Yorio NC, Sanwoo MM and Brown CS (1997): Photomorphogenesis, photosynthesis and seed yield of wheat plants grown under red light-emitting diodes (LEDs) with and without supplemental blue lighting. *Jounal of Experimental Botany* 48: 407- 413

Goncalves AN (1980): Reversion to juvenility and cloning of *Eucalyptus urophylla* S.T. Blake in cell and tissue culture systems. IUFRO Symposium and Workshop on Genetic Improvement and Productivity of Fast Growing Tree Species. S Lo Paulo, Brazil. pp 786-787

Gonzalez ER, Andrade A, Bertolo L, Lacerda G, Carneiro R, Defavari V, Labate M and Labate C (2002): Production of transgenic *Eucalyptus grandis* × *E. urophylla* using the sonication-assisted *Agrobacterium* transformation (SAAT) system. *Functional Plant Biology* 29(1):97–102

Gonzalez- Mendoza D, Quiroz Moreno A and Zapata-Perez O (2008): An improved method for the isolation of total RNA from *Avicennia germinans* leaves. *Verlag der Zeitschrift für Naturforschung* 63: 124-126

Grewal S, Ahuja A and Atal CK (1980): *In vitro* Proliferation of shoot apices of *Eucalyptus* citridora Hook. *Indian Journal of Experimental Biology* 18: 775-777

Gupta PK, Mehta UJ and Mascarenhas AF (1983): A tissue culture method for rapid clonal propagation of mature trees of *Eucalyptus torelliana* and *Eucalyptus camaldulensis*. *Plant Cell Reports*. 2: 296–299

Haigler CH, Ivanova-Datcheva M, Hogan PS, Salnikov VV, Hwang S, Marin K and Delmer DP (2001): Carbon partitioning to cellulose synthesis. *Plant Molecular Biology* 47:29–51

Haines R (1994): Biotechnology in Forest Tree Improvement. FAO, Rome

Haines R and Martin BE (1997): Biotechnology and sustainable production of tropical timber. *Forest Genetic Resources Information* (FAO) 25:52-58

Hall TA (1999): BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/ NT. *Nucleic Acids Symposium Series* 41: 95-98

Halpin C and Boerjan W (2003): Stacking transgenes in forest trees. *Trends in Plant Science* 8: 363-365

Haltiwanger RS and Lowe JB (2004): Role of Glycosylation in Development. *Annual Review of Biochemistry* 73: 491-537

Han GS, Siniossoglou S and Carman GM (2007): The cellular functions of the yeast lipin homolog PAH1p are dependent on its phosphatidate phosphatase activity. *Journal of Biological Chemistry* 282: 37026– 37035

Hansen G and Durham NC (2000): Plant transformation methods. – US Patent No. 6,162-965

Harcourt RL, Kyozuka J, Floyd RB, Bateman KS, Tanaka H, Decroocq V, Llewellyn DJ, Zhu X, Peacock WJ and Dennis ES (2000): Insect- and herbicide-resistant transgenic *Eucalyptus*. *Molecular Breeding* 6:307–315

Hardin SC, Tang GQ, Scholz A, Holtgraewe D, Winter H, Huber SC (2003): Phosphorylation of *sucrose synthase* at serine 170: occurrence and possible role as a signal for proteolysis. *Plant Journal* 35: 588–603

Hartman HT, Kester DE and Davies Jr. FT (1990): Plant propagation: Principles and practices. Fifth Edition. Prentice Hall International, Inc. New Jersey

Hazarika P and Rajam MV (2011): Biotic and abiotic stress tolerance in transgenic tomatoes by constitutive expression of S-adenosylmethionine decarboxylase gene. *Physiology and Molecular Biology of Plants* 17:115–128

Heid CA, Stevens J, Livak K J and Williams PM (1996): Real time quantitative PCR. *Genome Research.* 6: 986–994

Hellens RP, Edwards EA, Leyland NR, Bean S and Mullineaux PM (2000): pGreen: a versatile and flexible binary Ti vector for *Agrobacterium mediated* plant transformation. *Plant Molecular Biology* 42: 819–832

Henrissat B (1991): A classification of glycosyl hydrolases based on amino-acidsequence similarities. *Biochemical Journal 280:309*-316

Henrissat B and Bairoch A (1993): New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal* 293: 781-788

Henrissat B and Bairoch A (1996): Updating the sequence-based classification of glycosyl hydrolases. *Biochemical Journal* 316: 695-696

Henrissat B, Claeyssens M, Tomme P, Lemesle L and Mornon JP (1989): Cellulase families revealed by hydrophobic cluster analysis. *Gene* 81: 83–95

Henrissat B, Coutinho PH and Davies GJ (2001): A census of carbohydrate-active enzymes in the genome of *Arabidopsis thaliana*. *Plant Molecular Biology* 47: 55–72

Hernandey-Sebastia C, Piehe Y and Desjardins Y (1999): Water relations of whole strawberry plantlets *in vitro* inoculated with *Glomus intraradices* in a tripartite culture system. *Plant Science* 143: 81–91

Hibino T, Meng YL, Kawamitsu Y, Uehara N, Matsuda N, Tanaka Y, Ishikawa H, Baba S, Takabe T, Wada K, Iishii T and Takabe T (2001): Molecular cloning and functional characterization of two kinds of betaine-aldehyde dehydrogenase in betaine-accumulating mangrove *Avicennia marina* (Forsk.) Vierh. *Plant Molecular Biology* 45: 353-363

His I, Driouich A, Nicol F, Jauneau A and Hofte H (2001): Altered pectin composition in primary cell walls of *Korrigan*, a dwarf mutant of *Arabidopsis* deficient in a membrane-bound endo-1,4-beta-glucanase. *Planta* 212: 348-358

Ho CK, Chang SH, Tsay JY, Tsai CJ, Chiang VL and Chen ZZ (1998): *Agrobacterium tumefaciens*- mediated transformation of *Eucalyptus camaldulensis* and production of transgenic plants. *Plant Cell Reports* 17: 675-680

Hoekema A, Hirsch PR, Schilperoort PJJ and Hooykaas RA (1983): A binary vector strategy based on separation of *vir* and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. *Nature* 303:179 – 180

Holford P and Newbury HJ (1992): The effects of antibiotics and their breakdown products on the *in vitro* growth of *Antirrhinum majus*. *Plant Cell Reports* 11:93-96

Holland N, Holland D, Helentjaris T, Dhugga KS, Xoconostle-Cazares B and Delmer DP (2000): A comparative analysis of the plant cellulose synthase (CesA) gene family. *Plant Physiology* 123: 1313–1323

Holsters M, De Waele D, Depicker A, Messens E, Van Montagu M and Schell J (1978): Transfection and transformation of *Agrobacterium tumefaciens*. *Molecular Genetics and Genomics* 163:181–187

Hong YB, Liu SP, Zhu YP, Xie C, Jue DW, Chen M, Kaleri HA and Yang Q (2013): Expression of the MSI-99 m gene in ransgenic potato plants confers resistance to Phytophthora infestans and Ralstonia solanacearum. *Plant Molecular Biology Reporter* 31:418-424

Hood EE, Gelvin SB, Melchers LS and Hoekema A (1993): New *Agrobacterium* helper plasmids for gene transfer to plants. *Transgenic Research* 2:208–218

Huber SC, Huber JL, Liao PC, Gage DA, McMichael, Jr RW, Chourey PS, Hannah CL and Koch K (1996): Phosphorylation of Serine-15 of Maize Leaf Sucrose Synthase. *Plant Physiology* 2: 793-802

Humara JM, Lopez M and Ordas RJ(1999): *Agrobacterium tumefaciens* mediated transformation of *Pinus pinea* L. cotyledon: an assessment of factors influencing the efficiency of *uidA* gene transfer. *Plant Cell Reports* 19: 51-58.

Hutchinson IF and Zimmerman RH (1987): Tissue culture of temperate fruit and nut trees. *Horticultural Reviews* 9: 273–349

Ikemori, Y.K. 1987. Epicormic shoots from the branches of *Eucalyptus grandis* as an explant source for in vitro culture. *Commonwealth Foreign Review* 66:351-355

Itoh T, Hashimoto W, Mikami B and Murata K (2006): Crystal structure of unsaturated glucuronyl hydrolase complexed with substrate: molecular insights into its catalytic reaction mechanism. *The Journal of Biological Chemistry* 281: 29807-29816

Ivanova M and Van Staden J (2009): Nitrogen source, concentration, and NH_4^+:NO_3^- *ratio* influence shoot regeneration and hyperhydricity in tissue cultured *Aloe polyphylla*. *Plant Cell Tissue and Organ Culture* 99:167–174

Jain SM (2006): An update on overall recent progress on somatic embryogenesis in forest trees. In: Suzuki K, Ishii K, Sakurai S, Sasaki S (eds) Plantation technology in tropical forest science, Springer, Tokyo, pp 113–122

James DJ, Passey AJ and Rugini E (1988): Factors affecting high frequency plant regeneration from apple leaf tissues cultured *in vitro*. *Journal of Plant Physiology* 132:148-154.

James DJ, Uratsu S, Cheng J, Negri P, Viss P and Dandekar AM (1993): Acetosyringone and osmo-protectants like betaine or proline synergistically enhance *Agrobacterium*-mediated transformation of apple. *Plant Cell Reports* 12: 559–563

Jayashree B, Pradeep R, Anil K and Gopal B (2008): Correlation between the sucrose synthase protein subfamilies, variations in structure and expression in stress-derived expressed sequence tag datasets. *Journal of Proteomics and Bioinformatics* 1: 408-423

Jefferson RA, Kavanagh TA and Bevan MW (1987): GUS fusions: *β-glucuronidase* as a sensitive and versatile gene fusion marker in higher plants. *EMBO Journal* 6:3901–3907

Jeon MW, Ali MB, Hahn EJ and Paek KY (2005): Effects of photon flux density on the morphology, photosynthesis and growth of a CAM orchid, *Doritaenopsis* during post-micropropagation acclimatization. *Plant Growth Regulation* 45: 139–147.

Jha T and Sen SK (1992): Micropropagation of an elite Darjeeling tea clone. *Plant Cell Reports* 11: 101–104

Johansen, DA (1940): Plant Microtechnique. McGraw Hill Company, New York

Jones NB and van Staden J (1997): Micropropagation of Eucalypts. In: Bajaj YPS (ed) Biotechnology in Agriculture and Forestry, Vol. 39, High-Tech and Micropropagation, V. Springer-Verlag, pp 286–329

Jones P and Vogt T (2001): Glycosyl transferases in secondary plant metabolism: tranquilizers and stimulant controllers. *Planta* 213:164–174

Joshi CP and Mansfield SD (2007): The cellulose paradox - simple molecule, complex biosynthesis. *Current Opinion in Plant Biology* 10:220–226

Joshi CP, Bhandari S, Ranjan P, Kalluri UC, Liang X, Fujino T and Samuga A (2004): Genomics of cellulose biosynthesis in poplars. *New Phytologist* 164:53-61.

Joshi P and Dhawan V (2007): Assessment of genetic fidelity of micropropagated *Swertia chirayita* plantlets by ISSR marker assay. *Biologia Plantarum* 51: 22 - 26

Jube S and Borthakur D (2009): Development of an *Agrobacterium* mediated transformation protocol for the tree-legume *Leucaena leucocephala* using immature zygotic embryos. *Plant Cell Tissue and Organ Culture* 96:325–333

Jung M, Shin SH, Park JM, Lee SN, Lee MY, Ryu KH, Paek KY, Harn CH (2011): Detection of transgene in early developmental stage by GFP monitoring enhances the efficiency of genetic transformation of pepper. *Plant Biotechnology Reports* 5:157–167

Junghans DT, Alfenas AC, Brommonschenkel SH, Oda S, Mello EJ and Grattapaglia D (2003): Resistance to rust (*Puccinia psidii* Winter) in *Eucalyptus*: mode of inheritance and mapping of a major effect locus with RAPD markers. *Theoretical and Applied Genetics* 108: 175–180

Kato M (1985): Regeneration of plantlets from tea stem callus. *Japanese Journal of Breeding* 35: 317–322

Kaur R, Sood M, Chander S, Mahajan R, Kumar V and Sharma DR (1999): *In vitro* propagation of *Valeriana jatamansi. Plant Cell Tissue and Organ Culture* 59: 227–229

Kawazu T, Kondo K, Suzuki Y, Wada T and Koyama H (2003): Overexpression of mitochondrial citrate synthase in *Eucalyptus* trees improved growth when cultured by Alphosphate as a sole phosphate source. *Plant and Cell Physiology* 44: S91-S91

Kawiak A and Lojkowska E (2004): Application of RAPD in the determination of genetic fidelity in micropropagated *Drosera* Plantlets. *In Vitro Cellular and Developmental Biology-Plant* 40:592–59

Keegstra K and Walton J (2006): Plant science. Beta-glucans -brewer's bane, dietician's delight. *Science* 311:1872-1873

Keil M and Griffin AR (1994): Use of random amplified polymorphic DNA (RAPD) markers in the discrimination and verification of genotypes in *Eucalyptus. Theoretical and Applied Genetics* 89:442–450

Khan PSSV, Kozai T, Nguyen QT, Kubota C and Dhawan V (2002): Growth and net photosynthetic rates of *Eucalyptus tereticornis* Smith under photomixotrophic and various photoautotrophic micropropagation conditions. *Plant Cell Tissue and Organ Culture* 71: 141–146

Khuspe SS, Gupta PK, Kulkarni DK, Mehta V and Mascarenhas AF (1987): Increased biomass production by *Eucalyptus*. *Canadian Journal* of *Forest Research* 17:1361–1363

Kimura S, Laosinchai W, Itoh T, Cui X, Linder CR and Brown Jr RM (1999): Immunogold labeling of rosette terminal cellulose-synthesizing complexes in the vascular plant *Vigna angularis*. *Plant Cell* 11:2075-2085

King JL and Jukes TH (1969): Non-Darwinian evolution. *Science* 164:788-798

Knop W (1884): Bereitung einer concentrierten Nahrstofflosung fur Pflanzen. *Landwirtschaftliche Versuchsstation* 30: 292-294

Kozai T (1991): Acclimatization of micropropagated plants. In: Bajaj YPS (eds) High-Tech and Micro propagation V (Biotechnology in Agriculture and Forestry), Springer- Verlang, Berlin Heidelberg, 17:127–141

Krimi Z, Raio A, Petit A, Nesme X and Dessaux Y (2006): *Eucalyptus occidentalis* plantlets are naturally infected by pathogenic *Agrobacterium tumefaciens*. *European Journal of Plant Pathology* 116:237–246

Kumar A, Aggarwal D, Gupta P and Reddy MS (2010): Factors Effecting *in vitro* propagation and field establishment of *Chlorophytum borivillianum. Biologia Plantarum* 54: 601-606

Kumar A, Nandi SK, Chandra B and Pal M (2004): Conservation of some Himalayan medicinal plants using biotechnological approaches. *Indian Forester* 130:187-199

Kumar A, Palni LMS and Nandi SK (2003): The effect of light source and gelling agent on micropropagation of *Rosa damascene* Mill. and *Rhychostylis retusa* (L.) Bl. *Journal of Horticulture Science and Biotechechnology* 78: 786-792

Kumar A, Sood A, Palni LMS and Gupta AK (1999): *In Vitro* Propagation of *Gladiolus hybridus* Hort. Synergetic effect of heat shock and sucrose on morphogenesis. *Plant Cell Tissue and Organ Culture* 57:105-112

Kumar M, Thammannagowda S, Bulone V, Chiang V, Han KH, Joshi CP, Mansfield SD, Mellerowicz E, Sundber B, Teeri T and Ellis BE (2009): An update on the nomenclature for the cellulose synthase genes in *Populus*. *Trends in Plant Science* 14:248–254

Kumar SV and Rajam MV (2007): Induction of *Agrobacterium tumefaciens vir* genes by the green alga, *Chlamydomonas reinhadtii*. *Current Science* 92: 1727-1729

Kumari V and Das N (2013): Vacuolar invertases in potato (*Solanum tuberosum* L.): Molecular cloning, characterization, sequence comparison, and analysis of gene expression in the cultivars. *Acta Physiologiae Plantarum*: DOI 10.1007/s11738-013-1240-y

Kurek I, Kawagoe Y, Jacob-Wilk D, Doblin M and Delmer DP (2002): Dimerization of cotton fiber cellulose synthase catalytic subunits occurs via oxidation of the zinc binding domains. *Proceedings of the National Academy of Sciences of the USA* 99:11109-11114

Kutschera U and Heiderich A (2002): Sucrose metabolism and cellulose biosynthesis in sunflower hypocotyls. *Physiology Plantarum* 114:372–379

Ladiges PY, Udovicic F and Nelson G (2003): Australian biogeographical connections and the phylogeny of large genera in the plant family Myrtaceae. *Journal of Biogeography* 30:989–998

Lairson LL, Henrissat B, Davies GJ and Withers SG (2008): Glycosyltransferases: structures, functions and mechanisms. *Annual Review of Biochemistry* 77:521–55

Lal P, Kulkarni HD and Srinivasa K (1993): *Eucalyptus* improvement program of ITC Bhadrachalam Paper Board Ltd. In: Vivekanandhan K, Subramanian KN, Zabala NQ, Gurumurthy K (eds) Proceedings of workshop on production of genetically improved planting materials for afforestation programmes. RAS/91/004 field document no 7, pp 57– 63

Lane DR, Wiedemeier A , Peng LC, Hofte H, Vernhettes S, Desprez T, Hocart C, Birch R, Baskin T, Burn J, Arioli T, Betzner A, Williamson R (2001): Temperature-sensitive alleles of *rsw2* link the Korrigan endo-1,4- β -glucanase to cellulose synthesis and cytokinesis in *Arabidopsis*. *Plant Physiology* 126: 278 – 288

Lashbrook CC, Gonzalez-Bosch C and Bennett AB (1994): Two divergent endo-beta-1,4-glucanase genes exhibit overlapping expression in ripening fruit and abscissing flowers. *Plant Cell* 6 1485-1493

Lata H, Li XC, Silva B, Moraes RM and Halda-Alija L (2006): Identification of IAA-producing endophytic bacteria from micropropagated *Echinacea* plants using 16S rRNA sequencing. *Plant Cell Tissue and Organ Culture* 85: 353–359

Lattoo SK, Bamotra S, Sapru DR, Khan S and Dhar AK (2006): Rapid plant regeneration and analysis of genetic fidelity of *in vitro* derived plants of *Chlorophytum arundinaceum* Baker- an endangered medicinal herb. *Plant Cell Reports* 25:499–506

Lee SH, Tewari RK, Hahn EJ and Paek KY (2007): Photon flux density and light quality induce changes in growth, stomatal development, photosynthesis and transpiration of *Withania Somnifera* (L.) Dunal. Plantlets. *Plant Cell Tissue and Organ Culture* 90:141–151

Leifert C and Cassells AC (2001): Microbial hazards in plant tissue and cell cultures. *In Vitro Cellular and Devlopmental Biology- Plant* 37: 133–138

Letham DS (1978): Cytokinins. In: Letham DS, Goodwin PB, Higgins TJV (eds) Phytohormones and related compounds: A comprehensive treatise. Elsevier/North Holland Biomedical Press, Amsterdam, vol I, pp 205-26

Letham DS and Palni LMS (1983): The biosynthesis and metabolism of cytokinins. *Annual Review* of *Plant Physiology* 34: 163–197

Li J, Baga M, Hucl P and Chibbar RN (2011): Development of microsatellite markers in canary seed (*Phalaris canariensis* L.) *Molecular Breeding* 28:611–621

Li X, Krasnyanski SF and Korban SS (2002): Optimization of the *uidA* gene transfer into somatic embryos of rose via *Agrobacterium tumefaciens. Plant Physiology and Biochemistry* 40:453–459

Libertini E, Li Y and McQueen-Mason SJ (2004): Phylogenetic analysis of the plant endo-beta-1,4-glucanase gene family. *Journal of Molecular Evolution* 58:506- 515

Lin C (2002): Blue light receptors and signal transduction. *Plant Cell* 14:S207–S225

Lin DG, Chang JY, Chiou SY, Sung JM, Jeang CL and Wang CS (2009): Cloning and characterization of a cDNA encoding the endo-1, 4-beta-D-glucanase gene expressed in rapidly growing tuber and leaf of taro (*Colocasia esculenta* var. *esculenta)* *Botanical Studies* 50: 379-387

Liu X and Pijut PM (2008): Plant regeneration from *in vitro* leaves of mature black cherry (*Prunus serotina*). *Plant Cell Tissue and Organ Culture* 94:113–123

Livak KJ and Schmittgen TD (2001): Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta}C$T Method. *Methods* 25: 402–408

Lloyd G and McCown B (1981): Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia* by use of shoot tip culture. *Combined Proceedings of the International Plant Propagators Society* 30:421–427

Lopez SJ, Kumar RR, Pius PK and Muralidharan N (2004): *Agrobacterium tumefaciens*–mediated genetic transformation in Tea (*Camellia sinensis* [L.] O. Kuntze). *Plant Molecular Biology Reporter* 22: 201a–201j

Luna C, Collavino M, Mroginski L and Sansberro P (2008): Identification and control of bacterial contaminants from Ilex dumosa nodal segments culture in a temporal immersion bioreactor system using 16S rDNA analysis. *Plant Cell Tissue and Organ Culture* 95:13–19

Lynch M (2007) :The origin of genome architecture. Sinauer: Sunderland, MA

Machado R, Andrade D, Cid B, Penchel RM and Brasileiro M (1997): *Agrobacterium* strain specificity and shooty tumour formation in *Eucalyptus* (*Eucalyptus grandis* × *E. urophylla*). *Plant Cell Reports* 16:299–303

Mackenzie PI, Owens IS, Burchell B, Bock KW, Bairoch A, Belanger A, Fournel Gigleux S, Green M, Hum DW, Iyanagi T, Lancet D, Louisot P, Magdalou J, Chowdhury JR, Ritter JK, Schachter H, Tephly TR, Tipton KF and Nebert DW (1997): The UDP glycosyltransferase gene superfamily: recommended nomenclature update based on evolutionary divergence. *Pharmacogenetics* 7:255–269

Macrae S and Van Staden J (1999): Transgenic *Eucalyptus*. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry. Heidelberg, Springer. 44: 88–114

Maheswaran GM, Welander M, Hutchinson JF, Graham MW and Richards D (1992): Transformation of apple rootstock M26 with *Agrobacterium tumefaciens*. *Journal of Plant Physiology* 139:560–568

Maloney VJ and Mansfield SD (2010): Characterization and varied expression of a membrane-bound endo-beta-1,4-glucanase in hybrid poplar. *Plant Biotechnology Journal* 8: 294–307

Maloney VJ, Samuels AL and Mansfield SD (2012): The endo-1,4-beta-glucanase *Korrigan* exhibits functional conservation between gymnosperms and angiosperms and is required for proper cell wall formation in gymnosperms. *New Phytplogist*: 193:1076-87

Mandel M and Higa A (1970): Calcium-dependent bacteriophage DNA infection. *Journal of Molecular Biology* 53: 159-165

Manickavasagam M, Ganapathi A, Anbazhagan VR, Sudhakar B, Selvaraj N, Vasudevan A, and Kasthurirengan S (2004): *Agrobacterium* mediated genetic transformation and development of herbicide-resistant sugarcane (*Saccharum* species hybrids) using axillary buds. *Plant Cell Report* 23:134–143

Martin KP, Joseph D, Madassery J and Philip VJ (2003): Direct shoot regeneration from lamina explants of two commercial cut flower cultivars of *Anthurium andraeanum* hort. *In Vitro Cellular and Developmental Biology - Plant* 39:500–504.

Martin KP, Pachathundikandi S, Zhang CL, Slater A and Madassery J (2006): RAPD analysis of a variant of banana (*Musa* sp.) cv. grande naine and its propagation via shoot tip culture. *In Vitro Cellular and Developmental Biology – Plant* 42:188–192

Martins M, Sarmento D and Oliveira DD (2004): Genetic stability of micropropagated almond plantlets, as assessed by RAPD and ISSR markers. *Plant Cell Reports* 23:492–496

Master ER, Rudsander UJ, Zhou W, Henriksson H, Divine C, Denman S, Wilson DB and Teeri TT (2004): Recombinant expression and enzymatic characterization of PttCel9A, a *KOR* homologue from *Populus tremula* x *tremuloides*. *Biochemistry* 43: 10080 – 10089

Matic S, Akerlund HE, Everitt E and Widell S (2004): Sucrose synthase isoforms in cultured tobacco cells. *Plant Physiology and Biochemistry*, 42: 299-306

Matthysse AG, Thomas DL and White AR (1995): Mechanism of cellulose synthesis in *Agrobacterium tumefaciens*. *Journal of Bacteriology* 177:1076-1081

McComb JA and Bennett IJ (1986): *Eucalyptus* (*Eucalyptus* spp.). In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, trees I. Springer, Berlin Heidelberg New York, pp 340–362.

Melchers LS, Maroney MJ, den Dulk-Ras A, Thompson D V, Van Vuuren H A, Schilperoort RA and Hooykaas PJ (1990): Octopine and nopaline strains of *Agrobacterium tumefaciens* differ in virulence; molecular characterization of the vir F locus. *Plant Molecular Biology* 14: 249-259

Merkle S and Dean JFD (2000): Forest tree biotechnology. *Current Opinion in Biotechnology* 11: 298–302

Miguel CM and Oliveira MM (1999): Transgenic almond (*Prunus dulcis* Mill.) plants obtained by *Agrobacterium* mediated transformation of leaf explants. *Plant Cell Reports* 18: 387-393

Miyama M and Tada Y (2008): Transcriptional and physiological study of the response of Burma mangrove (*Bruguiera gymnorhiza*) to salt and osmotic stress. *Plant Molecular Biology* 68:119-129

Molhoj M, Johansen B, Ulvskov P and Borkhardt B (2001): Expression of a membrane anchored endo-1,4-beta-glucanase from *Brassica napus*, orthologous to KOR from *Arabidopsis thaliana*, is inversely correlated to elongation in light-grown plants. *Plant Molecular Biology* 45:93-105

Molhoj M, Pagant S and Hofte H (2002): Towards understanding the role of membrane-bound endo- β -1,4-glucanases in cellulose biosynthesis. *Plant and Cell Physiology* 43: 1399 – 1406.

Mullins KV, Llewellyn DJ, Hartney VJ, Strauss S and Dennis ES (1997): Regeneration and transformation of *Eucalyptus camaldulensis. Plant Cell Reports* 16: 787–791

Muralidharan EM and Mascarenhas AF (1987): *In vitro* plantlet formation by organogenesis in *E. camaldulensis* and by somatic embryogenesis in *Eucalyptus citriodora*. *Plant Cell Reports* 6: 256–259

Murashige T and Skoog F (1962): A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15:473-497

Murphy LD, Herzog CE, Rudick JB, Fojo AT and Bates SE (1990): Use of the polymerase chain reaction in the quantitation of mdr-1 gene expression. *Biochemistry* 29: 10351–10356

Mutwil M, Debolt S and Persson S (2008): Cellulose synthesis: A complex complex. *Current Opinion in Plant Biology* 11: 252 – 257

Nairn CJ and Haselkorn T (2005): Three loblolly pine CesA genes expressed in developing xylem are orthologous to secondary cell wall CesA genes of angiosperms. *New Phytologist* 166: 907-915

Nairn CJ, Lennon DM, Wood-Jones A, Nairn AV and Dean JFD (2008): Carbohydrate-related genes and cell wall biosynthesis in vascular tissues of loblolly pine (*Pinus taeda* L.). *Tree Physiology* 28: 1099–1110

Nakai T, Tonouchi N, Tsuchida T, Konishi T, Kojima Y, Tsuchida T, Yoshinaga F, Sakai F and Hayashi T (1999): Enhancement of cellulose production by expression of sucrose synthase in *Acetobacter xylinum. Proceedings of the National Academy of Sciences of USA* 96:14–18

Nandi SK, Palni LMS and Rikkari HC (1996): Chemical induction of adventitious root formation in *Taxus baccata* cuttings:. *Plant Growth Regulation* 19: 171-122

Nehra NS, Chibbar RN, Leung N, Caswell K, Mallard C, Steinhauer L, Baga M and Kartha KK (1994): Self-fertile transgenic wheat plants regenerated from isolated scutellar tissues following microprojectile bombardment with two distinct gene constructs. *Plant Journal* 5: 285–297

Neill SJ, Desikan R and Hancock JT (2003): Nitric oxide signalling in plants. *New Phytologist* 159: 11–35

Nicol F, His I, Jauneau A, Vernhettes S, Canut H and Hofte H (1998): A plasma membrane–bound putative endo-1, 4-beta-D-glucanase is required for normal wall assembly and cell elongation in *Arabidopsis*. *EMBO Journal* 17:5563–5576

Niu X, Li X, Veronese P, Hasegawa PM, Bressan RA and Weller SC (2000): Factors affecting *Agrobacterium tumefaciens*-mediated transformation of peppermint. *Plant Cell Reports* 19: 304-310

Noonan KE, Beck C, Holzmayer TA, Chin JE, Wunder, JS, Andrulis IL, Gazdar, AF, Willman CL, Griffith B, Hoff DD and Robinson IB (1990): Quantitative analysis of MDR1 (multidrug resistance) gene expression in human tumours by polymerase chain reaction. *Proceedings of the National Academy of Sciences of the USA* 87: 7160–7164

Norelli JL, Aldwinckle HS, Beltran LD and Jaynes JM (1994): Transgenic 'Malling 26' apple expressing the attacin E gene has increased resistance to *Erwinia amylovora*. *Euphytica* 77:123-128

Nugent G, Chandler SF, Whiteman P and Stevenson TW (2001a): Adventitious bud induction in *Eucalyptus globulus* labill. *In Vitro Cellular and Developmental Biology-Plant* 37: 388-391

Nugent G, Chandler SF, Whiteman P and Stevenson TW (2001b): Somatic embryogenesis in *Eucalyptus globulus. Plant Cell Tissue and Organ Culture* 6:785-788

Ohmiya Y, Nakai T, Park YW, Aoyama T, Oka A, Sakai F and Hayashi T (2003): The role of PopCel1 and PopCel2 in poplar leaf growth and cellulose biosynthesis. *Plant Journal* 33:1087-1097

Ohmiya Y, Samejima M, Shiroishi M, Amano Y, Kanda T, Sakai F and Hayashi T (2000): Evidence that endo-1, 4- beta-glucanases act on cellulose in suspension-cultured poplar cells. *Plant Journal* 24: 147 – 158

Padmanabhan P and Sahi SV (2009): Genetic transformation and regeneration of *Sesbania drummondii* using cotyledonary nodes. *Plant Cell Reports* 28:31-40

Palni LMS, Bag N, Nadeem M, Tamta S, Vyas P, Bisht MS, Purohit VK, Kumar A, Nandi S K, Pandey A and Purohit AN (1998): Micropropagation: Conservation through tissue culture of selected Himalayan Plants. In*:* Research for Mountain Development: Some Initiatives and Accomplishments. Gyanodaya Prakashan, Nainital, pp 431–452

Palombi MA and Damiano C (2002): Comparison between RAPD and SSR molecular markers in detecting genetic variation in kiwifruit *(Actinidia deliciosa* A. Chev). *Plant Cell Reports* 20:1061–1066

Pandey A, Bag N, Chandra B and Palni LMS (2002): Biological Hardening: A promising technology for tissue culture industry. In: Nandi SK, Palni LMS, Kumar A (eds) Role of Plant Tissue Culture in Biodiversity Conservation and Development. Gyanodaya Prakashan, Nainital, pp 565–577

Pandey A, Palni LMS and Bag N (2000): Biological hardening of tissue culture raised tea plants. *Biotechnol Letters* 22:1087– 1091

Park YW, Tominaga R, Sugiyama J, Furuta Y, Tanimoto E, Samejima M (2003): Enhancement of growth by expression of poplar cellulase in *Arabidopsis thaliana. Plant Journal 33*: 1099 – 1106

Patt R, Kordsachia O and Fehr J (2006): European hardwoods versus *Eucalyptus globulus* as a raw material for pulping. *Wood Science and Technology* 40: 39–48

Pear JR, Kawagoe Y, Schreckengost WE, Delmer DP and Stalker DM (1996): Higher plants contain homologs of the bacterial celA genes encoding the catalytic subunit of cellulose synthase. *Proceedings of the National Academy of Sciences of the USA* 93: 12637–12642

Pena L and Seguin A (2001): Recent advances in the genetic transformation of trees. *Trends in Biotechnology* 19: 500-506

Peng L, Kawagoe Y, Hogan H and Delmer D (2002): Sitosterol- beta- glucoside as primer for cellulose synthesis in plants. *Science* 295: 147 – 150

Perez-Tornero O, Egea J, Vanoostende A and Burgos L (2000): Assessment of factors affecting adventitious shoot regeneration from *in vitro* cultured leaves of apricot. *Plant Science* 158: 61–70

Pillonel C, Buchala AJ and Meier H (1980): Glucan synthesis by intact cotton fibers fed with different precursors at the stages of primary and secondary wall formation. *Planta* 149:306–312

Pinto G, Park YS, Silva, Neves L, Araujo C and Santos C (2008): Factors affecting maintenance, proliferation and germination of secondary somatic embryos of *Eucalyptus globulus* Labill. *Plant Cell Tissue and Organ Culture* 95:69–78

Pius J, George L, Eapen S and Rao PS (1993): Enhanced plant regeneration in pearl millet (*Pennisetum americanum*) by ethylene inhibitors and Cefotaxime. *Plant Cell Tissue and Organ Culture* 32: 91-96

Plomion C, Leprovost G and Stokes A (2001): Wood formation in trees. *Plant Physiology* 127(4): 1513-1523

Prakash MG and Gurumurthi K (2005): Somatic Embryogenesis and plant regeneration in *Eucalyptus tereticornis* Sm. *Current Science* 88:1311-1316

Prakash MG and Gurumurthi K (2009): Genetic transformation and regeneration of transgenic plants from precultured cotyledon and hypocotyl explants of *Eucalyptus tereticornis* Sm. using *Agrobacterium tumefaciens. In Vitro Cellular and Developmental Biology -Plant* 45:429-434

Quevillon E, Silventoinen V, Pillai S, Harte N, Mulder N, Apweiler R and Lopez R (2005): InterProScan: protein domains identifier. *Nucleic Acids Research* 33: W116–W120

Quoirin M and Quisen R (2006): Advances in genetic transformation of *Eucalyptus* species. *Molecular Biology of Tropical Plants* 2006: 41-56

Quoirin, M and Lepoivre P (1977): Improved medium for *in vitro* culture of *Prunus* sp. *Acta Horticulture* 78:437–442

Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J, Eckert CA, Frederick WJ Jr, Hallett JP, Leak DJ, Liotta CL, Mielenz JR, Murphy R, Templer R, Tschaplinski T (2006): The path forward for biofuels and biomaterials. *Science* 311:484-489

Rai VR and Chandra KSJ (1989): Micropropagation of Indian rosewood by tissue culture. *Annals of Botany* 64: 43– 46

Ramage CM and Williams RR (2002): Mineral nutrition and plant morphogenesis. *In Vitro Cellular and Developmental Biology - Plant* 38*:116*-124

Ramage CM, Borda AM, Hamill SD and Smith MK (2004): A simplified PCR test for early detection of dwarf off-types in micropropagated Cavendish Banana (*Musa* spp. AAA). *Scientia Horticulturae* 103:145–151

Rani V and Raina SN (1998): Genetic analysis of enhanced-axillary branching derived *Eucalyptus tereticornis* Smith and *E. camaldulensis* Dehn. Plants. *Plant Cell Reports* 17:236–242

Rani V, Parida A and Raina SN (1995): Random amplified polymorphic DNA (RAPD) markers for genetic analysis in micropropagated plants of *Populus deltoides* Marsh. *Plant Cell Reports* 14:459–462

Rao KS (1988): *In vitro* meristem cloning of *Eucalyptus tereticornis* Sm. *Plant Cell Reports* 7:546- 549

Rao RV, Shashikala S, Sreevani P, Kothiyal V, Sarma CR and Lal P (2002): Within tree variation in anatomical properties of some clones of *Eucalyptus tereticornis* Sm. *Wood Science and Technology* 36:271–285

Ray T, Dutta I, Saha P, Das S and Roy SC (2006): Genetic stability of three economically important micropropagated banana (*Musa* spp.) cultivars of lower Indo-Gangetic plains, as assessed by RAPD and ISSR markers. *Plant Cell Tissue and Organ Culture* 85:11-21

Read SM and Bacic T (2002): Prime time for cellulose. *Science* 295:59–60

Reiter WD (2002): Biosynthesis and properties of the plant cell wall. Curr Opin Plant Biol 5: 536–542

Richmond TA (2000): Higher plant cellulose synthases. *Genome Biology* 1:3001.1-3001.6

Richmond TA and Somerville CR (2000): The cellulose synthase superfamily. *Plant Physiology* 124:495-498

Robert S, Bichet A, Grandjean O, Kierzkowski D, Satiat-Jeunemaıtre B, Pelletier S, Hauser MT, Hofte H and Vernhettesa S (2005): An *Arabidopsis* endo-1,4- beta-d-glucanase involved in cellulose synthesis undergoes regulated intracellular cycling. *Plant Cell* 17: 3378–3389

Robert S, Mouille G and Hofte H (2004): The mechanism and regulation of cellulose synthesis in primary walls: lessons from cellulose deficient *Arabidopsis* mutants. *Cellulose* 11:351-364

Rochange F, Serrano L, Marque C, Teulieres C, Boudet AM (1995): DNA delivery into *Eucalyptus globulus* zygotic embryos through biolistics: optimization of the biological and physical parameters of bombardment for two different particle guns. *Plant Cell Reports* 14:674–678

Rockwood DL and Warrag EI (1994): Field performance of micropropagated and seed-derived propagules of three *Eucalyptus grandis* ortets. *Plant Cell Reports* 13:628–631

Rodrigues SM, Soares VL, De Oliveira TM, Gesteira AS, Otoni WC and Costa MG (2007): Isolation and purification of RNA from tissues rich in polyphenols, polysaccharides, and pigments of annatto (*Bixa orellana* L.). *Molecular Biotechnology* 37: 220-224

Rohini VK and Sankara Rao K (2001): Transformation of peanut (*Arachis hypogaea* L.) with tobacco chitinase gene: variable response of transformants to leaf spot disease. *Plant Science* 160 (5): 883-892

Rose JKC and Bennett AB (1999): Cooperative disassembly of the cellulose–xyloglucan network of plant cell walls: parallels between cell expansion and fruit ripening. *Trends in Plant Science 4*: 176 – 183

Rose JKC, Saladie M and Catala C (2004): The plot thickens: new perspectives of primary cell wall modification. *Current Opinion in Plant Biology* 7: 296-301

Ross J, Li Y, Lim EK and Bowles DJ (2001): Higher plant glycosyltransferases. Genome Biology 2:3004-3006

Rossi F, Baraldi R and Facini O (1993): Photomorphogenic effects on *in vitro* rooting of *Prunus* rootstock GF 655-2. *Plant Cell Tissue and Organ Culture* 32:145–151

Rout GR, Mahato A and Senapati SK (2008): *In vitro* clonal propagation of *Nyctanthes arbortristis. Biologia Plantarum* 52: 521-524

Ruan YL, Llewellyn DJ and Furbank RT (2003): Suppression of sucrose synthase gene expression represses cotton fiber cell initiation, elongation, and seed development. *Plant Cell* 15: 952–964

Rubio-Pina JA and Vazquez-Flota FA (2008): Isolation of functional total RNA from *Argemone mexicana*tissues. *Electronic Journal of Biotechnology* 11: 4-13

Rudd PM and Dwek RA (1997): Glycosylation: heterogeneity and the 3D structure of proteins. *Critical Reviews in Biochemistry and Molecular Biology* 32: 1–100

Saini R, Sonia and Jaiwal PK (2003): Stable genetic transformation of *Vigna mungo* L. Hepper via *Agrobacterium tumefaciens*. *Plant Cell Reports* 21: 851-859

Salnikov VV, Grimson MJ, Delmer DP and Haigler CH (2001): Sucrose synthase localizes to cellulose synthesis sites in tracheary elements. *Phytochemistry* 57: 823-833

Salnikov VV, Grimson MJ, Seagull RW and Haigler CH (2003): Localization of sucrose synthase and callose in freeze-substituted secondary-wall-stage cotton fibers. *Protoplasma* 221:175-184

Sanchez de la Hoz P, Vicente-Carbajosa J, Mena M, Carbonero P (1992): Homologous sucrose synthase genes in barley *(Hordeum vulgare)* are located in chromosomes 7H (syn.1) and 2H. Evidente for a gene translocation? *FEBS Letters* 310: 46-50

Sánchez MC, Martínez MT, Valladares S, Ferro E, Ana M and Vieitez (2003): Maturation and germination of oak somatic embryos originated from leaf and stem explants: RAPD markers for genetic analysis of regenerants. *Journal of Plant Physiology* 160: 699–707

Sanger F, Nicklen S and Coulson AR (1977): DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the USA* 74: 5463–5467

Sangwan RS, Bourgoies Y, Brow S, Vasseur G, Sangwan-Norreel BS (1992): Characterization of competent cells and early events of *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Planta* 188:439–456

Santos RL (1997): The *Eucalyptus* of California. Alley Cass Publications, Denair, California

Saritha KV and Naidu CV (2008): Direct shoot regeneration from leaf explants of *Spilanthes acmella. Biologia Plantarum* 52:334–338

Sartoretto LM, Cid LPB and Brasileiro ACM (2002): Biolistic transformation of *Eucalyptus grandis x E. urophylla* callus. *Functional Plant Biology* 29: 917-924

Sato S, Kato T, Kakegawa K, Ishii T, Liu YG, Awano T, Takabe K, Nishiyama Y, Kuga S, Sato S, Nakamura Y, Tabata S and Shibata D (2001): Role of the putative membrane-bound endo-1,4- β -glucanase Korrigan in cell elongation and cellulose synthesis in *Arabidopsis thaliana*. *Plant and Cell Physiology* 42: 251 – 263

Saurin AJ, Borden KLB, Boddy MN and Freemont PS (1996): Does this have a familiar RING? *Trends in Biochemical Sciences* 21**:** 208–214

Saxena B, Kaur R and Bhardwaj SV (2011): Assessment of Genetic Diversity in Cabbage Cultivars Using RAPD and SSR Markers. *Journal of Crop Science and Biotechnology* 14 (3): 191-196

Saxena IM, Brown RM Jr., Fevre M, Geremia RA and Henrissat B (1995): Multidomain architecture of beta -glycosyl transferases: Implications for mechanism of action. *Journal of Bacteriology* 177: 1419-1424

Schoofs J (1992): Rapid clonal propagation, storage and exchange of *Musa* species. In: Baker FWG (ed) Rapid propagation of fast growing woody species. CAB International, Oxford, pp 29–41

Scorza R, Cordts JM, Gray DJ, Gonsalves D, Emershad RL and Ramming DW (1996): Producing transgenic 'Thompson Seed- less' grape (*Vitis viniferu* L.) plants. *Journal of the American Society for Horticultural Science* 121: 616-619

Serrano L, Rochange F, Semblat JP, Marque C, Teulières C and Boudet AM (1996): Genetic transformation of *Eucalyptus globules* through biolistics: complementary development of procedures for organogenesis from zygotic embryos and stable transformation of corresponding proliferating tissue. *Journal of Experimental Botany* 47: 285–290

Shani Z, Dekel M, Cohen B, Barimboim N, Cohen O, Halay T, Kolosovski N and Shoseyov O (2004): *Eucalyptus* in the changing world. In: Borralho N (eds) Proceedings of the International IUFRO conference. Aveiro, Portugal. pp 668

Shani Z, Dekel M, Cohen B, Barimboim N, Kolosovski N, Safranuvitch A, Cohen O and Shoseyov O (2003): Cell wall modification for the enhancement of commercial *Eucalyptus* species. In: Sundberg B (ed) IUFRO tree biotechnology. Umea Plant Science Center, Umea. pp S10–S26

Sharma AK, Raghuram N, Chandhok MR, Das R and Sopory SK (1999a): Investigations on the nature of the phytochrome induced transmitters for the regulation of nitrate reductase in etiolated leaves of maize. *Journal of Experimental Botany* 45: 485-490

Sharma KK and Anjaiah V (2000): An efficient method for the production of transgenic plants of peanut (*Arachis hypogaea* L.) through *Agrobacterium tumefaciens* mediated genetic transformation. *Plant Science* 159: 7-19

Sharma M, Sood A, Nagar PK, Prakash O and Ahuja PS (1999): Direct rooting and hardening of tea microshoots in the field. *Plant Cell Tissue and Organ Culture* 58: 111–118

Sharma SK and Millam S (2004): Somatic embryogenesis in *Solanum tuberosum* L.: a histological examination of key developmental stages. *Plant Cell Reports* 23:115–119

Sharma SK and Ramamurthy V (2000): Micropropagation of 4-year-old elite *Eucalyptus tereticornis* trees. *Plant Cell Reports* 19: 511-518

Sharma SK, Bryan G and Millam S (2007): Auxin pulse treatment holds the potential to enhance efficiency and practicability of somatic embryogenesis in potato *Plant Cell Reports* 26:945–950

Shekhawat NS, Rathore TS, Singh RP, Deora NS and Rama Rao S (1993): Factors affecting i*n vitro* clonal propagation of *Prosopis cineraria*. *Plant Growth Regulation* 12: 273-280

Shenoy VB and Vasil IK (1992): Biochemical and molecular analysis of plants derived from embryogenic cultures of Napier grass (*Pennisetum purpureum* K. Schum.). *Theoretical and Applied Genetics* 83:947–955

Simms D, Cizdziel PE and Chomczynski P (1993): TRIZOL: a new reagent for optimal single-step isolation of RNA. *Focus* 15:99- 103

Singh M, Yadav A, Ma X and Amoah E (2010): Plasmid DNA transformation in *Escherichia Coli:* effect of heat Shock temperature, duration, and cold incubation of $CaCl_2$ treated cells. *International Journal of Biotechnology and Biochemistry* 6: 561–568

Smith MAL and McCown BH (1983): A comparison of source tissue for protoplast isolation from three woody plant species. *Plant Science Letters* 28: 149-156

Somerville CR (2006): Cellulose synthesis in higher plants. *Annual Review of Cell and Developmental* Biology 22:53-78

Song GQ and Sink KC (2004): *Agrobacterium tumefaciens* mediated transformation of blueberry (*Vaccinium corymbosum* L.). *Plant Cell Reports* 23:475–484

Sonia, Saini R, Singh RP and Jaiswal PK (2007): *Agrobacterium tumefaciens* mediated transfer of *Phaseolus vulgaris* *α*-amylase inhibitor-1 gene into mungbean: *Vigna radiata* (L.) Wilczek using *bar* as selectable marker. *Plant Cell Reports* 26:187–198

Sood A, Ahuja PS, Sharma M, Sharma OP and Godbole S (2002): *In vitro* protocols and field performance of elites of an important bamboo *Dendrocalamus hamiltonii* Nees et Arn. Ex Munro. *Plant Cell Tissue and Organ Culture* 71: 55–63

Spokevicius AV, Southerton SG, Mac Millan CP, Qiu D, Gan S, ibbits JFG, Moran GF and Bossinger G (2007): β-tubulin affects cellulose microfibril orientation in plant secondary fibre cell walls. *Plant Journal* 51:717–726

Stachel SE, Messens E, Van Montagu M and Zambryski P (1985): Identification of signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. *Nature* 318: 624–629

Stachel SE, Nester EW and Zambryski P (1986): A plant cell factor induces Agrobacterium tumefaciens vir gene expression. *Proceedings of the National Academy of Sciences of USA* 83:379–383

Strasser R, Bondili J, Vavra U, Schoberer J, Svoboda B, Glossl J, Leonard R, Stadlmann J, Altmann F Steinkellner H and Mach L (2007): A unique beta1, 3-galactosyltransferase is indispensable for the biosynthesis of N-glycans containing Lewis a structures in *Arabidopsis thaliana. Plant Cell* 19:2278–2292

Subbaiah MM and Minocha SC (1990): Shoot regeneration from stem and leaf callus of *Eucalyptus tereticornis*. *Plant Cell Reports* 9: 370–373

Sugimura Y, Kadotani N, Ueda Y, Shima K, Kitajima S, Furusawa T and Ikegami M (2005): Transgenic patchouli plants produced by *Agrobacterium*-mediated transformation. *Plant Cell Tissue and Organ Culture* 82: 251–257

Sutton WRJ (1999): Does the World need planted forests? *New Zealand Journal of Forestry* 44: 24–29

Swartz HJ (1990): Post culture behaviour: genetic and epigeneticeffects and related problems. In: Debergh PC, Zimmerman RH (eds) Micropropagation: technology and application. Kluwer, Dordrecht, pp 95–12

Szyjanowicz PMJ, McKinnon I, Taylor NG, Gardiner J, Jarvis MC and Turner SR (2004): The *irregular xylem 2* mutant is an allele of *korrigan* that affects the secondary cell wall of *Arabidopsis thaliana. Plant Journal 37*: 730 – 740

Tabachnik L and Kester DE (1977): Shoot culture for almond and almond-peach hybrid clones in vitro. HortScience 12:545–547

Takahashi J, Rudsander UJ, Hedenström M, Banasiak A, Harholt J, Amelot N, Immerzeel P, Ryden P, Endo S, Ibatullin FM, Brumer H, Campillo ED, Master ER, Scheller HV, Sundberg B, Teeri TT and Mellerowicz, EJ (2009): KORRIGAN1 and its aspen homolog PttCel9A1 decrease cellulose crystallinity in *Arabidopsis* stems. *Plant and Cell Physiology* 50: 1099–1115

Tanaka M, Takamura T, Watanabe H, Endo M, Yanagi T and Okamoto K (1998): *In vitro* growth of *Cymbidium* plantlets cultured under super bright red and blue light-emitting diodes (LEDs). *Journal of Horticultural Science and Biotechnology* 73:39-44

Tang GQ and Strum A (1999): Antisense repression of sucrose synthase in carrot (*Daucus carota* L.) affects growth rather than sucrose partitioning. *Plant Molecular Biology* 41: 465–479

Taylor NG (2008): Cellulose biosynthesis and deposition in higher plants. *New Phytologist* 178: 239-252

Taylor NG, Gardiner JC, Whiteman R and Turner SR (2004): Cellulose synthesis in the *Arabidopsis* secondary cell wall. *Cellulose* 11:329-338

Tazawa M and Reinert J (1969): Extracellular and intracellular chemical environments in relation to embryogenesis *in vitro*. *Protoplasma* 68:157– 173

Teasdale RD (1995): Optimising the application of biotechnology to forest plantations, In: Potts, BM, Borralho NMG, Reid JB, Cromer RN, Tibbitts WN and Raymond CA (Eds) Eucalypt Plantations: Improving Fibre Yield and Quality. Proceedings of the Cooperative Centre for Temperate Hardwood Forestry-International Union of

Forestry Research Organisations Conference. The Cooperative Centre for Temperate Hardwood Forestry, Hobart, Tasmania, Australia pp 427–429

Teixeira da Silva JA and Fukai S (2001): The impact of carbenicillin, Cefotaxime and vancomycin on chrysanthemum and tobacco TCL morphogenesis and *Agrobacterium* growth. *Journal of Applied Horticulture* 3: 318-27

Termignoni RR, Wang PJ and Hu CY (1996): Somatic embryo induction in *Eucalyptus dunnii*. *Plant Cell Tissue and Organ Culture* 45: 129–132

Teulieres C and Marque C (2007): *Eucalyptus*. In: Pua EC, Davey MR (eds) Biotechnology in agriculture and forestry 60, Transgenic Crops V. Springer, New York, pp 387–402

Teulieres C, Marque C and Boude AM (1994): Genetic transformation of *Eucalyptus*. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, vol 29, plant protoplasts and genetic engineering V. Springer, Berlin Heidelberg New York, pp 289–307

Thomas P, Kumari S, Swarna GK, Prakash DP and Dinesh MR (2007): Ubiquitous presence of fastidious endophytic bacteria in field shoots and index-negative apparently clean shoot-tip cultures of papaya. *Plant Cell Reports* 26:1491–1499

Thomas TD (2007): High-frequency, direct bulblet induction from rhizome explants of *Curculigo orchioides* Gaertn. an endangered medicinal herb. *In Vitro Cellular and Developmental Biology –Plant* 43: 442–448

Thomason P and Kay R (2000): Eukaryotic signal transduction via histidine- aspartate phosphorelay. *Journal of Cell Science* 113: 3141–50

Thorpe TA, Harry IS and Kumar PP (1991): Application of micropropagation to forestry. In: Debergh P and Zimmerman RH (eds). Micropropagation. Dordrecht, Netherlands : Kluwer Academic Publishers, pp311- 336

Tiwari V, Tiwari KN and Singh BD (2006): Shoot bud regeneration from different explants of *Bacopa monniera* (L.) Wettst. by trimethoprim and bavistin. *Plant Cell Reports* 25: 629–635

Torney F, Trewyn BG, Lin VSY and Wang K (2007): Mesoporous silica nanoparticles deliver DNA and chemicals into plants. *Nature Nanotechnology* 2:295–300

Tournier V, Grat S, Marque C, El Kayal W, Penchel R, de Andrade G, Boudet AM and Teulieres C (2003): An efficient procedure to stably introduce genes into an economically important pulp tree (*Eucalyptus grandis* x *Eucalyptus urophylla*). *Transgenic Research* 12:403-411

Trainotti L, Spolaore S, Pavanello A, Baldan B and Casadoro G (1999): A novel E-type endo-beta-1,4-glucanase with a putative cellulose-binding domain is highly expressed in ripening strawberry fruits. *Plant Molecular Biology* 40:323-332

Tripathi SB, Mathish NV and Gurumurthi K (2006): Use of genetic markers in the management of micropropagated *Eucalyptus* germplasm. *New Forest* 31:361–372

Trivedi P and Pandey A (2007): Biological hardening of micropropagated *Picrorhiza kurrooa* Royel ex Benth. an endangered species of medical importance. *World Journal of Microbiology and Biotechnology* 23:877–878

Tucker ML and Milligan SB (1991): Sequence analysis and comparison of avocado fruit and bean abscission cellulases. *Plant Physiology* 95: 928-933

Tzfira T & Citovsky V (2006): *Agrobacterium*-mediated genetic transformation of plants: biology and biotechnology. *Current Opinion in Biotechnology* 17: 147-154

Tzfira T, Zuker A and Altman A (1998): Forest-tree biotechnology: genetic transformation and its application to future forests. *Trends in Biotechnology* 16:1-10

Updegraff DM (1969): Semimicro determination of cellulose in biological materials. *Analytical Biochemistry* 32:420–424

Urbanowicz BR, Catalá C, Irwin D, Wilson DB, Ripoll DR and Rose JKC (2007): A tomato endo- beta-1,4-glucanase, SlCel9C1, represents a distinct subclass with a new family of carbohydrate binding modules (CBM49). *Journal of Biological Chemistry* 282: 12066 – 12074

Valerio L, Carter D, Rodrigues JC, Tourier V, Gominho J, Marque C, Boudet AM, Maunders M, Pereira H and Teulieres C (2003): Down regulation of cinnamyl alcohol dehydrogenase, a lignification enzyme, in *Eucalyptus camaldulensis*. *Molecular Breeding* 12:157–167

Vasanthaiah HKN, Katam R and Sheik MB (2008): Efficient protocol for isolation of functional RNA from different grape tissue rich in polyphenols and polysaccharides for gene expression studies. *Electronic Journal of Biotechnology* 11: 3-5

Vasil V and Vasil Ik (1980): Isolation and culture of cereal protoplasts. *Theoretical and Applied Genetics* 56: 97-99

Vengadesan G and Pijut PM (2009): *In vitro* propagation of northern red oak (*Quercus rubra* L.). *In Vitro Cellular and Developmental Biology –Plant* 45:474-482

Volkov RA, Panchuk II and Schoffl F (2003): Heat-stress-dependency and developmental modulation of gene expression: the potential of house-keeping genes as internal standards in mRNA expression profiling using real-time RT-PCR. *Journal of Experimental Botany* 54:2343–2349

Weir BJ, Ganeshan S, Lai KJ, Caswell K, Rossnagel BG and Chibbar RN (1998): Transforming spring barley using the enhanced regeneration system and microprojectile bombardment. *In Vitro Cellular and Devlopmental Biology* 34:54-A Abstract P-1047

Weisburg WA, Barns SM, Pelletier DA and Lane DJ (1991): 16S rDNA amplification for phylogenetic study. *Journal of Bacteriology* 173:697–703

Welander M (1988): Plant regeneration from leaf and stem segments of shoots raised *in vitro* from mature apple trees. *Journal of Plant Physiology* 132: 738-744

Wenck AR, Quinn M, Whetten RW, Pullman G and Sederoff R (1999): High-efficiency *Agrobacterium* mediated transformation of norway spruce (*Picea abies*) and loblolly pine (*Pinus taeda*). *Plant Molecular Biology* 39: 407–416

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA and Tingey SV (1990): DNA polymorphism amplified by arbitrary primers are useful as genetic marker. *Nucleic Acids Research* 18:6531–6535

Williamson RE, Burn JE, Birch R, Baskin TI, Arioli T, Betzner AS and Cork A (2001): Morphology of *rsw1*, a cellulose-deficient mutant of *Arabidopsis thaliana*. *Protoplasma* 215: 116–127

Winter H and Huber SC (2000): Regulation of sucrose metabolism in higher plants. *Critical Reviews in Biochemistry and Molecular Biology* 35: 253-289

Winter H, Huber JL and Huber SC (1997): Membrane association of sucrose synthase: changes during the graviresponse and possible control by protein phosphorylation. *FEBS Letters* 420: 151-155

Winter H, Huber JL and Huber SC (1998): Identification of sucrose synthase as an actin-binding protein. *FEBS Letters* 430: 205-208

Wu J, Xiao Q, Xu J, Li M, Pan J and Yang M (2008): Natural products from true mangrove flora: source, chemistry and bioactivities. *Natural Product Reports* 25: 955-981

Xu F and Joshi CP (2010): Overexpression of aspen sucrose synthase gene promotes growth and development of transgenic *Arabidopsis* plants. *Advances in Biosciences and Biotechnology* 1: 426-438

Yahiaoui N, Marque C, Corbiere H and Boudet AM (1998): Comparative efficiency of different constructs for down regulation of tobacco cinnamyl alcohol dehydrogenase. *Phytochemistry* 49: 295–305

Yanchuk AD (2001): The role and implications of biotechnological tools in forestry. *Unasylva* 52: 53-61

Yang G, Zhou R, Tang T and Shi S (2008): Simple and efficient isolation of high-quality total RNA from *Hibiscus tiliaceus*, a mangrove associate and its relatives. *Preparative Biochemistry and Biotechnology* 38: 257-264

Yao JL, Cohen D, Atkinson R, Richardson K, and Morris BAM (1995): Regeneration of transgenic plants from the commercial apple cultivar Royal Gala. *Plant Cell Reports* 14:407–412

Yao JL, Wu JH, Gleave AP and Morris BAM (1996): Transformation of citrus embryogenic cells using particle bombardment and production of transgenic embryos. *Plant Science* 113:175-183

Yasmeen A (2009): An improved protocol for the regeneration and transformation of tomato (cv Rio Grande). *Acta Physiologiae Plantarum* 31:1271-1277

Yasodha R, Sumathi R and Gurumurthi K (2004): Micropropagation for quality production in plantation forestry. *Indian Journal of Biotechnology* 3:159-170

Yepes LM and Aldwinckle HS (1994): Factors that affect leaf regeneration efficiency in apple, and effect of antibiotics in morphogenesis. *Plant Cell Tissue and Organ Culture* 37:257-269

Yevtushenko DP and Misra S (2010): Efficient *Agrobacterium*-mediated transformation of commercial hybrid poplar *Populus nigra* L. *x P. maximowiczii* A. Henry. *Plant Cell Reports* 29: 211-229

Yoshida K, Imaizumi N, Kaneko S, Kawagoe Y, Tagiri A, Tanaka H, NIshitani K and Komae K (2006): Carbohydrate-binding module of a rice endo- beta-1, 4- glycanase, OsCel9A, expressed in auxin-induced lateral root primordia, is post-translationally truncated. *Plant and Cell Physiology* 47: 1555 – 1571

Yu Y and Wei ZM (2008): Influences of Cefotaxime and carbenicillin on plant regeneration from wheat mature embryos. *Biologia Plantarum* 52: 553-556

Zambre M, Terryn N, Clercq JD, De Buck S, Dillen W, Montagu MV, Van Der Straeten D and Angenon G (2003): Light strongly promotes gene transfer from *Agrobacterium tumefaciens* to plant cells. *Planta* 216:580-586

Zeng HC, Deng LH and Zhang CF (2006): Cloning of salt tolerance-related cDNAs from the mangrove plant *Sesuvium portulacastrum* L. *Journal of Integrative Plant Biology* 48: 952-957

Zhang D, Xu B, Yang X, Zhang Z and Li B (2011): The sucrose synthase gene family in *Populus*: structure, expression, and evolution *Tree Genetics and Genomes: 443*–456

Zhang XQ and Chollet R (1997): Seryl-phosphorylation of soybean nodule sucrose synthase (nodulin-100) by a Ca^{2+}-dependent protein kinase. *FEBS Letters* 410:126–130

Zheng L, Dinga Z, Zhanga M and Jincai S (2011): Micrcroencapsulation of bayberry polyphenols by ethyl cellulose: Preparation and characterization. *Journal of Food Engineering* 104: 89–95

Zhou H (2005): Isolation and functional genetic analysis of *Eucalyptus* wood formation genes. Dissertation, Department of Genetics, University of Pretoria, Pretoria, South Africa

Zietjiewicz E, Rafalski Aand Labuda D (1994): Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* 20:176–183

Zobel BJ, Van WG and Stahi P (1987): Growing exotic forests. Wiley, New York

Zuker A, Ahroni A, Tzfira T, Meir HB and Vainstein A (1999): Wounding by bombardment yields highly yields highly efficient *Agrobacterium*-mediated transformation of carnation (*Dianthus caryophyllus L.). Molecular Breeding* 5:367-375

Zuo JR, Niu QW, Nishizawa N, Wu Y, Kost B and Chua NH (2000): Korrigan, an *Arabidopsis* endo-1, 4- β -glucanase, localizes to the cell plate by polarized targeting and is essential for cytokinesis. *Plant Cell* 12:1137 – 1152

Annexure 1

```
LOCUS       kor                     1860 bp    mRNA    linear   PLN 25-
JUL-2013
DEFINITION  Populus deltoides endo- 1, 4 -beta -glucanase(Kor) mRNA,
            complete cds.
ACCESSION
VERSION
KEYWORDS    .
SOURCE      Populus deltoides
ORGANISM    Populus deltoides
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta;
            Tracheophyta;Spermatophyta; Magnoliophyta; eudicotyledons;
            core eudicotyledons;rosids; eurosids I; Malpighiales;
            Salicaceae; Saliceae; Populus.
REFERENCE   1  (bases 1 to 1860)
AUTHORS     Aggarwal,D., Kumar,A. and Reddy,M.S.
TITLE       Cloning, characterization and over expression of Populus
            Deltoides endo- 1, 4 -beta -glucanase (Korrigan) gene in an
            elite clone of Eucalyptus tereticornis
JOURNAL     Unpublished
REFERENCE   2  (bases 1 to 1860)
AUTHORS     Aggarwal,D., Kumar,A. and Reddy,M.S.
TITLE       Direct Submission
JOURNAL     Submitted (25-JUL-2013) Deapartment of Biotechnology and
            Enviornmental Sciences, Thapar University, Bhadson Road,
Patiala,
            Punjab 147004, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES    Location/Qualifiers
source      1..1860
            /organism="Populus deltoides"
            /mol_type="mRNA"
CDS         1..1860
            /note="endo- 1, 4 -beta -glucanase"
            /codon_start=1
            /product="kor"

/translation="MYGRDPWGGPLEINAADSATDDDRSRNLNDLDRAALSRPLDETQ
QSWLLGPAEQKKKKKYVDLGCVIVSRKIFVWTVGSIVAAGLLVGLITLIVKTVPRHHHSHAPADNYTLALH
KALMFFNAQRSGKLPKHNNVSWRGSSCLGDGKGKQGSFYKDLVGGYYDAGDAIKFHFPASFSMTMLSWSVI
EYSAKYEASGELNHVKELIKWGADYFLKTFNSSADTIDRIVAQVGSGDTSGGSTTQNDHYCWMRPEDIDYD
RPVTECSSCSDLAAEMAAALASASIVFKDNKAYSQKLVHGAKTLFKFARDQRGRYSASGSEAAIFYNSTSY
WDEFIWGGAWLYYATGNNSYLQLATMPGLAKHAGAFWGGPDYGVLSWDNKLAGAQLLLSRLRLFLSPGYPY
EEILSTFHNQTSIIMCSYLPIFTKFNRTKGGLIELNHGRPQPLQYVVNAAFLATLFSGYLEAADTPGWYCG
PNFYSTDVLRDFAKTQIDYILGKNPRKMSYIVGFGNHYPKHLHHRGASIPKNKIRYNCKGGWKWRDTSKPN
PNTLVGAMVAGPDRHDGFHDVRTNYNYTEPTIAGNAGLVAALVALSGDKTTGIDKNTIFSAVPPMFPTPPP
PPAPWKP"
BASE COUNT      507 a    392 c    447 g    514 t
ORIGIN
  1 atgtacggaa gagatccatg gggaggaccc ctggagataa atgcagcaga ttcagcaaca
 61 gatgatgaca ggagcaggaa cttgaatgac ttagacaggg cagctctgtc aaggccattg
 121 gatgagactc agcagagctg gttgctgggt ccagccgagc aaaagaagaa gaagaaatat
181 gtggatctgg gctgtgtcat tgttagtcgc aagatctttg tgtggactgt tgggagtatc
241 gttgctgctg gtctattggt tggtcttatt actcttattg ttaaaactgt gcctcgccat
301 catcactctc atgctccagc tgataactac actcttgctc tacataaggc actcatgttc
```

```
361 ttcaatgctc aaagatcggg aaagcttccg aagcataaca acgtgtcatg gaggggaagc
421 tcgtgtttgg gcgatgggaa aggtaaacaa ggtagttttt acaaagatct ggtgggtgga
481 tattatgatg ctggggatgc aataaagttc cacttccctg cttctttttc catgacaatg
541 ttgagctgga gtgttattga atatagtgcg aaatatgaag cttctggtga gcttaaccat
601 gtcaaagaac ttataaagtg gggagctgac tactttctga agacatttaa tagttctgct
661 gataccatcg acaggatagt tgcacaggtt ggctcggggg atacttctgg tgggagtacc
721 actcaaaacg atcattattg ctggatgcgt cctgaggaca ttgattacga cagacctgta
781 actgaatgca gctcctgctc tgatcttgct gcagaaatgg ctgctgcctt agcttctgca
841 tctatcgttt tcaaagataa caaggcctac tctcagaagc ttgttcatgg tgccaaaact
901 ctctttaaat ttgcaaggga ccagcgaggt agatacagtg caagtggttc ggaagccgcc
961 attttctata attcaactag ttactgggat gaatttattt ggggtggagc ttggctatat
1021 tatgcaactg gaaataattc ctatcttcag cttgctacaa tgcctggctt agccaagcat
1081 gccggtgcat tttggggagg gccagactat ggtgtgctta gctgggacaa caagcttgct
1141 ggtgctcagc tacttctgag ccgcctaaga ttgttcttga gccctggtta tccatatgaa
1201 gaaatactga gtacatttca caaccagacc agtataatca tgtgctcata tctgccaatt
1261 ttcacaaagt ttaatagaac aaaaggaggc ttgatcgagt taaaccatgg aaggcctcag
1321 ccccttcagt atgtcgtcaa cgctgccttc ctagcaaccc tatttagtgg ttatcttgaa
1381 gctgctgata cacctggatg gtattgtgga ccaaatttct actctactga tgtgttgcgt
1441 gatttcgcca agactcaaat tgattacatc ctgggcaaaa atcctcgtaa aatgagctat
1501 attgtgggtt ttggtaatca ttacccaaaa catctccacc atagaggtgc atctatccct
1561 aagaacaaga tcaggtataa ctgtaaagga ggatggaaat ggagggacac ctcgaagcca
1621 aacccaaata cacttgttgg agccatggtt gcgggccctg accggcatga tggtttccat
1681 gatgttcgca ccaactataa ttatacagag ccaacgattg ctggaaatgc aggattagtt
1741 gcagcacttg tggccttgtc tggtgacaaa actactggga ttgacaagaa cacaatattc
1801 tctgcagttc ccccaatgtt tccaactcct ccaccaccac cagcaccttg gaaaccatga
//
```

```
LOCUS       sus                     2435 bp    mRNA    linear       26-
JUL-2013
DEFINITION  Populus deltoides sucrose synthase mRNA, complete cds.
ACCESSION
VERSION
KEYWORDS    .
SOURCE      Populus deltoides
ORGANISM    Populus deltoides
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta;
            Tracheophyta;Spermatophyta; Magnoliophyta; eudicotyledons;
            core eudicotyledons;rosids; fabids; Malpighiales;
            Salicaceae; Saliceae; Populus.
REFERENCE   1  (bases 1 to 2435)
AUTHORS     Aggarwal,D., Kumar,A. and Reddy,M.S.
TITLE       Cloning and characterization of sucrose synthase gene from
            Populus deltoides
JOURNAL     Unpublished
REFERENCE   2  (bases 1 to 2435)
AUTHORS     Aggarwal,D., Kumar,A. and Reddy,M.S.
TITLE       Direct Submission
JOURNAL     Submitted (26-JUL-2013) Department of Biotechnology &
            Environmental Sciences, Thapar University, Bhadson Road,
            Patiala, Punjab 147004, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES    Location/Qualifiers
source      1..2435
            /organism="Populus deltoides"
            /mol_type="mRNA"
CDS         1..2435
            /codon_start=3
            /product="sucrose synthase"

/translation="GSMTERALTRVHSIRERVDETLKAHRNEIVALLTRIEGKGKGIL
QHHQIVAEFEAIPEDTRKTLAGGAFAEVLRSTQEAIVVPPWIALALRPRPGVWEYIRL
NVQALVVEELRVAEYLHFKEELVDGGSNGNFVLELDFEPFNASLPRPTLSKYIGNGVE
FLNRHLSAKLFHDKESLHPLLAFLKVHCHKGKNMMLNDRIRNLDSLQYVLRKAEEFLS
TLEPDTPYSQFEHKFQEIGLERGWGDTAERVLETIRLLLDLLEAPDPCTLETFLGRIP
MVFNVVIMSPHGYFAQDNVLGYPDTGGQVVYILDQVRALENEMLQRIKKQGLDITSRI
LIITRLLPDAVGTTCGQRLEKVYGSEHCDILRVPFRDGKGMVRKWISRFEVWPYLETF
AEDVAAEIAKELQGKPDLIIGNYSDGNIVASLLAHKLGVTECTIAHALEKTKYPDSDI
YWKKFDEKYHFSCQFTADLFAMNHTDFIITSTFQEIAGSKDTVGQYESHTAFTLPGLY
RVVHGIDVFDPKLNIVSPGADESIYFPYTDEKRRLTSFHPEIDELLYSPVENEEHLCV
LKDRNKPILFTMARLDRVKNLSGLVEWYGKNTKLRELVNLVVVGGDRRKQSKDLEEQA
EMKKMYSHIEKYNLNGQFRWISSQMNRVRNGELYRYICDTKGAFVQPALYEAFGLTVV
EAMTCGLPTFATCNGGPAEIIVNGKSGFHIDPYHGEQAAELLVDFFEKCKVDPAHWDK
ISHGGLQRIQEKYTWQIYSQRLLTLTGVYGFWKHVSNLDRLESRRYMEMFYALKYRKL
AESVPLTKE*"

BASE COUNT      648 a    514 c    580 g    693 t

ORIGIN
  1 cgggatccatgactgaacgtgctcttactcgtgtccatagcattcgtgaacgtgtagatg
 61 agactctcaaggctcatcgcaatgaaattgttgcgttactcacaaggatcgaaggtaagg
121 gaaaaggaattctccaacaccaccagattgttgctgagtttgaagcaattcctgaagaca
181 ccaggaaaacattggcaggcggtgcttttgctgaagttctcagatccacacaggaagcaa
241 ttgttgtgcctccatggattgctcttgctctgcgcccgaggcctggtgtctgggagtaca
301 ttagactgaatgtccaagcacttgttgttgaggagctgcgtgttgctgagtatcttcatt
361 tcaaggaggagcttgttgatggaggctcgaacggcaactttgtgcttgaattggacttcg
```

```
421 aaccattcaatgcatctctccctcgcccaactctttcaaagtatattggtaatggtgttg
481 agttccttaatcgccacctttcggctaagttgttccatgacaaggaaagcctgcatcccc
541 tgcttgcatttctcaaagtgcactgtcacaaggggaagaatatgatgctgaatgacagaa
601 ttcgtaacctagactctctgcaatatgttctgagaaaggctgaggagtttctgtctactc
661 tggaacctgatactccgtattctcaatttgaacacaagttccaggagattggcttggaga
721 gaggatggggtgatactgccgagcgtgttcttgagacgattcgacttcttttggatcttc
781 ttgaggcaccagatccctgcactctcgaaacattcctaggcagaattcctatggtcttca
841 atgttgtgattatgtcccctcatggatactttgcccaagacaatgttttggggtatcctg
901 atactggaggccaggttgtttacattttggatcaagtccgtgccttggagaatgaaatgc
961  ttcagcgtatcaagaagcaaggacttgatattacctcccgaattctcattattactcgac
1021 tactccctgatgcagtaggaaccacttgtggtcaacgtttggagaaagtgtatggatctg
1081 agcattgtgatattcttcgagttcccttcagagatggaaagggtatggtccgcaaatgg
1141 tctctcgctttgaagtgtggccatacctagaaactttcgccgaggacgttgctgctgaaa
1201 ttgctaaggagttgcagggcaagcctgatcttatcattggaaattacagtgatggaaaca
1261 tcgttgcctccttgttagcacacaaattaggtgttacagagtgcaccattgcgcatgctc
1321 tagaaaaaacaaagtatccagactcagatatatactggaagaagtttgatgaaaagtacc
1381 acttctcatgccagtttacagctgatctttttgcaatgaaccatacagatttcattatca
1441 ccagcacattccaagagattgctggaagcaaggatactgttggacagtatgaaagccaca
1501 ctgctttcactctccctggcctctatagagttgttcatggtattgatgtctttgatccca
1561 aactcaacattgtatcccctggcgccgatgagagcatatacttcccctacactgacgaga
1621 aacgtaggttgacttctttccatccagaaattgacgagcttctttacagccctgttgaga
1681 atgaagagcacttatgtgttctaaaagaccgaaacaaaccaattctatttaccatggcaa
1741 ggctggacagagttaagaatttatctggtcttgtagaatggtatggaaagaacaccaagc
1801 tacgtgaattagttaatcttgttgtagttggtggagatagaagaaagcagtctaaagatc
1861 tagaagagcaagctgagatgaagaaaatgtacagtcatatagagaaatacaacttgaatg
1921 gccagttcagatggatttcttcccagatgaaccgtgtgaggaatggagagctttaccgtt
1981 acatttgtgataccaagggagctttcgtgcagcctgctttgtatgaggcttttggattga
2041 cagttgttgaggccatgacttgtggtttgccaacttttgctacctgcaatggtggtcctg
2101 ctgagatcattgttaatggaaaatccggattccacatcgatccttatcatggtgagcagg
2161 ctgctgagctccttgttgacttctttgagaagtgcaaggttgatcccgctcactgggaca
2221 aaatctcccacggaggtctgcaacgaatccaagagaagtatacctggcaaatttactctc
2281 aacggctcctgacgctcacaggagtttatggattctggaagcacgtttcgaaccttgatc
2341 gtcttgagagccgtcggtatatggaaatgttctatgcactcaaataccgcaaattggctg
2401 agtctgttcctttgaccaaggagtaacccggggga
  //
```