Agrobacterium mediated molecular breeding in *Hevea brasiliensis* for crop improvement

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By

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Dedicated to my beloved Mother

DECLARATION

I hereby declare that the thesis entitled **"*Agrobacterium* mediated molecular breeding in *Hevea brasiliensis* for crop improvement"** is an authentic record of original research carried out by me under the supervision and guidance of Dr. P. A. Nazeem, Professor and Co-ordinator, Centre for Plant Molicular Biology and Biotechnology, College of Horticulture, Vellanikkara, Thrissur and Dr. P. Venkatachalam, Associate Professor, Periyar University, Salem,Tamil Nadu in partial fulfilment of the requirement for the degree of DOCTOR OF PHILOSOPHY of the Mahatma Gandhi University and no part of this work has been presented for any degree, diploma or any other similar titles of any university.

RRII **Rekha K.**

July 2013

CERTIFICATE

This is to certify that the thesis entitled "***Agrobacterium* mediated molecular breeding in *Hevea brasiliensis* for crop improvement**" is an authentic record of original research work carried out by Smt Rekha.K at The Rubber Research Institute of India, Kottayam-9, under our joint supervision and guidance for the award of the Degree of **Doctor of Philosophy in Biotechnology**, under the Faculty of Science, Mahatma Gandhi University, Kottayam. It is also certified that the work presented in the thesis has not been submitted earlier for any other degree, diploma or any other similar titles of any university.

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ABSTRACT

Hevea brasiliensis Muell. Arg, (Para rubber tree) is a perennial out-breeding species and is the major source of commercial natural rubber (cis-1, 4-polyisoprene). Global projected gap between the demand and supply of natural rubber necessitates production enhancement. However, adverse environmental conditions such as drought, high and low temperatures, high solar radiation, low atmospheric humidity, poor soils, etc. limit the expansion of cultivation in several rubber producing countries including India. Crop improvement through conventional breeding in *H. brasiliensis* has limitations due to the narrow genetic base of cultivated clones, long gestation period (6-7 years), high heterozygosity, brief and seasonal nature of flowering, poor seed set etc.

In this context, *Agrobacterium* mediated transformation of *Hevea* was attempted with the gene coding for osmotin protein in order to impart abiotic and biotic stress tolerance. *Agrobacterium* strain *GV 2260* harboring the plasmid osm/BinAR with osmotin gene under the control of CaMV35S promoter containing kanamycin resistance (*nptII*) as the selectable marker was employed for genetic transformation. Seven different target tissues, and two different protocols were tried to bring about genetic transformation. Experiments were carried out at various stages of plant regeneration by changing the growth regulator combinations.

In the present study, successful integration of osmotin gene into *Hevea* callus followed by regeneration of transgenic plants has been achieved. High frequency transformation was achieved with clonal (48%) as well as zygotic target tissues (76 %). A callus proliferation frequency of 60 per cent could be obtained with a growth regulator combination of 1.5 mg l^{-1}

2, 4- D and 1 mg l^{-1} NAA in modified MS medium. The highest embryo induction frequency was 67 per cent in the presence of growth regulators ABA (0.1mg l^{-1}, kinetin (0.3 mg l^{-1}), NAA (2 mg l^{-1}), GA_3 (0.3 mg l^{-1}) and mannitol. Embryo maturation (63%) was achieved with a combination of 75 g l^{-1} sucrose and 4 g l^{-1} phytagel in presence of 0.8 mg l^{-1} ABA. A growth regulator combination of 1.5 mg l^{1-} BA and 1.5 mg l^{-1} GA_3 favoured embryo germination (27%), in presence of 5 g l^{-1} mannitol. Plant conversion could be achieved with a frequency of 23 per cent in a combination of 3 mg l^{-1} BA and 1.5 mg l^{-1} IBA. Transgenic plants of *Hevea* were developed and hardened. Transgene integration and expression have been confirmed by molecular analysis. Evaluation of stress tolerance on transgenic callus showed positive indications towards drought and salinity tolerance. The *In silico* analysis confirmed the uniqueness of the transgene over the native osmotin gene. These osmotin transgenic plants are expected to perform well under stressful environments.

PREFACE

Hevea brasiliensis, the prime source of natural rubber accounts for 99% of global production .The demand for natural rubber continues to rise inspite of the development of synthetic substitutes. In order to meet the ever increasing demand for natural rubber, its production as well as productivity needs to be enhanced through crop improvement. Rubber, being a perennial tree crop with narrow genetic base, low fruit set, long gestation period, heterozygous nature and absence of fully reliable early selection parameters, progress of conventional breeding is limited to a greater extent. In this context, plant genetic transformation offers a viable alternative for crop improvement.

Thirty years ago, the results of first experiments describing the successful transfer and expression of foreign genes in plant cells were published. Since then, transgenic technology has become an essential tool for studying the plant biology and for the development of novel plant varieties that have been cultivated extensively in some regions of the world. Transgenic technology has had a profound impact on the development of plant biology in the past 25 years by providing the means to produce gene – tagged populations, cell markers to study the plant development and the technology to study the gene function. The production of transgenic plant involves the marriage of two critical yet distinct basic technologies. The first directs the introduction of new genetic material into plant cells (transformation), whereas the second uses methods based in tissue culture to regenerate the resulting transformed cells into transgenic plants. Of the various methods developed to introduce DNA into plant cells, transformation t mediated by *Agrobacterium tumefaciens* is more prevalent.

In the present study, *Agrobacterium* mediated transformation of *Hevea* was attempted with a gene coding for osmotin protein with a view to impart abiotic and biotic stress tolerance. The scope of the study, review of literature, methodologies used, results obtained and its implications are described in detail in six different chapters of the thesis.

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ABBREVIATIONS

ABA	Abscisic acid
BA	Benzyl adenine
B5	Gamborg B5
CTAB	Hexadecyl trimethyl ammonium bromide
2,4-D	2,4-Dichlorophenoxy acetic acid
DEPC	Diethyl pyrocarbonate
dNTPs	Deoxy nucleotide triphosphates
EDTA	Ethylene diamine tetra acetic acid
EtBr	Ethidium bromide
GA_3	Gibberellic acid
MnSOD	Manganese Superoxide Dismutase
HAS	Human Serum Albumin
IAA	Indole-3-acetic acid
IBA	Indol-3-butyric acid
Kb	Kilobase
LB	Luria-Bertani broth
MS	Murashige and Skoog
NAA	Naphthalene acetic acid
NCBI	National Centre for Biotechnological Information

PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PVPP	Poly-vinyl-polypyrrolidone
RRII	Rubber Research Institute of India
NR	Natural rubber
TPD	Tapping Panel Dryness
T-DNA	Transfer DNA
CaMV	Cauliflower Mosaic Virus
npt II	Neomycin phospho transferase II
rpm	Revolutions per minute
g	Gram
mg	Milligram
µ g	Microgram
M	meter
nm	nanomètre
l	litre
µ l	Microlitre
ml	milli litre
mM	milli molar
µM	micro molar
w/v	weight/volume

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Hevea brasiliensis Muell. Arg, (Para rubber tree) popularly known as the 'weeping tree' or 'rubber tree' is a perennial out breeding species and is the major source of commercial natural rubber (cis-1, 4-polyisoprene). Natural Rubber (NR), produced in specialized cells called laticifers is one of the most important biological molecules used for the manufacture of about 40,000 products indispensible for the economic and commercial development of a nation. Among the various end uses of NR, the transport sector, which is considered as the engine of economic development, enjoys dominance. The higher strength, low heat buildup, better resistance to wear, and flex cracking make natural rubber a suitable raw material for the manufacture of heavy duty automobile tyres. There is always a gap between the demand and supply of NR which necessitates a production enhancement to meet the demand. *H. brasiliensis* has a gestation period of 6 to 7 years and an economic life span of about 25 years. Crop improvement through conventional methods is laborious and time consuming due to these reasons.

Investment in rubber plantation has long term implications and the high initial development cost further signifies its gravity. In India, rubber is predominantly a small growers' crop. Eighty six percent of the total area under rubber is occupied by the small growers who in turn contribute 86 per cent of the total production. The predominance of resource-poor small holders in the industry makes the investment irreversible. Once the planting is done, the growers lack the flexibility to adopt newly developed clones as they have to wait until the existing tree reaches the replanting stage, which normally takes 30 years. Owing to these rigidities, the technology adopted in the selection of planting materials has long-term implications on the yield profile of the crop as well as the income stream of growers.

The ever increasing demand for natural rubber can be met either by increasing the area under cultivation or by increasing the productivity. The decline in NR production occurs mainly due to unfavorable weather conditions. Adverse environmental conditions such as drought, high and low temperature, high solar radiation, low atmospheric humidity, poor soils, etc., limit the expansion of cultivation to newer areas in several rubber producing countries including India. Stressful environment is a limiting factor in rubber productivity in the traditional rubber growing areas too. Climate change and global warming are generating rapid changes in the atmosphere that are unprecedented. Atmospheric CO_2 concentrations also increased significantly in the past two centuries. Unpredicted climatic conditions such as prolonged drought and heat episodes affect plant growth and yield, causing annual loss estimated at billions of dollars. Hence, importance of genetic improvement in a perennial tree crop like rubber facing the vagaries of environment needs no further emphasis.

The identification and fixation of a particular gene through conventional methods of breeding requires several generations of crosses and field trials carried out over many years. Recent developments in recombinant DNA technology and *in vitro* plant regeneration techniques have opened up new avenues for fast development of new varieties with desirable characters. Gene or genes for a particular character can be isolated from any organism and could be introduced into any crop species by genetic transformation.

Recombinant DNA technology has become a powerful tool for crop improvement since it provides a means of genetic manipulation that can bypass sexual barriers and to some extent circumvent the limitations of the long breeding cycle of perennial trees. Over the past decades, the value of

introducing alien gene into plants has been well documented. This technology could minimize the difficulties associated with traditional breeding methods and reduce the time necessary to produce genetic changes in woody species like *Hevea.* The ability to obtain the expression of specific foreign or native genes in *Hevea* opens up the possibility of improving *Hevea* commercially by genetic manipulation. A wide range of traits can be introduced without compromising the genetic background of the elite clones, provided a reliable method of plant regeneration from the transformed cell is available.

For the introduction of a transgene into a cell, several methods have been developed. Among them, *Agrobacterium* mediated gene transfer is the most widely used and powerful technique for the production of transgenic plants in the recent past. In nature, *Agrobacterium tumefaciens* is the causative agent of crown gall disease and was discovered at the turn of the last century. The ability of *Agrobacterium* to genetically transform a wide variety of plant species, contributed much in modern biotechnology. Transformation results from the production of a single stranded copy of T-DNA molecule by the bacterial virulence machinery, its transfer into the host cells followed by the integration into the host genome.

In *H. brasiliensis,* plant regeneration *via* somatic embryogenesis using a variety of explants like integument, immature anther, immature inflorescence and leaf explants are well standardized. Many genes controlling important agronomic traits and tissue-specific promoters have also been characterized in rubber. *Agrobacterium* and biolistic-mediated genetic transformation systems have been successfully carried out in this crop. Since the major harvested products such as latex, wood and rubber seed

oil are not used as food material, the biosafety concerns are less for the genetically modified rubber plants. Therefore, genetic engineering could play an important role in the future of rubber industry.

Osmotin is a stress responsive multifunctional protein belonging to PR-5 protein family, providing osmotolerance to plants (Amjad and Malik, 2008). It is a basic 24 kDa protein that accumulates in sodium chloride (NaCl) and desiccation adapted tobacco cells. The gene has been cloned and the expression has been studied extensively. The gene expression pattern indicates that its transcription can be activated by several factors like sodium chloride, desiccation, ethylene, wounding, abscisic acid, tobacco mosaic virus, fungi and UV light (Liu *et al.,* 1994). Using *in vitro* assay, it has been demonstrated that osmotin has antifungal activity against a variety of fungi, including *Phytophthora candida, Neurospora crassa* etc. Osmotin could be involved in osmotic adjustments by the cells, either by facilitating the accumulation or compartmentalization of solutes by involving in the metabolic or structural alterations during osmotic adjustments. Transgenic plants integrated with osmotin gene have been produced in different crops like tobacco (LaRosa *et al.*,1992 ; Barthakur *et al.*, 2001), potato (Liu *et al.*, 1994, Li *et al.,* 1999), tea (Bhattacharya, 2006 a & b), olive (Angeli and Altamura, 2007), wheat (Noori and Sokhansanj, 2008), strawberry (Amjad and Malik, 2008), cotton (Parkhi *et al.*, 2009),tomato (Goel *et al.,* 2010), chilli pepper (Subramanyam *et al.*, 2011) and soybean (Subramanyam *et al.,* 2012) and imparted biotic / abiotic stress tolerance.

In this context, *Agrobacterium* mediated genetic manipulation was attempted in *Hevea brasiliensis*, using the gene coding for osmotin protein isolated from tobacco, with the following objectives

Objectives:

- Integration of tobacco osmotin gene into *Hevea* tissue
- Optimisation of somatic embryogenesis for developing transgenics
- Development of transgenic plants and their validation through molecular tools

CHAPTER 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 History and origin

Hevea brasiliensis (Willd. ex Adr. Juss.) Muell.-Arg is a native of Amazon river basin of South America. It is one of the recently domesticated crops in the world. The successful transfer and the subsequent establishment of commercial rubber plantations to Asia were in response to the growing demand for this strategic raw material. *H. brasiliensis* was introduced to Tropical Asia in 1876 through Kew Gardens mainly from the seeds brought from the upper Amazon region of Brazil by Sir Henry Wickam (Dijkman, 1951). Kew Gardens, UK played a special role in the domestication of wild plants. It was in the Kew Gardens, the planting materials were assembled from the native land, propagated and then distributed to other botanical gardens around the world (Baulkwil, 1989). *H. brasiliensis* is now commercially cultivated in the tropical regions of Asia, Africa, and South America in countries like Indonesia, Thailand, Malaysia, India, China, Sri Lanka, Philippines, Vietnam, Nigeria, Cameroon, Ivory Coast, Liberia, Brazil and Mexico. However, the major share of rubber comes from Tropical Asia. The genus *Hevea,* which comprises of 10 species, occurs in the wild, covering the whole of the Amazon basin and extending to parts of Brazil, Bolivia, Peru, Colombia, Ecuador, Venezuela, French Guiana, Surinam and Guyana (Webster and Paardekooper, 1989). According to them, *Hevea brasiliensis* occurs naturally over about half the range of the genus. The seeds which formed the foundation of the rubber industry of the East were collected by Wickham in a very small area at the confluence of the river Tapajos. All the eastern clones of *H.brasiliensis* originated from this relatively very narrow genetic base, refered to as Wickham base (Simmonds, 1989).

Fig. 2.1 Rubber Plantation

2.2 Taxonomy

The Para rubber tree (*Hevea brasiliensis* (Willd. ex Adr. Juss) Mueller-Argoviensis), named after Para State, Brazil from where the first natural rubber was produced belongs to the Euphorbiaceae family.

2.3 Cytology

The chromosome counts made by various investigators showed variations and were reported as 2n=16, 34, and 36. However, detailed cytological investigations have confirmed the chromosome complement of rubber tree in the somatic cells as $2n = 2x = 36$ (Ramaer, 1935; Saraswathyamma *et al.*, 1984). The chromosomes are small and vary in length and the total chromosome length of the species is 89.7μm. Meiotic division is regular and pollen fertility is over 80% (Saraswathyamma, 1997). Critical analysis of karyomorphology revealed significant differences between clones with reference to centromeric position and total chromosome length (Sankariammal and Saraswathyamma, 1995). There are no chromosomal or genetic barriers between the 10 *Hevea* species. Triploid plants with $2n = 3x = 54$ (Nazeer and Saraswathyamma, 1987) and induced tetraploids with $2n = 4x = 72$ (Saraswathyamma *et al.*, 1990a) were also reported. Wide range of meiotic abnormalities was noticed in the triploids and tetraploids (Saraswathyamma, 1997). The cytophotometric determination of DNA content of various cytotypes revealed 44.2 pg (picogram) in the diploids, 62.4 pg in the triploid, and 89.37 pg in the tetraploids (Saraswathyamma *et al.*, 1990b).

2.4. Economic importance

H.brasiliensis is the most important industrial crop for Natural Rubber (NR) production (Fig.2.1). It is a renewable, sustainable, non-polluting and environment friendly source of natural rubber. The global area under rubber cultivation is about 9.6 million hectares producing 11.6 million tons annually valuing about US$18 billion as raw material alone (IRSG, 2012). Chemically, natural rubber is *cis*-1, 4-polyisoprene, having molecular weight of 200,000 to 8,000,000 and with viscous elastic properties. The flexibility of natural rubber to undergo vulcanization with sulphur under high temperatures is an important attribute. A major quantity of natural rubber produced is consumed by the automobile tyre industry. Natural rubber is a good insulator and can be easily manipulated. Being water resistant, it finds use in the manufacture of water proofing materials as well. More than 35,000 rubber-based products such as hand gloves, toys, balloons, hoses, footwear, etc. are manufactured from this raw material. Besides, rubber is also useful in soil stabilization, vibration absorption, road surfacing, etc (Thulaseedharan *et al.*, 2007).

The world supply of NR is barely keeping up with an expected global demand for 12 million tons by 2020. Rubber content of raw *Hevea* latex accounts for 30-40 per cent of the dry weight of latex. Most of the latex is processed at the site of production by coagulating the colloidal rubber with acetic or formic acid, and converted into sheets, crepe, blocks or granules which are then manufactured into rubber goods such as tyres, shoe soles, tubing and so on. This process eliminates most of the cytoplasm proteins before manufacture. About 10 per cent of *H. brasiliensis* latex is kept in the colloidal form, with ammonia added as an anticoagulant/ preservative and the rubber particles concentrated by centrifugation to around 60 per cent dry

rubber content. The latex concentrate thus contains rubber particles suspended in ammoniated cytoplasm and is kept in this form for at least 3-4 weeks before manufacture into gloves, catheters, elastic bands, foam or other latex goods. Apart from the latex, there are some important by products of economic importance such as rubber seed oil, rubber honey, and rubber wood. The estimated seed production potential in India is about 150 kg/ha. The production of rubber seed oil and cake is more than 3000 t and 5000 t respectively. Organized commercial exploitation of honey from rubber plantations in India is under the auspices of Khadi and Village Industries Commission. The mature rubber plantations have the potential to produce about 70000 t/year of rubber honey. However, the extent of exploitation is much less. The emergence of rubber wood as the alternative for eco-friendly wood is noteworthy. The development of processing technologies like standardization of preservative treatment and drying procedures had contributed to this. Rubber wood is now extensively used in furniture manufacture, structural applications and interior decoration (George *et al.*, 1997).

2.5 Botany

Rubber tree (*H. brasiliensis*) is a sturdy, fast growing one, which attains a height of 25–30 m. The tree is deciduous with an annual leaf fall (wintering). Refoliation and flowering follow wintering. Rubber tree is monoecious with diclinous flowers arranged in a pyramid-shaped panicle. The flowers are short stalked and fragrant. The flower has one whorl of bell-shaped yellow colored perianth with five lobes. Male flowers are smaller in size but more in number than the female flowers. In the male flower, there are 10 sessile anthers arranged on a slender staminal column in two whorls of five each. Each anther contains two pollen sacs that split

longitudinally on dehiscence. Pollen grains are trifoliate, smooth, and sticky. Female flowers are seen at the tip of the panicle and its branchlets. When fully developed, they are recognizable by their relatively bigger size and the green basal disc, torus. The gynoecium is tricarpellary and syncarpous with an ovule in each locule. The stigma is short styled and tri lobed. Pollination is mediated by insects. Sticky pollen and stigmatic surfaces indicate the typical entomophilous nature of the flower. After fertilization, the ovary develops into a three-lobed dehiscent capsule, regma, with three large mottled seeds. Fruits ripen 5–6 months after fertilization (Mydin and Saraswathyamma, 2005).

2.6 Breeding

As in any other perennial tree crop, introduction, selection and hybridization are the conventional methods adopted in *Hevea* for crop improvement. So far, 127 clones have been introduced to India from different countries and this serves as a source of valuable genes for breeding programmes. From these introductions, selected clones have been used as parents in hybridization programmes, which have resulted in the evolution of some very successful cultivars like RRII 105. Ortet selection / mother tree selection or plus tree selection is the oldest selection method adopted in *H. brasiliensis*. Primary clones were developed through ortet selection where ortet is the original tree from which members of clones have descended. Ortet selection is based on natural genetic recombination. Seedling plantations having a good average performance is selected and systematically screened for individual trees with high yield or secondary characters. Selected trees are cloned and the clones are evaluated in small scale, large scale and block trials and the promising ones are released for commercial cultivation. In Indonesia, ortet selection resulted in classical

primary clones like Tjir 1, PR107, and GT 1. Gl 1 is one of the earlier ortet selections from Malaysia. In India, the plus tree selection led to the evolution of 46 primary clones designated as RRII series (Marattukalam *et al.,* 1980; Mydin and Saraswathyamma, 2005).

Hybridization programmes are aimed at combining genes controlling desirable characters from different clones. The process involves artificial pollination between selected parents, evaluation of F_1 hybrids, selection of promising recombinants and multiplication by bud grafting (Varghese and Mydin, 2000). Major objective of rubber tree breeding is to develop potential clones with high rubber yield combined with secondary characters such as high initial vigour, smooth and thick bark with good latex vessel system, good bark renewal, good growth after initiation of latex harvest and tolerance to major diseases and wind (Annamma *et al.*, 1990; Varghese *et al.*, 1992). The early hybrid clones developed by the Rubber Research Institute of India (RRII) include RRII 100, 200 and 300 series. RRII 105 is the most popular clone cultivated in the traditional rubber growing tract of India. In India, the clone RRII 105 was developed through hybridization of Tjir 1 and Gl 1, both having several desirable characters, and released for commercial cultivation by Rubber Research Institute of India in 1980. Few other clones of the RRII 400 series obtained by hybridization between RRII 105 and Srilankan clone RRIC 100 have been released since 2005. Five clones of this series viz, RRII 414, RRII 417, RRII 422, RRII 429 and RRII 430 exhibited significant yield improvement in various evaluation trials (Licy, *et al.,* 2003, Meenakumari *et al.*, 2010; Mydin *et al.,* 2011) over the female parent and ruling check variety RRII 105. Crop improvement activities are being continued further (Chandrasekhar and Gireesh, 2009; Gireesh and Pravitha, 2010) using RRII

400 series clones with selected Wickham clones as parents aiming at improving the yield and secondary traits of present varieties.

Crop improvement through conventional breeding in *H. brasiliensis* has limitations due to narrow genetic base of the cultivated clones, long breeding cycle, long juvenile period, highly heterozygous nature of the seed propagated plants, poor seed set etc. The brief and periodic nature of flowering impedes the progress of pollination programme. Very often, it becomes impossible to attempt sufficient number of artificial pollinations to obtain families large enough to conduct effective seedling selection in the nursery. In hybridization programmes, the low fruit set results in loss of numerous potentially good cross combinations, thus limiting the progress of genetic improvement. Low fruit set also reduces the size of legitimate families on which selection is to be applied for the evolution of new clones with the desired attributes. The inaccessibility of flowers also makes the hand pollination programme labour intensive and limits the numbers of crosses that can be performed in a season. Efforts to overcome the problem have been in vain and fruit set could not be raised to more than 5% (Mydin *et al.,* 1989). Hence, it is necessary to conduct intensified hand pollination programme bringing about heavy input involvement. Gene transfer *via* biotechnological approaches may solve these problems to a larger extent.

2.7 *Hevea* tissue culture

In *Hevea*, dependence on bud grafting has become inevitable for the propagation of selected clones and maintenance of clonal integrity. Since the seedlings are genetically divergent, it is often implicated as the source of large tree to tree variation in growth and yield of bud grafted *Hevea* trees. Isozyme polymorphism between different trees of the same clone due to the

variable rootstocks was also reported. Consequently, cloning the rootstock is a major challenge in *Hevea* breeding. Tissue culture techniques may be a viable option in this context.

2.7.1 Micropropagation

Plant tissue culture is based on the cell doctrine which states that a cell is capable of autonomy and is potentially totipotent. In 1902, the German botanist Gottlieb Haberlandt developed the concept of *in vitro* cell culture. He isolated single cells from palisade tissue of leaves, pith parenchyma, epidermis of various plants and cultured on Knop's salt solution. Following this, many workers such as Gautheret (1939) cultured cambial tissue of carrot root, for prolonged periods of time. The first known work on *in vitro* culture of *H. brasiliensis* was carried out by Bouychou (1953) of the Institute Francais Cautchouc, with the aim of using calli to obtain convenient material for the study of the laticeferous system. This line of research was taken up again by Wilson (Wilson and Street, 1975) and later by Chua (1966) of the Rubber Research Institute of Malaysia with the backing of the Malaysian Rubber Producers' Research Association. Over the past two decades, considerable progress has been made on *in vitro* techniques for multiplication and improvement of *H. brasiliensis.* Most of such reports are directed towards micro propagation through shoot tip culture, nodal culture, somatic embryogenesis and genetic transformation (Senivirtnae and Nayanakantha, 2007). Paranjothy and Gandhimathy (1975) had attempted shoot tip culture from 2 to 4 weeks old asceptic seedlings. They could introduce rooting among some of seedling derived cultures; but failed to do so with clonal materials. Micro propagation was investigated later by Enjalric and Carron (1982) and led to the production of several hundreds of plantlets by micro cuttings from 1-3 year old green house grown

seedlings. Carron and Enjalric (1983) also reported that the propagation of elite *H. brasiliensis* stock material from stem cutting was a failure due to inadequate rooting system, necessary for tree stability. Later, plantlets with shoot and root development were successfully obtained from seedlings by different investigators (Gunatillege and Samaranayake, 1988; Carron *et al.,* 1989; Sompong and Muanghaewngam, 1992 and Seneviratnae and Flegmann, 1996). At Rubber Research Institute of India, experiments were carried out with elite *H. brasiliensis* clones using shoot tip explants derived from mature trees (Sinha *et al.,* 1985; Sobhana *et al.*, 1986). Shoots have been regenerated from axillary bud explants of a few *H. brasiliensis* clones by Sinha *et al.,* (1985), but failed to obtain rooting. Asokan *et al.,* (1988) have successfully produced self rooted clonal plants of *H. brasiliensis* by shoot tip culture but there still existed problems of juvenility and root formation, since explants derived from elite clones of mature *H. brasiliensis* are highly recalcitrant. This prevented the system from being commercialized. The major problem is the failure of producing adequate root system with tap root quality necessary for tree stability (Carron and Enjarlic, 1983). Another problem is the presence of bacterial and fungal contamination in the field grown mature plant derived explants. Effective sterilization techniques for obtaining contamination free initial explants were developed (Enjalric *et al.,* 1987; Asokan *et al.*, 1988). The effect of different fungicides and antibiotics to control microbial contamination in *H. brasiliensis* cultures were also examined (Kala *et al.*, 2004). Physiological juvenility of the explants was found to have significant role in micro propagation. Seneviratnae and Flegmann (1996) reported multiple shoot production from nodal explants of juvenile origin and root production from elongated shoots. Seneviratnae and Wijesekara, (1997) demonstrated that

axillary bud development can be accelerated by application of cytokinins. Mendanha *et al.*, (1998) described shoot development from the axillary buds in MS medium containing phytohormones. Carron *et al.*, (2000) have demonstrated the rooting capacity of *in vitro* propagated plantlets of *H. brasiliensis.* In spite of all these efforts over several years, no reliable technique is currently available for *H. brasiliensis* micro propagation on a commercial scale. Rejuvenated explants by micro grafting (Perrin *et al*, 1994) and buds of nodal explants taken from dormant branches were found to exhibit better *in vitro* response (Seneviratnae and Wijesekara, 1997; Lardet *et al.*, 1998). Attempts were also made at RRII for the standardization of an *in-vitro* micro grafting technique for the induction of explant rejuvenation as well as for the rescue of important difficult-to-root plant materials (Kala *et al.*, 2002). A technique for the rescue of immature embryos (Rekha *et al.*, 2010 a) and induction of multiple embryos (Rekha *et al.*, 2010 b) have been developed recently in *Hevea.*

2.7.2. Somatic embryogenesis

Somatic embryogenesis is defined as a process in which a bipolar structure resembling a zygotic embryo develops from a non-zygotic cell without vascular connection with the original tissue (Arnold *et al.*, 2002). Attempts to develop somatic embryogenesis as an *in vitro* propagation technique in *Hevea* was started in the early 1970s by a Chinese team at the Rubber Cultivation Research Institute (Baodao) and a Malaysian team at the Rubber Research Institute of Malaysia, simultaneously. The Institute de Recherche sur le Caoutchouc in France began work in this field in 1979 (Carron, 1980). The Rubber Research Institute of Sri Lanka initiated callus culture in 1972 (Anon, 1972) and expanded work on somatic embryogenesis in 1984 (Anon, 1984). The first anther derived callus was developed at the

Rubber Research Institute of Sri Lanka in 1972 (Satchuthananthavale and Irugalbandara, 1972). Later, this line of work was followed by the Chinese and Malaysian teams. Paranjothy in India achieved differentiation of embryoids from anther wall derived callus for the first time. Subsequently, shoot development was also achieved (Paranjothy and Gandhimathi, 1975; Paranjothy and Rohani, 1978). Initiation and growth of *Hevea* cell suspension cultures has been studied by Wilson and Street (1975). Chen *et al.,* (1979) reported the production of first batch of anther derived plants (Anon, 1977). Wang *et al.,* (1980) reported the field establishment of *in vitro* cultured *Hevea brasiliensis* plants of anther-wall derived callus for the first time. However, according to Rahaman *et al.,* (1981), clonal differences had been observed in the frequency of embryogenesis of anther-wall derived callus and therefore the reported technique was not applicable to all clones of *H. brasiliensis*. Carron (1981) used inner integument tissue of seeds for somatic embryogenesis and was successful in plantlet development. Carron and Enjarlic, (1982) also achieved differentiation of embryoids from the anther wall derived callus. During 1979-1989, Shiji *et al.,* (1990) produced 1700 plants out of 52,896 embryoids by inoculating 31,584 anthers in tubes. They successfully transplanted and established 539 plants of 13 clones. However, great variation among the clones for their induction frequency was reported. Some clones such as Haiken 2 had given high induction rates for embryoid and plantlet formation, while on the same media, other clones had not responded at all. Extensive experiments were carried out by many researchers to enhance the frequency of somatic embryo induction and plant regeneration in *Hevea*. Studies were conducted to optimize culture conditions, nutritional and hormonal requirements during somatic embryogenesis. Hadrami *et al.,* (1989) compared the effect of polyamines

on two embryogenic clones with two non-embryogenic clones and revealed quantitative differences in the polyamine (PA) contents. Effect of hormonal balance (Michaux-Ferrier and Carron, 1989), moisture content of the medium and explants (Etienne *et al.,* 1991), interaction of growth regulators, sucrose and calcium on callus friability (Montoro *et al.*, 1993, 1995), role of sucrose and abscisic acid on embryo induction (Veisseiere *et al.,* 1994 a & b; Linossier *et al.,* 1997) and carbohydrate types (Blanc *et al.*, 2002) have also been evaluated. Wang *et al.,* (1998) attempted to regenerate plantlets through somatic embryogenesis from stamen cultures. They optimized the temperature requirements for callus induction, somatic embryogenesis and plant regeneration at 26^0C, 24-25^0C and 26-27^0C respectively and regeneration frequencies up to 40.5 per cent were obtained. In spite of all the above studies, the plant regeneration frequency remains very low and the technology could not reach a commercial scale. In most of the above studies, inner integument tissue was used as the explant. It is reported that the calli obtained from the integuments of immature seeds frequently displayed browning (necrosis), leading to tissue degeneration and loss of embryogenic competence (Housti *et al.,* 1991; Veisseier *et al.,* 1994b). Carron *et al.,* (2000) have also studied the field performance of the *in vitro* plants regenerated *via* somatic embryogenesis and found clearly better growth of the *in vitro* plants compared with the seedling control, and the annual increment was consistently higher giving an increasing gap between the two treatments. Several factors such as the developmental stage and type of the explant, quantity of growth regulators and other growth enhancing substances, basal media composition, light intensity, etc. appear to play a crucial role in the induction and maintenance of somatic embryogenesis in many plants including *Hevea*. A high frequency of secondary embryogenesis

was induced from isolated early cotyledonary-stage somatic embryos of *H. brasiliensis* (Cailloux *et al.*, 1996). Repetitive embryogenesis was also induced from primary somatic embryos derived from integument tissue. Secondary embryogenesis has also been developed in *H. brasiliensis* for improving the efficiency of former somatic embryogenesis technology, which was based on the use of budded-tree integuments as primary explants, mainly by reducing subculture number and duration (Lardet *et al.*, 2008). Despite these improvements, the success rate of the secondary embryogenesis technology based on explants from budded-tree integuments remained very low , unpredictable and highly dependent on the composition of the medium (Lardet *et al*, 2008).

For the past 15 years, research is being carried out at the Rubber Research Institute of India to develop a plant regeneration system through somatic embryogenesis. In order to identify a suitable explant source, a variety of explants such as leaf, tender shoots, inner integumental tissues of immature fruit, immature anther, immature inflorescence etc., have been tried. Protocols have been developed for somatic embryo induction and plant regeneration in clone RRII 105 from different explants such as immature anthers i.e. before microsporogenesis (Jayasree *et al.,* 1999), immature inflorescence (Sushamakumari *et al.,* 2000) and leaf (Kala *et al.*, 2005, 2006 and 2008). Jayasree *et al.,* (1999) reported a standard protocol for the induction of friable embryogenic callus, somatic embryogenesis and further plant regeneration from the immature anthers. Optimum callus induction was obtained in modified MS medium supplemented with 2.0 mg l^{-1} 2, 4-D and 0.5 mg l^{-1} kinetin. Somatic embryo induction was found to be better with 0.7 mg l^{-1} kinetin and 0.2 mg l^{-1} NAA. Further development of the embryos into plantlets was achieved on a hormone free medium. Cytological analysis

revealed that all the plantlets were diploid. Sushamakumari *et al.*, (2000) developed a technique for somatic embryogenesis and plant regeneration using immature inflorescence as explants. They also studied the role of sucrose and abscisic acid on embryo induction. A higher sucrose level was found to be essential for effective embryo induction as well as maturation. However, lower levels were found to be beneficial for plant regeneration. Further, efforts have been made to enhance the embryo induction and plant regeneration frequency by the manipulation of nutrients and hormonal combinations. Sushamakumari *et al.*, (1999) have attempted induction of multiple shoots from germinating somatic embryos thereby enhancing the efficiency of plantlet formation. They could induce an average of 3.45 micro-shoots per explant by manipulation of the levels of BA and thidiazuron in the medium, as well as by wounding of the shoot primordia of the somatic embryos. Culture conditions and other nutritional requirements for improving the efficiency of somatic embryo induction and germination were investigated by Jayasree and Thulaseedharan (2001). A study on the gibberellic acid requirement on embryo induction and maturation revealed that incorporation of GA_3 up to 2.0 mg l^{-1} increased the embryo induction frequency. Germination percentage was also significantly enhanced by higher concentrations. However, further plant development was affected by increased GA_3 levels (Jayasree and Thulaseedharan, 2001). An isozyme study revealed a clear difference between embryogenic and non-embryogenic calli, as well as between different stages of embryogenesis where markers could be developed (Asokan *et al.*, 2002). Repetitive embryogenesis was also induced from primary somatic embryos derived from integumental tissue. Somatic embryos cultured on B-5 medium supplemented with 0.5 mg l^{-1} NAA, 2.0 mgl^{-1} KIN, 0.5 mgl^{-1} IAA and 4.0

mgl^{-1} 2,4-D enhanced repetitive embryogenesis and 5 per cent sucrose was found to be optimum (Asokan *et al.*, 2002). Embryogenic cultures had been maintained for over three years for retaining the embryo induction and plant regeneration potential (Jayasree and Thulaseedharan, 2004). Several *in vitro* plants have been raised through somatic embryogenesis from immature anther as well as immature inflorescence of the *Hevea* clone RRII 105 (Thulaseedharan, 2002) and it has been confirmed that those plants were morphologically as well as genetically uniform. Attempts were made to regenerate plants through somatic embryogenesis from callus cultures derived from leaf explants (Kala *et al.,* 2005). Leaf explants were cultured on modified MS medium supplemented with different combination of phytohormones. Embryo induction was achieved in modified MS medium containing BA, GA_3 and NAA and maturation occurred in WPM medium containing BA, GA_3. Plant regeneration occurred in half MS medium free of growth regulators. However the repeatability of the technique is yet to be studied for further utilization of the technique for gene transfer experiments. So far floral explants had proved to be ideal for genetic transformation. Genetic stability of somatic embryo derived plantlets was examined using RAPD technique by Jayasree *et al.,* (2010). Ten somatic plants were subjected to RAPD analysis along with their mother plant and 10 monoclonal seedlings. Fifteen decamers displayed monomorphic banding pattern with all the somatic plants and the mother tree where as polymorphism was observed among the monoclonal seedlings with the same primers. This shows genetic uniformity of somatic plants. At present, reliable somatic embryo formation is limited to only a few genotypes of *Hevea, i.e.,* RRII-105 (Jayasree *et al.,* 1999, Sushamakumari *et al.,* 2000, Kala *et al.,* 2005), SCATC 93/114, PB 260, PB 235, PR 107, RRIM 600 and GT 1

(Montoro *et al.*, 1993), Haiken, 2, Haiken 1 and SCATC 88-13 (Shiji *et al.*, 1990). Somatic embryogenesis in *Hevea* is highly fugacious (genotypic dependent and strictly specific to genotype-medium interaction), non-synchronous and its germination is very difficult. There is a very low rate of conversion of the embryos into plantlets (Carron *et al.*, 1995; Linossier *et al.*, 1997) which necessitates optimization of culture conditions for each genotype of *Hevea*. Commercial plant production has not yet been achieved, owing to the poor regeneration rates of the somatic embryos and problems in hardening.

2.8. Genetic transformation for crop improvement

Transgenic technology has a profound impact on the rapid development of plant biology in the past two decades. The development of technologies that allow the introduction and expression of functional genes in plant cells has extended to the production of transgenic plants with improved traits including insect and disease resistance (Leroy *et al.*, 2000; Perthuis *et al.*, 2005; Ismail *et al.*, 2010) seeds and fruits with enhanced nutritional qualities and plants that are better adapted to adverse environmental conditions (Jayashree *et al.*, 2003., Sobha *et al.*, 2003 a &b). Vaccines against human diseases have also been developed using transgenic plants. The emergence of new functional genomic strategies for the identification and characterization of genes promises to provide a wealth of information with an enormous potential to enhance traditional plant breeding and to genetically engineer plants for specific purposes. Plant biotechnology is at the threshold of an exciting new era in which emphasis is on the trans-kingdom gene transfer and production of elite genotypes of different crop species (Das *et al.*, 2011, Kancharla, 2011). For tree species, this technology is especially important because their long breeding cycles delay and restrict

improvement programmes by conventional breeding techniques. There are different methods of transformations including biolistic direct gene transfer (Alpeter, 2005), liposome/ PEG mediated gene transfer, and *Agrobacterium* mediated indirect gene transfer. *Agrobacterium* mediated gene transfer is the most popular one, due to the simplicity of the transformation system and precise integration of transgenes. *Agrobacterium* based vectors continue to offer the best system for plant transformation (Veluthambi *et al.*, 2003; Dandekar and Fisk, 2004; Khan *et al.*, 2009).

2.8.1 *Agrobacterium* mediated genetic transformation

The story of *Agrobacterium* is a century old. First written report of crown gall disease was given by Fabre and Dunal (1853). Later *Agrobacterium vitis* was identified as the causal agent of crown gall in grape (Cavara, 1897). *A. tumefaciens*, a member of Rhizobiaceae, is the organism responsible for the elicitation of crown gall in plants. In 1974, Zaenen, Schell and Montagu at the University of Ghent, Belgium, identified a mega plasmid that was present only in the virulent strain of *Agrobacterium* and named as Ti plasmid or Tumor inducing plasmid. Three year later, Eugin Nester, Milton Gorden and Mary Dell Chilton at the University of Washington demonstrated that only some genes of the Ti plasmid were transferred to the chromosomes of the plant cell and were responsible for inducing tumors. The DNA segment transferred to the host cell was called T DNA and is delimited by left and right borders which are 25 base pairs imperfect direct repeats. Researchers reasoned that any piece of DNA between these borders could be transferred into the plant cell and randomly integrated into the genome of the plant. Taking this into consideration, a research team at the University of Ghent, the Monsanto Company, and the University of Washington, inserted heterologous genes with the appropriate

regulatory regions into the T-DNA region and showed the integration and expression of foreign genes in plant cells. Later, disarmed plasmids were created which contained a T DNA, lacking genes for tumor formation and were used to produce the first transgenic plant (Zambryski *et al.*, 1983). Transgenic tobacco conferring insect tolerance by expression of genes encoding insecticidal proteins from *Bacillus thuringiensis* (Bt) were developed (Vaeck *et al.*, 1987). Since then, great progress have been achieved in understanding *Agrobacterium* mediated genetic transfer as a practical and common method for introducing specific DNA fragments into the plant genome and several transgenic plants harboring agronomically important genes were regenerated. The first genetically modified crop approved for sale in the U.S, in 1994 was the FlavrSavr tomato, which had a longer shelf life (Bruening and Lyons, 2000). In 1994, the European Union approved tobacco engineered to be resistant to the herbicide bromoxynil, making it the first commercially genetically engineered crop marketed in Europe (Debora MacKenzie, 1994). The year 1996 can be considered a landmark in agricultural biotechnology in general and crop protection in particular as four transgenic crops comprising three insect-resistant crops and a herbicide tolerant soybean, developed by Monsanto Company, received regulatory approvals and these were commercially grown and harvested for the first time in the USA (Manjunath, 2004).

2.9. Gene transfer attempts in *Hevea*

The possibility of genetic transformation in rubber trees was first explored by Arokiaraj and Rahaman (1991). They employed co-cultivation of *in vitro*- and *in vivo*-propagated plantlets with *A.tumefaciens* (Strain 541) and subsequently cultured in the Murashige and Skoog (MS) medium without growth regulators. Co-cultivated explants developed tumors and

produced octopine, indicating effective transformation. Subsequently, transformation protocols were developed for direct DNA delivery through microprojectile bombardment as well as through *A. tumefaciens* mediated methods (Arokiaraj *et al.*, 1994; 1996). In their studies, anther-derived calli were used as explants and binary vectors harbouring *GUS* as reporter gene and either *nptII* or chloramphenicol acetyl transferase (*CAT*) as the selection gene. Genetic transformation was confirmed by histochemical staining and flourometric assay for GUS activity. ELISA (Enzyme-Linked Immuno Sorbent Assay) for detecting expression of the *nptII* gene and direct enzyme assay for detection of *CAT* gene expression were carried out. The presence of alien gene in the transformed calli, embryoids and transgenic plants was further confirmed by PCR analysis. Arokiaraj *et al.,* (1998) studied the constitutive promoter (CaMV 35S) directed β-glucuronidase expression in the laticifer system of transgenic rubber and the stability of expression of the transgene in successive vegetative generations raised through bud grafting of the original transformants. Anther-calli were genetically transformed using *Agrobacterium* strain GV2260 harboring the β-glucuronidase (*uid A*) and *nptII* genes. B-glucuronidase protein was expressed in the serum fraction of latex. Transverse sections of the leaf petiole from a transformed plant revealed *GUS* expression, especially an enhanced expression in the phloem and laticifers. *GUS* expression was detected in three successive vegetative cycles propagated from the original transformants (Arokiaraj *et al.,* 1998).

Studies were also undertaken to enhance the efficiency of *Agrobacterium*-mediated genetic transformation of rubber callus. It is reported that the virulence capacity of the *Agrobacterium* strain and the combination of the *Agrobacterium* strain and the type of binary vector used significantly influenced the transient expression of the *GUS* gene (Montoro

et al., 2000). Out of the five *A. tumefaciens* strains viz. C58pMP90, C58 pGV2260, AGL1, LB4404 and EHA 105 and the two binary vectors, pGIN and pCAMBIA 2301 tested, the combination of EHA 105 and the binary plasmid vector pCAMBIA 2301 showed the highest transient expression. It is also reported that the transfer of friable callus from maintenance medium containing 9.0mM $CaCl_2$, to a calcium-free medium before *Agrobacterium* infection as well as use of a calcium-free *Agrobacterium* resuspension medium to inoculate friable calli, significantly enhanced the transformation efficiency. Paramomycin proved more effective than kanamycin for the selection of transformed cells (Montoro *et al.*, 2003). The influence of cryopreservation of explants and co cultivation temperature on transformation efficiency of the *Agrobacterium*-mediated genetic transformation of rubber callus was studied by the above group. It was reported that the transformation efficiency and competence of the embryogenic calli improved after two cycles of cryopreservation. When the co-cultivation temperature was reduced from 27^0C to 20^0C and the duration of this phase was increased up to 7 days, the *GUS* activity was increased (Blanc *et al.*, 2006).

After developing efficient plant regeneration protocols through somatic embryogenesis, RRII has initiated active research for the development of transgenic plants integrated with genes for desirable agronomic traits. Initial focus was to develop transgenic plants tolerant to abiotic stresses like elevated light and temperature, drought and TPD. In nature, plants encounter a wide range of environmental stresses that detrimentally affect their growth and development. Plants exposed to environmental stress generate excess reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2 O_2) and hydroxyl radicals (OH^-)

(Asada and Takahashi, 1987). Superoxide dismutase (SOD) is the first enzyme involved in the detoxifying process of reactive oxygen species (Fridovich, 1986). Significant yield loss occurs in plantations across the rubber-growing countries due to TPD, a physiological disorder, characterized by the browning of bark followed by the cessation of latex flow. Since no pathogens are unequivocally proved as the causative organism yet, this is considered as a physiological disorder. Increased free radical accumulation and a reduced level of SOD enzyme and cytokinins were reported in the TPD-affected trees (Das *et al.,* 1998). Therefore, attempts were made to develop transgenic rubber plants by incorporation of the genes coding for SOD and *ipt* (isopentanyl transferase). Transgenic plants with over expression of SOD were regenerated and the stable integration of the transgene was confirmed through molecular analysis (Jayashree *et al.*, 2000; 2003; Sobha *et al.,* 2003b). The preliminary biochemical studies conducted with SOD-transformed callus cultures showed enhanced activity of SOD and related enzymes such as catalase and peroxidase (Sobha *et al.,* 2003b). The gene expression of SOD and drought related traits in MnSOD transgenic plants were confirmed in polybag plants (Jayashree *et al.,* 2011). Physiological performance of 1 year old transgenic plants were evaluated in a dry humid climate, by withholding water and the results showed that one of the transgenic line showed increased drought tolerance and better recovery after re watering, compared to control RRII 105 plants (Sumesh *et al.,* 2012).

Genetic transformation with *ipt* gene for the over expression of cytokinin was also attempted. The putative transformed calli were able to grow without external supply of hormones and showed increased cytokinin levels compared to the controls. The embryos showed developmental abnormalities and most of the transformants were severely deformed (Kala

et al., 2003). An efficient protocol for high frequency transformation using embryogenic calli as the explant has been developed at RRII and a transformation frequency of 63 per cent has been achieved with 2 constructs of SOD (Rekha *et al.,* 2006). Sobha *et al.,* (2009) studied the expression and stability of transgene in the system and confirmed that the gene is stably integrated in the genome. Again various improvements have been made in the transformation protocols for increasing the transformation frequency including preculturing of calli, addition of phenolic compounds silver nitrate in the co- cultivation medium and vacuum infiltration and considerable improvement in the transformation frequency have been achieved with different gene constructs (Sobha *et al.,* 2010, 2012; Kala *et al.*, 2012).

Chemically, natural rubber is *cis*-1, 4-polyisoprene and its biosynthesis is through a cascade of reactions catalyzed by various enzymes. A few enzymes involved in the conversion of acetate into rubber in rubber tree latex have been demonstrated which indicates that the activity of 3-hydroxy-3-methyl glutaryl Coenzyme A reductase (*hmgr l*) is low, compared to the other enzymes suggesting that the constitutive levels of this enzyme may be a limiting factor in rubber biosynthesis (Lynen, 1969) . Arokiaraj *et al.,* (1994) over expressed *hmgr l* in transgenic rubber, where *hmgr l* activity of transformed anther derived callus ranged from 70 to 410 % of the value of wild-type control and the activity in the transformed embryos ranged from 250 to 300%. In order to enhance the rubber biosynthesis, experiments were initiated at RRII to develop transgenic plants integrated with the genes coding for important enzymes involved in this pathway. Initially, the genes coding for *hmgr l*, farnesyl-diphosphate synthase (*FDP*) and rubber elongation factor (REF) were selected. The *hmgr l* and *REF* genes were cloned into the binary vector pBIB121 for the genetic transformation which contains the antibiotic

genes *nptII* and *hpt* (hygromycin phosphotransferase). The *Farnecyl Diphosphate* gene was cloned into the binary vector pCAMBIA under the control of CaMV 35S promoter. Three *Agrobacterium* strains such as EHA 105, LBA 4404 and pGV 3101 were used for infecting the tissue for the transformation of hmgr 1 gene while EHA 105 alone was used for FDP and REF genes. Two-month-old calli were infected with different *Agrobacterium* strains carrying the above genes and the transgenic lines were selected. Transgenic embryos and few plantlets integrated with the FDP gene were regenerated. Further work is progressing (Venkatachalam *et al.,* 2007).

2.10 Osmotin gene

Among the several stress-related proteins, osmotin is one of the unique proteins isolated from tobacco cell cultures (Singh *et al.*, 1985) which is induced in response to both biotic and abiotic stresses (Singh *et al.*, 1985, 1989; LaRosa *et al.*, 1992; Raghothama *et al.*, 1997) (Fig. 2.1). Osmotin and osmotin-like proteins or the genes encoding these proteins have been extensively studied in several laboratories (Singh *et al.,* 1987; LaRosa *et al.,* 1989; Meeks-Wagner *et al.,* 1989; Grosset *et al.,* 1990a, 1990b; Neale *et al.,* 1990; Roberts and Selitrennikoff, 1990 and Woloshuk *et al,* 1991). Osmotin is a cationic protein that belongs to the PR5 family proteins with a molecular mass of 26 kDa and exists in at least two forms, one with a pI of greater than 7.8 and the other with a pI of greater than 8.2 which differ slightly in molecular weight (Singh *et al.,* 1985). Osmotin exhibits a very high level of sequence homology with other proteins, including the sweet protein thaumatin, potato pathogenesis-related protein PR-C, tobacco PR-S and the maize α-amylase/trypsin inhibitor (Richardson *et al.,* 1987; Singh *et al.,* 1987).

Osmotin synthesis is regulated mainly by abscisic acid, but the accumulation depends on adjustment of the cells to NaCl or to water stress. Osmotin is concentrated in dense inclusion bodies within the vacuole (Singh *et al.,* 1987). The expression of osmotin mRNA has been linked to development, particularly flowering and as the consequences of hormone treatments. The abundance and distribution of isoforms of osmotin vary among tissues and organs. At least six hormonal or environmental signals, including abscisic acid, ethylene, tobacco mosaic virus infection, salinity, stress, and desiccation and wounding induce accumulation of osmotin mRNA in both cultured cells and whole plants of tobacco (Singh *et al.,* 1987). Stress conditions such as NaCl, desiccation, ethylene, wounding, abscisic acid, tobacco mosaic virus, fungi, and UV light were tipped to be inducers of this protein (LaRosa *et al.*, 1992). The large spectrum of cues, both abiotic and biotic gave, good indications that osmotin gene could always be activated under field conditions. It was found that osmotin is positively involved in the programmed cell death, in blocking the cold-induced calcium signaling, and in affecting cytoskeleton in response to cold stimuli (Angeli and Altamura, 2007). It has been proved that the expression of Tbosm (Tobacco osmotin) provides salinity tolerance in tobacco (Barthakur *et al.*, 2001), strawberry (Husaini and Abdin, 2008), wheat (Noori and Sokhansanj, 2008), cotton (Parkhi *et al.*, 2009), tomato (Goel *et al.*, 2010), chilli pepper (Subramanyam *et al.,* 2011), and mulberry (Das *et al.,* 2011). It was reported that Tbosm provides the salinity stress tolerance by accumulating the osmolytes such as proline and glycine betaine (Barthakur *et al.*, 2001; Singh *et al.*, 1987; Subramanyam *et al.*, 2011), by facilitating the compartmentation of solutes or by being involved in metabolic or structural alterations during salinity (Husaini and Abdin, 2008).

This PR-5 family member had been a hotspot for biotechnologist, since it was suspected to play an important role in enhancing the level of resistance to secondary challenges by pathogens, a phenomenon referred to as SAR (Systemic Acquired Resistance). The first evidence that osmotin and other PR-5 family proteins had antifungal properties came from the works of Vigers *et al.,* (1991) indicating the N-terminal sequence of zeamatin, an antifungal protein from corn, which was very similar to osmotin. *In vitro* assays, demonstrated that osmotin had antifungal activity against a variety of fungi, including *P. infestans, Candida albicans, Neurospora crassa, and Trichoderma reesei* (Woloshuk *et al.*, 1991; Vigers *et al.,* 1992). The constitutive expression of osmotin led to enhancement of potato resistance to late blight (Liu *et al.,* 1994) its role in resistance consist of causing sporangia lysis of *P. infestans* (Woloshuk *et al.*, 1991. It also has a role in plant protection against osmotic stress (Konowicz *et al.*, 1992) and freezing tolerance (Hon *et al.*, 1995). It was previously unveiled that, at high concentration, it causes the lysis of hyphae tips of fungi (Vigers *et al.,* 1991). A more conclusive antifungal potential of osmotin was shown by Abad *et al.,* (1996). They concluded that osmotin induces spore lysis, inhibit spore germination or reduce its viability in seven fungal species that exhibited some degree of sensitivity in hyphal growth inhibition tests. These broad spectral mechanisms of action validated osmotin gene, as potential sentinel candidate against *P. infestans* in transgenic potatoes. Overexpression of PR-5 (or thaumatin like proteins) in potato delayed development of disease symptoms of *P. infestans* (Liu *et al.,* 1994*) in vitro*, whereas trials in transgenic potato plants over expressing antisense PR-5 did not exhibit any higher susceptibility (Zhu *et al.*, 1996). Tbosm also displays antifungal property both *in vitro* and *in vivo* conditions (Liu *et al.,* 1996) by altering the

permeability of fungal membrane and ultimately causes cell apoptosis (Yun *et al.,* 1998; Narasimhan *et al.*, 2001, 2005). A plant expressing the complete open reading frame of Tbosm accumulates osmotin protein mostly in the vacuole, so that those plants are unable to exhibit effective antifungal activity (Liu *et al.,* 1996). In plants expressing a C-terminal 20-amino acid-truncated Tbosm, osmotin (24 kDa) protein was totally secreted into the extracellular matrix which may retard the fungal infection most effectively (Liu *et al.*, 1996). The constitutive expression of the Tbosm confers resistance against several fungal pathogens including *Candida albicans*, *Neurospora crassa*, *Phytophthora infestans* (Liu *et al.*, 1994), *Fusarium pallidoroseum*, *Colletotrichum gloeosporioides*, *Colletotrichum dematium* (Das *et al.,* 2011) and *Rhizoctonia solani* (Parkhi *et al.*, 2009).

Transgenic plants integrated with osmotin gene have been produced in different crops like tobacco (Barthakur *et al.,* 2001), potato (Liu *et al.*, 1994; Li *et al.,* 1999), tea (Bhattacharya *et al.*, 2006 a&b), olive (Angeli and Altamura, 2007), strawberry (Amjad and Malik, 2008), tomato (Goel *et al.*, 2010), soybean (Subramanyam *et al.,* 2012) which imparted tolerance to biotic as well as abiotic stress tolerance.

CHAPTEI

MATERIALS &METHO

3. MATERIALS AND METHODS

3.1 Target tissues for transformation

In earlier studies on genetic transformation in *Hevea*, with different gene constructs, target tissues derived from different explants have been used and transformation frequencies reported so far ranged from 2-63% (Arokiaraj *et al.*,1994; Jayashree *et al.*, 2003; Rekha *et al.*, 2006). In the present study, with a view to get efficient transformation using the gene coding for osmotin protein under the control of CaMV 35S promoter, seven different target tissues derived from three explants including both clonal and zygotic origin were used as detailed below (Table 3.1) (Fig 3.1).

Table.3.1 Different target tissues used for the integration of osmotin gene in *H. brasiliensis*

Source	Explant	Target tissues
Clonal	A. Immature anther	Intact anther Primary calli Embryogenic calli
	B. Immature ovule	Intact ovule Primary calli Embryogenic calli
Zygotic	Immature zygotic embryo	Embryogenic calli

a. Immature inflorescence
b. Female flower bud
c. Male flower bud
d. Ovule
e. Anther

Fig. 3.1 Sources of explant

The methods of raising the target tissues are given below:

3.1.1 Anther& anther derived target tissues

Young male flower buds (Fig.3.1.c) were separated from immature inflorescences (Fig.3.1.a) collected from 15-year-old rubber (clone RRII 105) trees growing in the experimental fields of Rubber Research Institute of India. The flower buds were washed thoroughly in running tap water for 10 min to remove surface contaminants, surface sterilized with 0.1% (w/v) mercuric chloride solution containing two drops of Tween-20 for three minutes and washed extensively in sterile distilled water. Immature anther, at diploid stage (before microsporogenesis) was dissected out aseptically under a stereomicroscope from the male flower buds. Sterile ascorbic acid (0.1%) solution was spread over the flower buds during dissection to prevent oxidation and browning of the anther. The intact anthers were used as target tissues for transformation (Fig. 3.1.e).

For the induction of primary calli, anthers were inoculated on callus induction medium. MS (Murashige and Skoog 1962) medium was modified by lowering the NH_4NO_3 concentration to 1.0 g l^{-1} and replacing MS vitamins with B5 (Gamborg *et al.*, 1968) vitamins. This basal medium was supplemented with growth regulators 2, 4-D (2 mg l^{-1}) and Kinetin (0.5 mg l^{-1}) (Jayasree *et al.*, 1999) (Fig. 3.2.a).

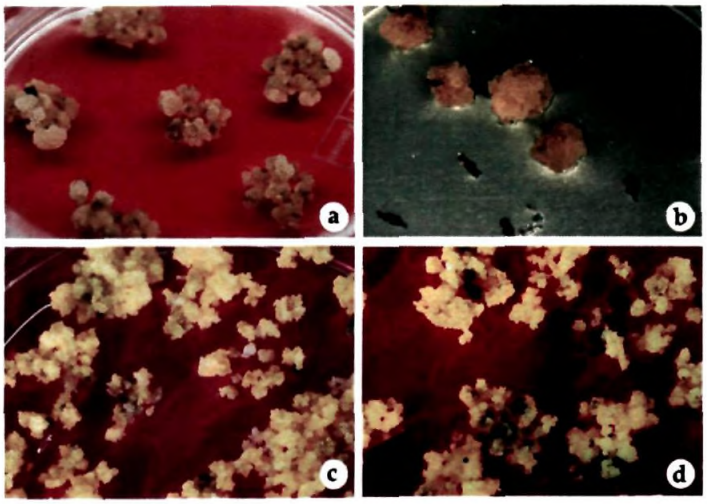
A portion of the primary calli was cultured over modified MS medium supplemented with 2, 4, D (0.2 mg l^{-1}), Kinetin (0.2 mg l^{-1}), NAA (0.2 mg l^{-1}) BA (0.2 mg l^{-1}) and GA (0. 5 mg l^{-1}) (Jayashree *et al.*, 2003) for embryogenic callus induction. After 3-4 subcultures in the same medium, cultures were transferred to the media with high phytagel and charcoal. Other

components were constant. Embryogenic calli emerged in this medium was used as another target tissue for transformation (Fig.3.2.a).

3.1. 2 Ovule and ovule derived target tissues

Young female flower buds (Fig. 3.1.b) were separated from inflorescence collected from 15-year-old *Hevea brasiliensis* (clone RRII 105) trees growing in the experimental field of Rubber Research Institute of India. The flower buds were sterilized as described in section 3.1.1. The ovules before megasporogenesis were dissected out from the immature female flowers (Fig.3.1.d). The intact ovules were used as target tissue for transformation.

Primary calli was induced from the ovule in the same basal medium used for anther, but with a growth regulator combination of BA (0.8 mg l^{-1}) and 2, 4-D (1.0 mg l^{-1}). Embryogenic calli were raised from this primary calli, after sub culturing repeatedly in the embryogenic calli induction medium as mentioned in the section 3.1.



a. Primary calli from ovule
b. Primary calli from anther
c. Embryogenic calli from ovule
d. Embryogenic calli from anther

Fig. 3.2 Target tissues used for transformation

3.1.3 Induction of embryogenic calli from immature zygotic embryo following *in ovulo* embryo culture

Immature fruits (8-10 weeks old) were collected from field grown trees of *Hevea* (clone RRII 105), washed thoroughly in distilled water and allowed to dry (Fig.3.3.a). Fruits were then dipped in 80% alcohol for 15 minutes and allowed to dry on a sterile filter paper in a petriplate. The developing seeds were isolated (Fig.3.3.b) aseptically from the fruits and inoculated in the nutrient media. Since embryos are not visible at early stages and are difficult to dissect, the immature seeds were cut into two halves and placed in the medium with the micropylar end touching the medium (Fig.3.3.c). Nitsch (1960) basal medium fortified with GA_3 (3mg l^{-1}), kinetin (3 mg l^{-1}) and Zeatin (0.4 mg l^{-1}) was used for inducing calli. Embryogenic calli emerged within 30 days (Fig.3.3.d) after inoculation. Further proliferation was obtained by replacing the growth regulators with NAA (0.2 mg l^{-1}). This proliferated embryogenic calli was used as target tissue for transformation.

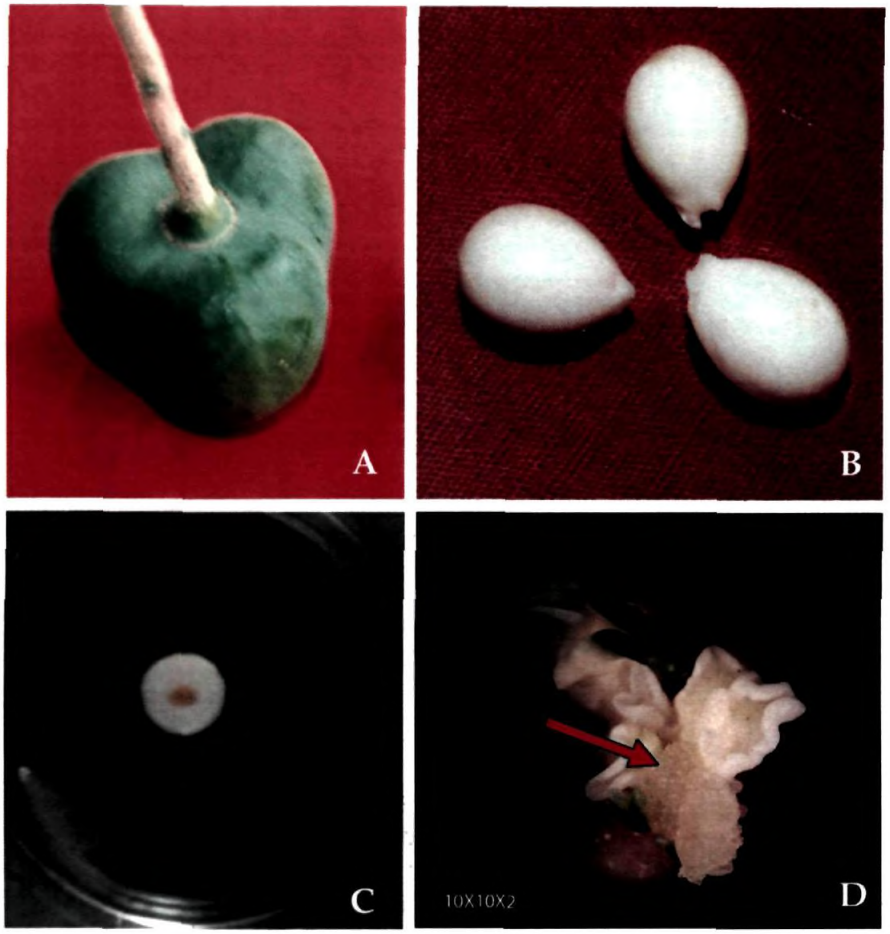
The composition of the media used for induction of primary as well as embryogenic calli from different sources is given in Table 3.2.

Table. 3.2 Growth regulator combinations for callus induction from different explants.

Growth regulators (mg l^{-1})	Anther *		Ovule *		Zygotic embryo **
	Primary calli	Embryogenic calli	Primary calli	Embryogenic calli	Embryogenic calli
2, 4- D	2	0.2	1.0	0.2	-
kinetin	0.5	0.3	-	0.3	3.0
NAA	-	0.2	-	0.2	0.2
BA	-	0.2	0.8	0.2	-
GA	-	0.2	-	0.5	3.0
Zeatin	-	-	-	-	0.4

* Basal medium used for callus induction from anther and ovule is modified MS (Annexure-1)

** The basal medium used for callus induction from immature zygotic embryo is Nitsch medium (Annexure11)



A. Immature fruit
B. Developing seeds
C. *In ovulo* embryo culture
D. Embryogenic callus from zygotic embryo

Fig. 3.3 Different stages in developing embryo derived callus

Among the different target tissues attempted for transformation, only the most responsive ones namely embryogenic calli from anther as well as embryogenic calli from zygotic embryo were used for subsequent experiments. Optimization of different stages of somatic embryogenesis for transgenic plant regeneration was done using the clonal explant anther derived embryogenic calli.

3.2 Osmotin gene construct

Agrobacterium strain GV 2260 harbouring the plasmid osm/BinAR under the control of CaMV35 S promoter containing kanamycin resistance as the selectable marker (Barthakur *et al.,* 2001) was employed for genetic transformation (Fig.3.4). This gene construct was obtained from Dr. Kailash Bansal, Director, NBPGR, on Material Transfer Agreement.

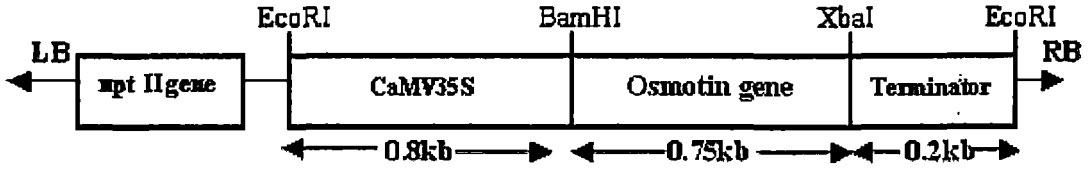


Fig.3.4. Details of the osmotin gene construct

3.3 Genetic transformation protocol

Two different protocols were attempted to bring out *Agrobacterium* infection. 1) The protocol reported earlier by Jayashree *et al.*, (2003) and 2). A modified version of the method followed in California University (Personal communication).

3.3.1 Method-1

The bacterial culture for *Agrobacterium* infection was prepared according to Jayashree *et al.,* (2003). A single colony of *Agrobacterium* GV2260 harbouring the binary vector osm/BinAR was streaked on solid AELB medium (Tryptone 10 g l^{-1}, yeast extract 5g l^{-1}, pH-7) supplemented with kanamycin (50 mg l^{-1}), 75 mg l^{-1} rifampicin and 100 mg l^{-1} carbenecillin and grown at 28°C. After 48 hours, a single colony of these actively growing bacteria was transferred to 20 ml liquid AELB medium containing the above antibiotics in the same concentration. The *Agrobacterium* was allowed to

grow overnight at 28°C in a rotary shaker at 250 rpm to get a density of 10^8 cells/ml (an optical density of 0.5 at 420 nm). 10 ml of this culture was centrifuged at 2500 rpm for 10 min for separating the bacteria. The bacteria were resuspended in 100 ml sterile MS basal medium containing 20 mg l^{-1} acetosyringone, 115 mg l^{-1} proline and 153 mg l^{-1} glycine betaine to get a bacterial density of 10^8 cells/ml. The bacterial cells were allowed to grow in this medium in a rotary shaker at 250 rpm for four hours at 28°C. This bacterial culture was used for callus infection.

Approximately 1.0 g of target tissue was taken in sterile (30mm) glass Petri plates containing 5 ml of the *Agrobacterium* culture. The callus clumps immersed in bacterial culture (inoculum) were cut into small pieces with sterile scalpel blade and kept in the *Agrobacterium* inoculum for 10 min. The infected calli were then blotted dry with sterile filter paper to remove the excess bacterial suspension and carefully transferred to a filter paper placed over the solid co-culture medium. For co-culture, the same basal medium as mentioned above was used. This was supplemented with 20 mg l^{-1} acetosyringone, 115 mg l^{-1} proline and 153 mg l^{-1} glycine betaine for increasing the virulence of the bacterium. Co-culture was done for 3 days at 28°C. The calli were then dried on a filter paper and transferred to selection medium (Jayashree *et al.*, 2003; Rekha *et al.*, 2006).

3.3.2 Method 2

Agrobacterium glycerol stock 50µl was added to 5 ml MGL* (Annexure IV) medium with pH 7 (Jones *et al.*, 2005) containing 50 mg l^{-1} kanamycin, 75 mg l^{-1} rifampicin and 100 mg l^{-1} carbenecillin. This was kept in an incubator shaker overnight at 23°C at 250 rpm. After 24 hours of growth, the bacterial culture was subjected to a pH shock by diluting 2.5 ml of the culture with 7.5 ml of TY* (Annexure V) medium (pH 5.5) containing

the respective antibiotics and 200 µM acetosyringone. The cultures were incubated overnight at 23°C at 250 rpm. The next day, 1.5 ml of the bacterial culture was diluted to 20 ml with TY medium (pH 5.5), containing 200 µM acetosyringone. The optical density (OD) was measured against TY blank at 600 nm and adjusted to the optimal level (0.1-0.2) and was used for transformation.

Table. 3.3 Protocols followed for *Agrobacterium* mediated transformation

Steps followed	Method-1	Method-2
Inoculum preparation	5 days procedure	3 days procedure
Temperature at which bacteria was grown	28^0C	23^0C
Bacterial density	$5x10^8$ cells/ml	$5x10^8$cells/ml
Optimum O.D	0.5 at 420nm	0.1-0.2 at 600nm
Inoculation medium	*AELB followed by MS Acetosyringone (20 mg l^{-1})	*MGL followed by TY Acetosyringone (40 mg l^{-1})
Co-culture medium	Solid Acetosyringone (20 mg l^{-1})	Liquid Acetosyringone (40 mg l^{-1})
Co-culture temperature	28^0C	23^0C

* Compositions of AELB, MGL, TY and Co-culture medium are given in Annexures IV &V.

The explants were kept in a sterile filter paper placed in a 30 mm petriplate and soaked with *Agrobacterium* suspension using a micropipette. After 5 minutes, the calli were blotted dry and transferred to co- culture

media and were incubated at 23°C for 3 days. For co-culture, liquid medium was used in this method. The filter paper kept in a sterile petriplate was wetted using 5 ml of liquid co-culture medium and the infected calli were placed over it (Fig.4.1.a). The co culture media contained 40 mg l^{-1} acetosyringone, 115 mg l^{-1} proline and 113mg l^{-1} glycine betaine along with the other ingredients. The two protocols are summarized in Table.3.3

3.4 Development of transgenic callus

3.4.1 Selection of transgenic cell lines

After the co- culture period, infected tissues were transferred to media designed for the inhibition of the growth of *Agrobacterium* as well as selection of transformants. In *Hevea,* cefotaxime has been reported to be an ideal antibiotic for suppressing *Agrobacterium.* The *npt II gene* imparting kanamycin resistance is incorporated in the gene construct along with the gene of interest. The concentration of kanamycin for effective selection is already standardized in *Hevea* (Jayashree *et al.*, 2003; Sobha *et al.*, 2003b; Rekha *et al.,* 2006). After 3 days of co culture, the infected calli were blotted dry using a sterile filter paper and were transferred to selection medium containing 500 mg l^{-1} cefotaxime and 300 mg l^{-1} kanamycin and maintained at 25±2°C in the dark and subculturing was done at three weeks interval.

After 30 days, cefotaxim was omitted from the medium and selection for putative transgenic cell lines in the presence of kanamycin was continued. The cultures were observed weekly and those cultures with overgrowth of the bacteria were discarded and the rest of the cultures were carried forward. Putatively transformed cell lines emerging from these cultures were selected and transferred to proliferation medium.

3.4.2 Proliferation of the transformed putative cell lines

Proliferation of the transformed putative calli was observed to be a difficult task since growth of the transgenic cell lines was very slow in the already established proliferation medium. Experiments were designed with different growth regulators individually and in combinations for faster proliferation and growth of the calli. Two auxins, NAA and 2, 4- D were tried individually and in combinations. The basal medium used was modified MS supplemented with 50 g l^{-1} sucrose, 10% coconut water, and 400 mg l^{-1} casein hydrolysate (Jayashree *et al.,* 2003). The gelling agent used was phytagel (0.2%). Each experimental unit consisted of 10 calli clumps and the experiment was repeated 5 times. Percentage proliferation was calculated based on the number of calli responded for growth.

3.4.3 Confirmation of the transgene integration by PCR analysis

Osmotin gene integration was tested in five randomly selected cell lines developed from independent transformation events by PCR analysis using osmotin gene specific primers as per the standard procedure as described in the session 3.8.1

3.5 Development of transgenic plants

All the experiments described below are with anther derived transgenic cell lines unless and otherwise mentioned. In the optimized media combinations, comparison of the clonal transgenic cell lines with that of zygotic origin was attempted.

3.5.1 Embryo induction from transgenic cell lines

The proliferated embryogenic callus was subcultured on to different media combinations for achieving embryo induction. The basal medium used was the one already reported for embryo induction from transgenic cell lines of *Hevea* (EI medium)* (Jayashree *et al.*, 2003). Based on the results of the preliminary studies, experiments were designed with varying concentrations and combinations of growth regulators *viz.*NAA (0-5 mg l^{-1}), Kin (0.1- 0.5 mg l^{-1}), ABA (0.1- 0.5 mg l^{-1}) and GA (0.5-2 mg l^{-1}) along with 50 g l^{-1} sucrose and B5 vitamins. Embryo induction was attempted in media without charcoal. All the ingredients other than the growth regulators were kept the same as mentioned above.

The culture medium was solidified with 0.3% phytagel. Each treatment contained 25 calli groups and the experiments were repeated 5 times. Observations on embryo induction were recorded after two months and percentage of embryo induction was worked out.

3.5.2 Embryo maturation

Embryos were transferred to the maturation medium one month after embryo induction. For maturation, the same basal medium used for embryo induction, devoid of growth regulators other than ABA were used. Since ABA is an established growth regulator for achieving embryo maturation in several crops, it was tried along with other components. The combined effect of ABA with phytagel and phytagel with sucrose were studied in two separate experiments. Different levels of ABA (0.2-1.0 mg l^{-1}) were supplied with varying levels of phytagel (0.2- 0.6%) in the hormone free basal

* Annexure 3

medium. Similarly, the same range of phytagel as mentioned above was tried along with varying concentrations of sucrose ranging from 50 g l^{-1} to100 gl^{-1}.

Organic supplements such as coconut water (CW) 10%, 150 mg l^{-1} banana powder, 100mg l^{-1} malt extract and 300 mg l^{-1}casein hydrolysate (Jayashree *et al.,* 2003) were also supplemented in the maturation medium. The maturation medium was also provided with 0.2% charcoal as it is necessary in *Hevea* for the maturation of somatic embryos (Carron *et al.,* 1995). A mixture of amino acids containing glutamine (200 $mgl^{-1)}$, proline (100 mg l^{-1}), glycine (10 mg l^{-1}) (Sobha *et al.,* 2003), was also added for embryo maturation.

3.5.3 Germination of embryos

3.5.3.1 Effect of growth regulators

The mature somatic embryos with well-developed cotyledons were transferred to the germination medium. The poorly developed and abnormal embryos were discarded. MS medium, supplemented with different proportions of growth regulators BA and, GA (0.5-5 mg l^{-1}) were tried for germination. Embryos transferred to these media combinations were incubated in the dark condition for a period of 2 weeks and then transferred to light for shoot development. Organic supplements such as CW (5%), malt extract (50 mg l^{-1}), casein hydrolysate (400 mg l^{-1}) and banana powder (200 mgl^{-1}) were added generally in all germination media since they have already proved beneficial for germination of *Hevea* somatic embryos. Sucrose concentration was reduced to 2% in all the media tried for germination, since it was reported earlier (Rekha *et al.,* 2010a).

Experiments were repeated 5 times with 20 mature embryos per set. The pH of all media was adjusted to 5.7 prior to autoclaving at 121°C for 10

minutes. Media were solidified with 0.2% phytagel. Cultures were raised in culture tubes containing 20 ml medium and incubated at 25 ± 2° C under cool white fluorescent lamps. Subculture was done every 3 weeks unless otherwise mentioned.

3.5.3.2 Effect of desiccation on germination of embryos

Embryos were subjected to desiccation for improving germination and conversion to normal plants. Two methods of desiccation were tried. Rapid drying of mature embryos for 1-6 hrs in the laminar flow hood was followed. Alternatively, slow drying of embryos for different duration ranging from 6 to 72 hr, in closed petridishes was also attempted.

3.5.3.3 Influence of suspension cultures

For improving germination, suspension culture was also tried. The matured embryos with well-developed cotyledons were cultured in ½ MS hormone free liquid media for 1 to 5 days and transferred to solid media after blotting over a sterile filter paper.

3.5.4 Plant regeneration

The bipolar differentiated embryos were transferred to MS medium for plant regeneration. For plant regeneration, BA (1-5 mg l^{-1}) and IBA (0.5-2.5 mg l^{-1}) combinations were tried in presence of GA_3 (1.5 mg l^{-1}).

3.5.5 Effect of sugars and sugar alcohols in different stages of regeneration

For improving plant regeneration pathway, experiments were performed by incorporating different sugars and sugar alcohols in all stages of plant regeneration in presence of sucrose. Sugars such as glucose (20-100g l^{-1}), maltose (20-100 g l^{-1}) and sugar alcohols like sorbitol (2-10 g l^{-1}) and mannitol (2-10 g l^{-1}) were added in the medium at different levels.

3.6 Acclimatization of transgenic plants.

Plantlets after complete development and medium leaf maturation were carefully removed from the culture tubes and washed gently in running tap water to remove adhering medium. Dead tissues, if any, near the cotyledons were also removed. Plantlets were dipped in 0.2% bavistin for 3 minutes in order to reduce fungal infection and were blotted to remove the adhering water particles by keeping for 2 minutes in tissue paper. Then they were planted in earthenware pots (5cm diameter x 8 cm height) containing sterile sand, soil and soil-rite mixture and kept in the controlled conditions in growth chamber with a RH 90% and temperature 25°C . Humidity was decreased by 2 units at 2 days interval. Simultaneously, temperature was gradually increased from 25°C to 30°C. These plants were supplied with dilute Hoagland mixture weekly once and watering was done at two days interval. After about three weeks, the surviving plants were transferred to poly bags filled with garden mixture (1:1:1) and kept in the growth chamber itself. After the emergence and maturation of a new flush, the hardened plants were taken out from the growth chamber and maintained in the shade house. These plants were irrigated at two days interval. NPK Mg (20:20: 0: 15) mixture was applied fortnightly.

3.7 Micrografting

Because of the difficulty in hardening, *in vitro/in vivo* micrografting was attempted in order to rescue the transgenic shoots. The transgenic shoots were grafted on to seedlings having the same girth. For *in vitro* micrografting, zygotic embryo derived plantlets raised *in vitro* were used as the root stocks. The scions were placed in the incisions made in the root stocks with the cut surface in good contact. The stock and scion were held

together at the point of graft with sterile parafilm. For *in vivo* micrografting, young seedlings raised *in vivo* in polybags were used as the root stocks. Grafted plants were kept in environment controlled growth chamber. Alternately, seedlings were raised in *vitro* and planted in polybags, few days before grafting.

3.8 Molecular analysis of transgenic plants

In order to confirm the gene integration and expression, PCR and Southern hybridization and RT-PCR analysis was performed.

3.8.1 PCR analysis

3.8.1.1 Isolation of genomic DNA

Soft friable callus obtained after 3 or 4 weeks of culture in the proliferation medium were used for DNA isolation. Genomic DNA was extracted from the transformed as well as untransformed callus according to the modified CTAB method (Doyle and Doyle, 1990). The modified CTAB procedure consists of the following steps:

i. One gram tissue was ground to a fine powder in liquid nitrogen using a sterile, pre-cooled mortar and pestle. The powdered tissue was homogenized with 20 ml 2X CTAB extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 0.1 M Tris-HCl pH-8.0, 0.1% Polyvinyl polypyrrolidone (PVPP) and 0.1% β-mercaptoethanol).

ii. The samples were incubated at 60°C for 30 min. in a 50 ml centrifuge tube.

iii. Centrifuged at 8,000 rpm for 10 min. The supernatant was transferred to a new sterile tube and the pellet was discarded.

iv. Equal volume of Tris-saturated phenol-chloroform-isoamyl alcohol (25:24:1) was added and mixed gently by inverting the tubes.

v. The sample was spun at 10,000 rpm for 10 min and the aqueous phase was transferred to a fresh tube. The organic phase containing the denatured proteins was discarded.

vi. 5.0 µl of *DNase* free *RNase* (10 mg/ml stock) was added and incubated at 37°C for 1 h to remove RNA.

vii. Equal volume of chloroform: isoamyl alcohol (24:1) was added to the sample, mixed gently and centrifuged at 10,000 rpm for 10 min.

viii. The aqueous phase was transferred to a fresh tube and the organic phase containing the lipids and carbohydrates were discarded.

ix. To the sample, equal volume of chloroform and isoamyl alcohol was added, mixed gently and centrifuged at 10,000 rpm for 10 min.

x. Aqueous phase was transferred to a fresh tube and the organic phase was discarded.

xi. To the sample, 0.6 volume ice-cold isopropyl alcohol was added to precipitate the DNA and incubated in ice for 20 min.

xii. The precipitated DNA was pelleted by centrifuging at 8,000 rpm for 10 min at 4°C.

xiii. The DNA was washed in 70% ethanol.

xiv. The pellet was air-dried and dissolved in 1.0 ml of TE buffer (10:1).

The quality as well as the quantity of genomic DNA was checked on agarose gel in a UV illuminator (Beckman USA). DNA quantification was carried out in a Nano drop spectrophotometer. A ratio 1.8 indicates good quality DNA without protein contamination. After checking the quantity and quality in a spectrophotometer, the DNA samples were stored at –20°C.

3.8.1.2 Isolation of plasmid DNA for using as a positive control in molecular studies.

Recombinant binary vector with the osmotin gene insert was isolated from *Agrobacterium* following the alkaline lysis method (Birnboim and Doly, 1979).

i. An overnight grown 3 ml bacterial culture was inoculated into 100 ml of LB broth containing the respective antibiotic and kept under constant shaking for 12–16 h at 37°C.

ii. 1.5ml of the culture was taken in a microfuge tube. Centrifuged at 12,000 rpm for 5 min at 4°C. The medium was decanted completely leaving the pellet undisturbed.

iii. The bacterial pellet was resuspended in 100 µl of solution I by vigorous vortexing and the tubes were stored on ice for 5 min. (Solution I: 50 mM glucose, 25 mM Tris.Cl pH 8 and 10 mM EDTA pH 8. Autoclaved at 121°C, 15 lb and stored at 4°C).

iv. 200 µl of freshly prepared solution II was added to the tubes and mixed five times rapidly by inversion and the tubes were stored at room temperature for 5 min. (Solution II: 0.2 M NaOH (freshly diluted from 10 M stock and 1% SDS).

v. 150 µl of Solution III was added to the tubes and the contents were vortexed gently to disperse the solution through the viscous bacterial lysate. The tubes were returned to ice for 5 min. (Solution III: 5 M potassium acetate 60 ml, glacial acetic acid 11.5 ml and distilled water 28.5 ml).

vi. Centrifuged at 12,000 rpm for 5 min at 4°C to collect the clear supernatant into a fresh tube.

vii. An equal volume of phenol: chloroform was added to the collected supernatant, kept for 10 min, centrifuged at 8,000 rpm for 10 min. at room temperature and the upper aqueous phase was collected.

viii. RNA in the sample was degraded by incubating at 37°C for 1 h after the addition of 1 µl *DNase* free *RNase* (10 mg/ml stock).

ix. Plasmid DNA was precipitated by the addition of 2Vof ethanol and spun at 8,000 rpm for 10 min at 4°C.

X. The DNA pellet was washed with 70% alcohol (v/v), dried and was dissolved in 50 µl TE buffer (10:1, pH 8).

3.8.1.3 Primer designing

Based on a previously published cDNA sequence of osmotin (Kumar and Spencer,1992) and npt *II*, gene specific and marker specific oligonucleotide primers (forward and reverse) were designed with the help of 'Primer3' programme of 'Lasergene' software (DNASTAR, USA). The primer sequences and their Tm value are shown below

osmotin

Forward primer- 5'-ATG GGC AAC TTG AGA TCT TCT-3'Tm- 55.9
Reverse primer- 5'-CTA CTT AGC CAC TTC ATC- 3' Tm- 53.2

npt II

Forward primer- 5'-GAG GCT ATT CGG CTA TGA CT 3'Tm-58
Reverse primer- 5'-AAT CTC GTG ATG GCA GGT TG 3' Tm-58

3.8.1.4 Polymerase chain reaction

The synthesized primers (M/S Metabion, Deutschland) were dissolved in sterile double distilled water to get a concentration of 100 pmols/µl. The primer stock solutions were stored at –20°C.

PCR analysis was carried out with the osmotin and nptII specific primers designed to amplify the DNA fragments of 0.75 kb and 0.7kb respectively, using the standard procedure. PCR amplification was performed with 50 ng of genomic DNA as templates. Plasmid DNA was used as the positive control, whereas DNA from the untransformed calli was used as the negative control. Amplifications were carried out in 20 µl reactions, which contained the following components:

Table 3.4 Components of PCR reaction

Component	Volume	Final concentration
Template DNA	1.0 µl	50 ng
Reaction buffer *	2.0 µl	1 X
dNTP mix	2.0 µl	100 µM of each dNTPs
Forward primer	1.0 µl	10 pmol
Reverse primer	1.0 µl	10 pmol
Taq DNA polymerase **	0.15 µl	0.75 U
Sterile dist. water	12.85 µl	
Total volume	**20 µl**	

* (Tris-HCl, pH- 9 – 10 mM, KCl- 50 mM, $MgCl_2$- 15 mM)

** AmpliTaq® from Roche, USA

The reaction mix was overlaid with a drop of mineral oil and amplification was carried out in a Perkin-Elmer 480 DNA thermal cycler. The PCR conditions were as follows:

Step I	-	Initial denaturation	-	4 min.	94°C
Step II	-	Denaturation	-	30 sec.	94°C
		Annealing	-	1 min.	58°C
		Extension	-	2 min.	72 °C
Step III	-	Repeat step II	-	35 times	
Step IV	-	Final elongation	-	10 min.	72°C

The PCR products were analyzed in 1.5 % agarose gels. Gel images were captured using 'EDAS 290' (Electrophoresis Documentation and Analysis System- Kodak, USA). Molecular weight of the amplified products was determined using Kodak I D Image Analysis software.

3.8.1.5 Clonning and sequencing of PCR amplicon

Cloning using pGEM-T Easy vector (M/S Promega).

Ligation reaction set up: In a 1.5 ml tube the following components were added

5X ligation buffer – 5 µl

PCR product - 2 µl

pGEM-T Easy vector – 1 µl

DNA ligase enzyme – 1 µl

Water – 1 µl

Incubated at room temperature for one hour.

Cloning

1. Competent cells were thawed on ice.

   1) 2 µl of the ligated product was transferred into 50 µl competent *E.coli* cells and kept in ice for 20 min.

   2) Heat shocked the tubes at 42°C for 1 minute and returned to ice for about 2-3 min.

   3) Added 250 µl SOC medium and grown at 37°C for an hour; plated on LB Agar plates containing ampicillin (100 µg/ml) and 40 µl X-gal (2%).

   4) After overnight incubation, transformed white colonies grown were analyzed.

3.8.2 Southern blot analysis

For the confirmation of osmotin gene integration Southern hybridization was performed as per the standard procedure (Southern 1975) using the PCR products as well as *Eco*RI digest of genomic DNA.

3.8.2.1 Genomic DNA isolation

Isolation of DNA from the transgenic leaves and non-transgenic control leaves using the CTAB procedure as described earlier in section 3.8.1.1.

3.8.2.2 Restriction digestion of genomic DNA

Sufficient quantity of genomic DNA with good quality was isolated from transgenic leaf as well as non-transgenic leaf. Around 30µg of genomic DNA was digested with *Eco*R1 in separate reactions. The reaction mix (60 µl) was prepared as follows

DNA	-	30 µl
Enzyme buffer	-	6.0 µl
Restriction enzyme	-	4.0 µl (20 U)
ddH_2O	-	20 µl

The digestion was performed overnight at 37^0C. The fragments were size fractionated on 1.0 % agarose gel.

3.8.2.3 Blotting

Blotting was carried out following the standard procedure developed by Southern (1975). DNA digests were loaded in 1.0 % agarose gel and electrophoresis was carried out at 40V for 5h. The gel was processed after electrophoresis as follows:

i. DNA in the gel was de-purinated by soaking in a solution of 0.25 N HCl for 15 min and rinsed the gel twice with distilled water briefly.

ii. Denaturation of the DNA was carried out by treating the gel in denaturation solution (0.2M NaOH, 1.5M NaCl) for 25 min with gentle shaking followed by rinse with water.

iii. Gel was neutralized by soaking in neutralization [1M Tris-HCl (pH-8.0), 1.5M NaCl] buffer for 30 min.

iv. DNA was transferred from the treated gel to nylon membrane (Hybond N^+, Amersham, UK) through capillary blotting method (Sambrook and Russell, 2001). The gel after neutralization was briefly washed in 10X SSC [20 X SSC: 3M NaCl, 0.3M Sodium citrate (pH.7.0)]

v. A tray was filled to a height of 5 cm with 10X SSC. A suitable platform with dimensions slightly bigger than the gel was placed in the tray.

vi. The surface of the platform was covered with whatman No. 3 filter paper presoaked in 10X SSC in such a way that the ends of the paper are immersed in the SSC. Three sheets of Whatman No.1 filter paper trimmed to the same dimensions of the gel and presoaked in 10X SSC was placed on top of the platform. Air bubbles, if trapped any, were removed by rolling the surface with a glass rod.

vii. The gel was placed upside down carefully on top of this and then a Hybond N+ nylon membrane, presoaked in 10X SSC was placed on top of the gel.

viii. Two sheets of pre-soaked whatman No.1 filter papers were placed on top of this assembly. Three more sheets of clean dry filter paper were then stacked on it over which ordinary filter papers cut to the gel dimensions were stacked to a height of 10 cm. Over this, a suitable weight of around 200-300 g was placed in such a way that the weight should not crush the gel but should be sufficient to keep the papers tight. The transfer was allowed to proceed overnight for a period of 12-16 h.

ix. After transfer, the assembly was disassembled and the nylon membrane was briefly washed in 5X SSC and air-dried.

x. The membrane was fixed using a UV cross linker (M/S Hoefer, USA) at 12000 J/cm^2.The membrane was covered with saran wrap and stored between ordinary filter papers in the refrigerator.

3.8.2.4 Preparation of labeled probes

The osmotin gene from the construct was used as the probe for southern hybridization here. It was amplified from the cloned binary vector (osm/Bin AR) through PCR using gene specific primers, purified and radio-labeled using 'Multiprime DNA labeling system' from M/S Amersham (UK) following manufacturer's instructions. It utilizes random hexanucleotides to prime DNA synthesis on denatured template DNA at numerous sites along its length. The procedure is as follows:

i. About 50 ng of template DNA was diluted to 5 µl with dist. H_2O and boiled for 5 min. to denature.

ii. Chilled immediately on ice for 5 min and centrifuged briefly.

iii. Added 2.5 µl of buffer, which contains all the dNTPs except dCTP.

iv. Then 2.5 µl of random primer solution was added.

v. To this 2.5 µl of α-^{32}P labeled dCTP (sp. activity ~ 3000 Ci/mM or 10 µCi/µl) was added.

vi. Then 11.5 µl of nuclease free autoclaved water was added.

vii. Finally 1 µl of the enzyme (Klenow fragment of DNA polymerase I) was added and mixed gently by pipetting up and down.

viii. Spun for few seconds and incubated at 37°C for 30 min.

ix. The reaction was stopped by adding 0.5 µl of 0.5 M EDTA and the probe was diluted to 100 µl with distilled water.

The labeled probe was purified by passing through a Sephadex G-50 column as follows:

i. Sephadex G-50 was added to dist. water to form slurry (10 g of dry powder yields around 160 ml of slurry).

ii. Glass wool was placed at the bottom of a 1 ml column and 1 ml of the slurry was added without trapping air bubbles.

iii. The column was spun at 3000 rpm for 3 min in a swinging bucket rotor.

iv. More slurry was added until the Sephadex was tightly packed up to 1 ml level.

v. The column was equilibrated first with STE buffer and then with dist. water.

vi. The labeled probe was then passed through the column and purified.

vii. The column purified probe was denatured by boiling at 100°C for 3 min and immediately chilled in ice. It was stored in the freezer till use.

Such purification helps to remove all the small nucleotides and other unincorporated nucleotides to avoid background signals in the blot.

3.8.2.5 Hybridization of the blot

Hybridization of the labeled probe to the nylon membrane was performed according to Sambrook and Russell (2001).

i. The blotted membrane was placed in a hybridization tube and appropriate amount of pre-hybridization solution (0.2 ml/cm^2 of the blot, 25 ml for the 13x10 cm membrane) was added.

Pre-hybridization solution - 6 X SSC, 5 X Denhardt's reagent, 0.5 % SDS

50 X Denhardt`s reagent - 1.0 % BSA, 1.0 % Ficoll, 1 % polyvinyl pyrrolidone

ii. The pre-hybridization was carried out at 65 ^{0}C for 1 h in a hybridization oven (M/S Amersham, UK) with rotary movement at very low speed

iii. The pre-hybridization solution was poured out and hybridization solution (pre-hybridization solution containing denatured probe DNA labeled with α-^{32}P) was poured into the tube and then incubated with slow rotation for 12 – 16 h at 65 ^{0}C.

3.8.2.6 Washing the blot and autoradiography

i. After hybridization, the membrane was washed twice at room temperature for 5 and 15 min respectively with solution I.

Washing solution I - 2X SSC, 0.1 % SDS

ii. Then the blot was subjected to two high stringent washes at 65 ^{0}C for 30 min. each with solution II.

Washing Solution II - 0.1 X SSC, 0.5 % SDS

iii. The membrane was then floated briefly in 0.1 X SSC at room temperature, air-dried, wrapped in a cling film and subjected to autoradiography with the Phosphor image analyzer FLA 5000 (M/S Fujifilm, Japan). The blot was exposed to the image plate BAS IP (MS 2025) (M/S Fujifilm, Japan) and kept sealed in the BAS cassette for 4h. The image plate was removed and read in the Phosphor image analyzer with red laser beam (635 nm). The presence of the gene

insert in the transgenic *Hevea* genome was determined by visualizing the net intensity of the band in the southern blot.

3.8.3 Reverse Transcription PCR analysis

In order to study the expression of osmotin gene in *Hevea,* total RNA was isolated from the transgenic tissues and non-transgenic tissues (both callus and leaves) and cDNA synthesis was carried out using standard procedure. PCR was performed using osmotin specific primers with the cDNA as the template as explained in section 3.8.1.4

3.8.3.1 Isolation of RNA from leaf samples

RNA was isolated from transgenic tissues as well as from control leaves according to the procedure of Venkatachalam *et al.,* (1999) with suitable modifications. All the reagents required were prepared in DEPC treated H_2O. The protocol involved the following steps:

i) One g of callus tissue/ leaf was rinsed with DEPC treated H_2O and ground to a fine powder in liquid nitrogen.

ii) 10 ml of extraction buffer was added and the homogenate was transferred to a centrifuge tube.

iii) Equal volume of extraction buffer saturated phenol was also added, mixed gently and centrifuged at 10,000 rpm for 15min.

(Extraction buffer -0.2 M NaCl, 0.1 M Tris-HCl (pH-8.5), 0.01 M EDTA, 1.5% SDS, 0.1%, β-mercaptoethanol (added immediately before use) and insoluble PVPP (added to the homogenate).

iv) The upper aqueous phase was transferred to a new tube and re-extracted with equal volume of chloroform.

v) Centrifuged at 10,000 rpm for 10 min. and the aqueous phase were recovered.

vi) 1/3 volume of 8 M LiCl was added and the precipitation was continued overnight in ice.

vii) The RNA was pelleted by centrifugation at.10,000 rpm for 10 min at 4 °C.

viii) The pellet was washed first with 2 M LiCl followed by 100% ethanol, air-dried and dissolved in 1 ml DEPC treated sterile H_2O.

ix) For further purity, the RNA was re-precipitated with 0.1 volume 3 M sodium acetate (pH- 5.2) and 2.5 volume of ethanol.

x) The precipitated RNA was pelleted at 10,000 rpm for 10 min at 4°C and washed twice with 70% ethanol.

xi) Pellet was air-dried, re-suspended in 200 µl of sterile H_2O.

xii) The quantity of RNA was estimated spectrophotometrically and its quality and contamination, if any, with DNA was assessed in 1% agarose gel.

xiii) The isolated RNA was used immediately for subsequent analysis or stored in 3 vol. of ethanol at –70°C.

3.8.3.2 First strand cDNA synthesis

First strand cDNA was synthesized from the RNA isolated from tissues by reverse transcription reaction with oligo-(dT) primers using the 'Improm-II™ Reverse Transcription System' (M/S Promega, USA) as per the following procedure.

i) 1 µl of total RNA isolated from transgenic and non-transgenic tissues(1 µg each) was combined with 1 µl oligo-(dT) primers (0.5 µg). The reaction was made up to 5 µl by the addition of nuclease free water.

ii) The tube was incubated for 5 min at 70°C in a pre-heated block and immediately chilled on ice for 5 min.

iii) The tube was then spun down for 10 sec in a micro-centrifuge to collect the condensate and maintain the original volume. This RNA-primer combination was kept on ice

iv) The RT-reaction mix was prepared in a 1.5 ml tube on ice. 4 µl of reaction buffer supplied by the manufacturer along with 1.5 µl $MgCl_2$ (1.8 mM), 1 µl d NTP mix (0.5 mM of each d NTP) and 1 µl reverse transcriptase was made up to 15 µl with nuclease free water.

v) The RNA-primer mix (5 µl) was added to the reaction mix to form the final volume of 20 µl.

vi) Annealing was done by incubating the reaction at 25°C for 5 min.

vii) Primer extension was carried out at 42°C for 1 h in a heating block.

viii) The reaction was stopped by inactivating the reverse transcriptase by keeping the tube at 70°C for 15 min.

ix) The synthesized first strand cDNA was stored at –20°C for subsequent PCR amplification.

3.8.3.3 PCR analysis of cDNA

i) PCR amplification was carried out as described in section 3.8.1.4 using osmotin gene specific primers with non-transgenic cDNA as

the negative control, plasmid as the positive control and transgenic cDNA as the test sample.

3.9. Evaluation of stress tolerance in transgenic cell lines

3.9.1 Evaluation of drought tolerance

Free proline content was estimated in transgenic and non transgenic calli using the following the procedure of Bates *et al.*, (1973).

0.5g plant tissue was taken in a pre-washed mortar and pestle and homogenized in 5ml of 3% sulphosalycylic acid. The homogenate was filtered through Whatman No.1 filter paper and collected the filtrate. 2ml of extract was taken in a test tube and 2ml of glacial acetic acid and 2ml ninhydrin reagent was added. The reaction mixture was heated in a boiling water bath for 1 hour till a brick red colour developed. After cooling the reaction mixture, 4 ml of toluene was added and then transferred to a separating funnel. After thorough mixing, the chromospheres containing toluene is separated and the absorbance was measured at 520 nm in spectrophotometer against toluene blank. The standard curve of proline was prepared by taking 5 to 100 µg/ml concentrations. Free proline content in sample was estimated by referring to a standard curve made from known concentrations of proline by following the formula.

Calculation

Proline content was expressed on fresh-weight-basis as follows:

$$\mu M \text{ per g tissue} = \frac{\mu g \text{ proline/ml x ml toluene}}{115.5} \times \frac{5}{\text{Wt. of sample (g)}}$$

Where, 115.5 is the molecular weight of proline.

The transgenic and one non transgenic cell lines were cultured over callus proliferation media simultaneously. After a period of 3 weeks, both the calli were transferred to the same media supplemented with 6 % PEG in order to induce water stress. After 3 weeks of culture in this medium, the proline content was estimated in all the cell lines as mentioned above.

3.9.2 Evaluation of salt tolerance of transgenic cell lines

The transgenic cell lines along with the non transgenic control were subcultured to callus proliferation media. After 3 weeks, the calli were subjected to salt stress by culturing the callus over medium containing different concentrations of sodium chloride (50-200mM) for a period of 20 days and observations were recorded. 50 callus clumps were kept in each treatment and the treatments were replicated 5 times.

All the experiments were carried out according to the Biosafety guide lines DBT, Govt. of India for recombinant-DNA work. Transgenic plants were grown in containment facility.

3.10 *In silico* analysis of transgene (osmotin)

Since a few reports were available in the literature for the presence of osmotin protein in *Hevea, in silico* analysis of the osmotin gene sequence was performed, in order to confirm the presence/absence of similar gene sequence in *Hevea.* The sequence of the inserted gene was blasted against the NCBI database to identify similar sequences, and with the sequence contigs of recently published *Hevea* whole genome sequence data.

CHAPTER 4

RESULTS

4. RESULTS

Agrobacterium mediated genetic transformation experiments resulted in successful integration of osmotin gene into *H. brasiliensis* callus. The transgenic cell lines were developed, proliferated, embryos induced, matured and germinated to develop transgenic plants integrated with osmotin gene. The transgene integration and expression were confirmed by molecular and biochemical analysis. The results of the experiments carried out for developing transgenic plants integrated with osmotin gene are given below.

The first step in the development of a transgenic plant is the generation of transgenic cell lines, which involves the identification of suitable explants, transformation protocol and an efficient selection procedure. The explants were raised as described in the previous session. Transformation experiments were carried out following two methods and the negative selection system based on kanamycin resistance was followed.

4.1 Development of transgenic cell lines

4.1.1 Influence of target tissue

Explants have a significant role in transformation efficiency as different explants behaved differently (Table 4.1). Among the different types of explants used for transformation, highest transformation frequency (76.4%) was obtained for the embryogenic calli derived from immature zygotic embryo, followed by embryogenic calli derived from anther (48%). Transformation frequency was very low for the two-month-old primary calli from anther. The intact explants as well as 2-month-old calli from the ovule failed to give any positive results and dried after two weeks in the selection medium. It was clearly indicated that embryogenic calli produced higher

number of transformants compared to primary calli. It was also observed that further proliferation of transgenic lines was better for embryogenic calli, irrespective of the source. Even though a few transgenic lines were obtained from anther derived two-month-old calli, they failed to proliferate.

Table.4.1 Influence of target tissue on transformation efficiency

No.	Explants	Transformation frequency (%)	
		Method 1	Method 2
1.	2 month old anther calli	6.00	11.60
2.	Embryogenic calli from ovule	10.4	19.20
3.	Embryogenic calli from anther	32.2	48.00
4.	Embryogenic calli from zygotic embryo	44.8	76.40
	CD (0.05)	6.11	5.88

4.1.2 Genetic transformation protocol

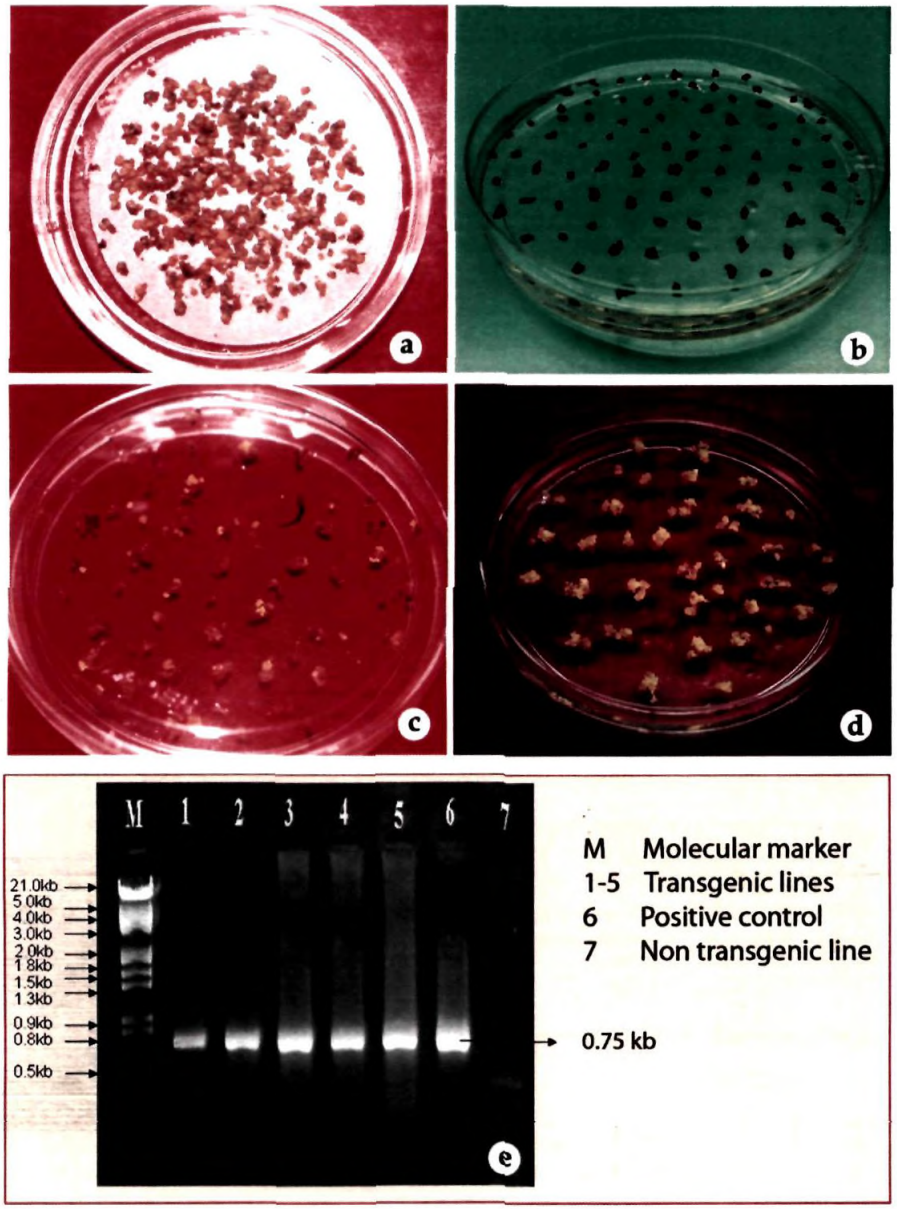
Among the two methods of transformation tried, method 2 was found to be ideal than the method 1 in three aspects. The transformation frequencies were significantly higher in method 2 compared to that of method 1 irrespective of the explants used. Number of transgenic lines could be enhanced from 32.2 to 48 % and 44.8 to 76.4% for the anther derived and zygote derived embryogenic calli respectively. The results are given in table.4.1 Moreover, in the method 2, overgrowths of bacteria in the selection medium were considerably reduced and this method required less time for inoculum preparation (3 days instead of 5).

4.1.3 Selection of putative transgenic cell lines

After co-culture for three days, the infected calli were transferred to selection medium with appropriate antibiotics (kanamycin 300 mg l^{-1} and cefotaxime 500 mg l^{-1}). In the selection medium, most of the calli clumps gradually turned brown initially. Fresh creamy yellow cell clusters started emerging from the clumps after 40 to 60 days of culture in the selection medium (Fig 4.1b). With a view to eliminate false positives, fresh cell clusters were recovered from dying explant tissue and transferred to fresh selection medium. After 2 to 3 weeks of culture in the fresh medium with kanamycin, putative transformed cell lines, showing resistance to kanamycin emerged (Fig.4.1c). Emergence of transgenic cell lines from the cultures with liquid co-culture system was faster than the solid co-culture system. This may be due to the increased access of nutrients from the liquid co-culture medium. The putative transgenic lines were separated individually and transferred to proliferation medium fortified with kanamycin. Since each line represents a single transformation event, each transgenic cell line showing kanamycin resistance was handled individually.

4.1.4 Proliferation of transgenic cell lines

Proliferation of transgenic calli integrated with osmotin gene was found to be very difficult. Even though the calli appeared to be yellow and friable, the growth was very slow in the medium already standardized for *Hevea* callus proliferation. Different growth regulators were tried in modified MS medium. Positive results and better proliferation were obtained in the presence of 2, 4-D and NAA. Table 4.2 shows the effect of different levels of 2, 4-D in presence of 1 mg l^{-1} NAA.



a. Co-culture with *Agrobacterium*
b. Selection in kanamycim medium
c. Emergence of transgenic cell lines
d. Proliferation of transgenic callus
e. Confirmation of gene integration by PCR

Fig. 4.1 Development on transgenic cell lines from *Hevea* callus

Table.4.2. Effect of 2, 4-D and NAA (1 mg l^{-1}) on callus proliferation

2,4-D (mg l^{-1})	Callus proliferation (%)	Appearance of calli
0.5	19.53 (11.2)	Yellow friable calli
1.0	34.57 (32.2)	Yellow embryogenic calli
1.5	60.00 (75)	Yellow embryogenic calli
2.0	73.38 (91.8)	Loose watery calli
2.5	93.2 (74.94)	Loose white calli
	CD (0.05) = 1.39	

Data were subjected to square root transformation and transformed means are given in parenthesis

A steady increase in the callus proliferation rate with increase in 2, 4-D was observed. However, up to 1.5 mg l^{-1} 2, 4-D, the calli were yellow and friable whereas at higher concentrations the calli turned white and watery. Hence, 1.5 mg l^{-1} 2, 4-D along with 1 mg l^{-1} NAA was selected as the optimum concentration for callus proliferation and thereby a proliferation rate of 60% could be achieved with the transgenic lines derived from embryogenic calli (Fig.4.1.d). However, the cell lines derived from primary calli failed to proliferate in all the media tried. Actively proliferating lines were subjected to PCR analysis and the PCR positive lines were further used for embryo induction. Rest of the lines showing minimal growth was discarded.

4.1.5 PCR analysis of transgenic cell lines

Since the gene construct is not having any reporter gene, the only way for the preliminary assurance of transformation is PCR. DNA isolated from the proliferated transgenic cell lines were subjected to PCR analysis for the confirmation of gene integration with non-transgenic calli as the negative control and plasmid as the positive control. Results shows that all the lines tested were PCR positive. The 0.75 kb osmotin gene insert could be positively amplified from all the transgenic cell lines whereas no amplification was obtained from the non-transgenic cell (Fig. 4.1.e). The lines 1-5 in the figure are transgenic cell lines, line 6 is the positive control and line 7 is the non-transgenic calli. This indicates the presence of the transgene in the transformed cell lines of *H. brasiliensis* genome. The PCR positive lines were used for further experiments. These lines were sub cultured to different formulations of embryo induction medium.

4.2. Developing transgenic plants

4.2.1. Embryo induction

Embryos could be developed from the proliferated calli after subculture in to embryo induction medium. From the different experiments carried out for embryo induction, it was clearly observed that growth regulators play a pivotal role in embryogenesis from the osmotin transgenic cell lines. The optimum level of the growth regulators, NAA and Kinetin for embryo induction were derived from a factorial experiment and the results are presented in table.4.3

Table 4.3 Effect of NAA and Kinetin on embryo induction from transgenic cell lines derived from anther derived embryogenic calli.

	Embryo induction percentage					
NAA → Kin mg l^{-1} ↓	1.0	2.0	3.0	4.0	5.0	10
0.1	14 (21.97)	25.5 (30.18)	25.0 (30.34)	15 (22.79)	15.75 (23.39)	12.25 (20.44)
0.2	17.5 (24.74)	30 (33.13)	25.25 (30.18)	27.25 (27.46)	17.75 (24.93)	15 (22.79)
0.3	20.75 (27.11)	**43.5 (41.29)**	38 (38.01)	32.75 (34.93)	27.75 (31.80)	19.5 (26.21)
0.5	21 (27.29)	31.5 (34.16)	37 (37.48)	33.25 (35.23)	27 (31.32)	20.75 (27.11)
1.0	17.25 (24.55)	31.75 (34.31)	27.75 (31.80)	25.75 (3 0.5)	23 (28.67)	7.75 (16.17)
				CD (0.05)	(Kin x NAA)	= 1.23

The data were subjected to arcsine transformation and transformed means are given in parenthesis

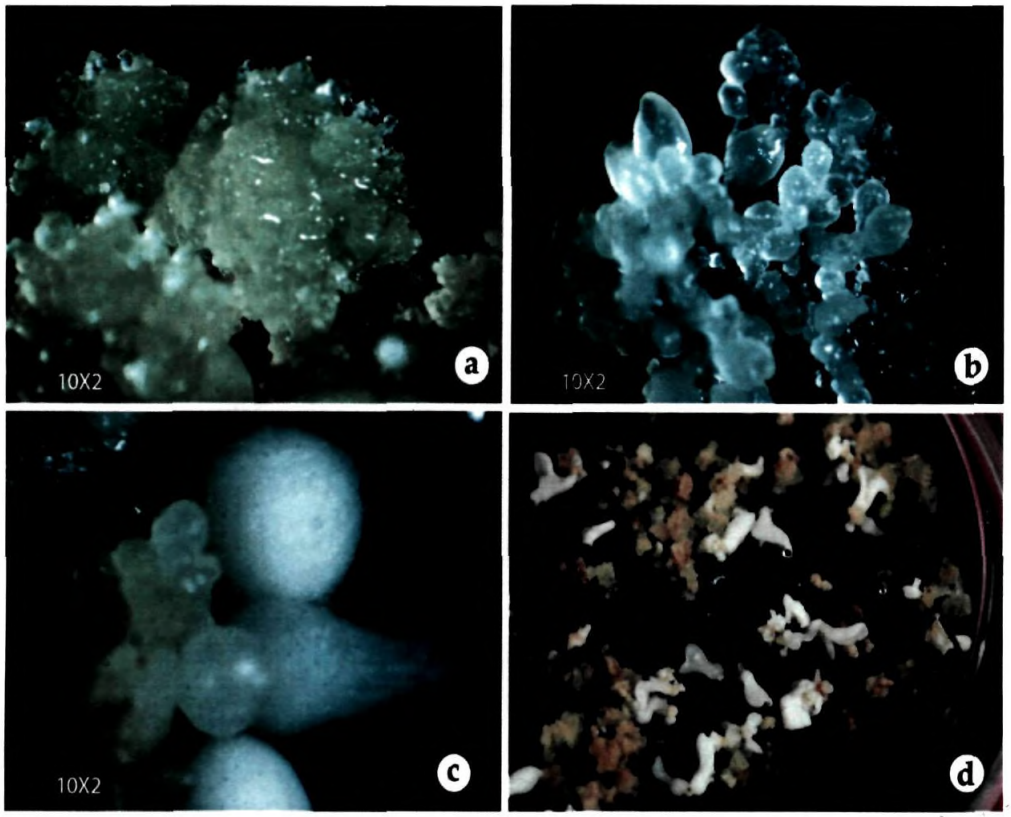
It can be observed that NAA and kinetin influenced induction of somatic embryos from transgenic cell lines integrated with osmotin gene. The highest percentage of embryo induction was obtained in a combination of 2 mg l^{-1}NAA and 0.3 mg l^{-1} kinetin. Further increase in concentration of NAA resulted in excess proliferation of the callus and reduction of embryo

induction frequency. Higher concentrations of kinetin also showed significant reduction in embryo induction from transgenic cell lines. The time taken for embryo induction was about 80-100 days in this medium. Initially the embryos were translucent, then turned milky white and gradually turned to ivory colour (Fig.4.2.a-d) The induced embryos were asynchronous in nature (Fig. 4.2.c).

Table 4.4 Effect of ABA and GA_3 in presence of NAA (2 mg l^{-1}) and kinetin (0.3 mg l^{-1}) on embryo induction

GA_3 (mg l^{-1}) Embryo induction percent					
ABA (mg l^{-1})	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**
0.1	9 (17.2)	28.5 (32.21)	**50 (45)**	42 (40.4)	37.5 (37.76)
0.2	15.25 (22.98)	30.5 (33.5)	42 (40.39)	39.5 (38.94)	26.25 (30.76)
0.3	20 (26.56)	34.5 (35.97)	39.5 (38.09)	33.5 (35.3)	21.5 (27.62)
0.4	17 (24.32)	27 (31.3)	32.75 (34.91)	24.5 (29.66)	20 (26.55)
0.5	12.5 (20.69)	16 (23.55)	28.5 (32.26)	15 (22.76)	12 (20.23)
			CD(0.05)	(ABA X GA_3) =	1.5

Data were expressed in mean percentage and were subjected to arcsine transformation and transformed values are given in parenthesis



a. Embrygenic callus
b. Globular embryos
c. Asynchronus embryo development
d. Syncronus development of embryo

Fig. 4.2 Embryo induction from transgenic cell lines in *Hevea*

Embryo induction frequency could be further enhanced when GA_3 and ABA were supplemented along with NAA and kinetin (Table 4.4). An enhanced embryo induction frequency (50%) was obtained in EI medium fortified with 0.3 mg l^{-1} kinetin, 0.1mg l^{-1} ABA, 0.3 mg l^{-1} GA3 and 2 mg l^{-1} NAA. ABA at higher concentrations reduced the frequency of embryo induction. Similarly increasing concentration of GA_3 beyond 0.3 mg l^{-1} also inhibited embryo induction. Moreover, supplementing ABA in the medium could reduce precocious germination of the embryos as a result of which the development of embryos was more synchronous (Fig. 4.2.d)

4.2.2. Embryo maturation

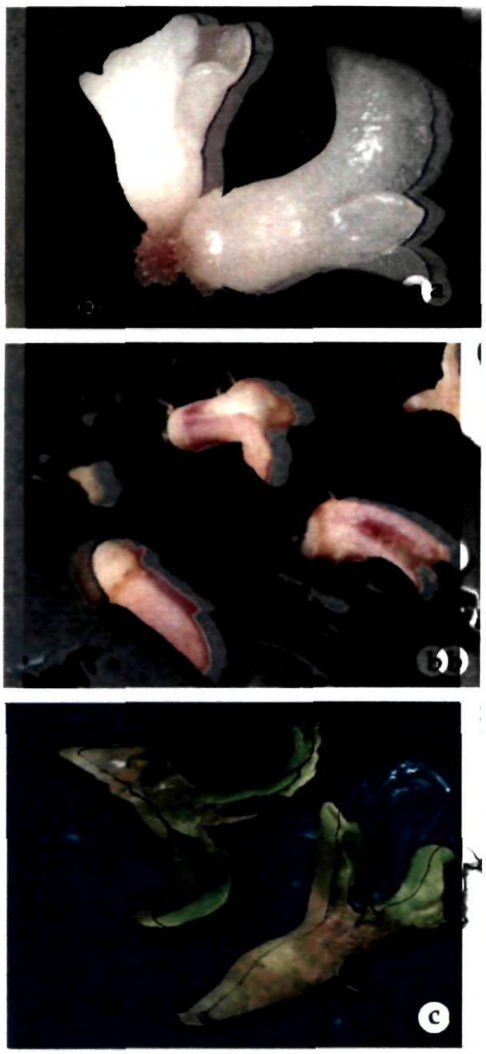
The globular embryos developed into cotyledonary embryos in the embryo induction medium itself. The cotyledonary embryos were transferred to embryo maturation medium. For embryo maturation, the effect of ABA in combination with different levels of phytagel was studied in hormone free EI medium along with organic supplements and charcoal. The effect of ABA and phytagel on maturation of transgenic embryos is given in Table.4.5. It was observed that the maturation of embryos improved with higher concentration of phytagel as well as ABA. Maximum maturation was observed in a combination of 0.5% phytagel along with 0.8 mg l^{-1} ABA. The embryos enlarged in size and the cotyledons turned opaque. Maturation of the embryos needed four to six weeks under dark incubation.

Table 4.5 Effect of phytagel and ABA on maturation of transgenic embryos integrated with osmotin gene

	Embryo maturation percentage				
ABA (mg l^{-1}) → Phytagel (%) ↓	**0.2**	**0.4**	**0.6**	**0.8**	**1.0**
0.2	14.75 (22.58)	17.75 (24.91)	21.75 (27.79)	27.25 (31.47)	30.75 (33.68) *
0.3	13.75 (21.75)	22.75 (28.48)	28.25 (32.105)	32.00 (34.443)	35.0 (36.27)
0.4	20.75 (27.08)	27.75 (31.78)	31.75 (34.29)	34.0 (35.67)	35.75 (36.72)
0.5	28.0 (31.94)	35.0 (36.27)	41.25 (39.963)	**46.0 (42.70)**	38.0 (38.055)
0.6	29.5 (32.86)	24.0 (29.33)	24.5 (29.66)	22.5 (28.30)	20.25 (26.74)
			CD(0.05)	ABA X phytagel	=1.16

Data were subjected to arcsine transformation and transformed means are given in parenthesis.

Embryo maturation in *Hevea* is reported to be improved by the addition of high levels of sucrose. Hence, different levels of sucrose and phytagel were tried along with 0.8 mg l^{-1} ABA in order to evaluate the combined effect of these two on embryo maturation. Result of this experiment is presented in table 4.6.



a. Cotyledonary embryos
b. Embryos showing accumulation of protein reserve
c. Fully matured embryos

Fig. 4.3 Transgenic embryos at different stages of maturation

Table 4.6 Effect of sucrose and phytagel in presence of ABA (0.8 mg l^{-1}) on embryo maturation

	Embryo maturation percentage				
Phytagel (%) → Sucrose g l^{-1} ↓	0.2	0.3	0.4	0.5	0.6
30	10.0 (18.43)	14.75 (22.589)	17.75 (24.93)	22.00 (27.99)	32.75 (34.93)
50	25 (30.013)	28 (31.97)	32 (34.62)	45 (42.15)	38.00 (38.53)
75	28 (31.96)	34 (35.84)	**51 (45.88)**	45 (42.44)	40 (39.25)
100	21 (27.80)	28 (31.963)	36 (45.88)	46.0 (42.87)	38 (38.07)
150	16.75 (24.16)	19.00 (25.85)	17.75 (24.92)	20.0 (26.57)	17.0 (24.36)
			CD **(0.05)**	Phytagel x Sucrose	= 0.89

A combination of 75 g l^{-1} sucrose and 4 g l^{-1} phytagel favoured embryo maturation and a high maturation frequency of 51% could be obtained. Further increase in both the components had a negative trend in embryo maturation. Under dark incubation, the embryos imparted light pink colour indicating the accumulation of storage proteins (Fig. 4.3.b) and turned green when transferred to light incubation (Fig. 4.3.c). The matured embryos were transferred to germination medium.

4.2.3 Embryo germination

Germination of the embryos proceeded sequentially in two steps 1- Emergence of root and shoot 2- Development of leaves and plantlet regeneration.

4.2.3.1 Effect of growth regulators

Among the growth regulators tried, a maximum germination of 23.6% was obtained in a combination of 1.5 mg l^{-1} BA and 1.5 mg l^{-1} GA_3 in MS basal medium. Higher concentrations had a negative effect on the germination of embryos (Table 4.7) (Fig. 4.5 a-d).

Table 4.7 Effect of BA and GA_3 on germination of transgenic embryos

BA (mg l^{-1}) → GA_3 (mg l^{-1}) ↓	0.5	1	1.5	2	5
0.5	10.40 (3.22)	12.40 (3.64)	12.8 (3.57)	14.40 (3.78)	10.80 (3.28)
1	10.80 (3.27)	14.40 (3.79)	17.20 (4.14)	15.60 (3.94)	12.0.00 (3.46)
1.5	14.40 (3.78)	18.40 (4.23)	**23.6 (4.86)**	17.20 (4.15)	14.40 (3.46)
2	13.6 (3.78)	17.2 (3.68)	17.20 (4.14)	16.40 (4.05)	9.60 (3.08)
5	9.60 (3.08)	10.00 (3.15)	14.40 (3.78)	14.40 (3.78)	7.60 (2.74)
			CD(0.05)	interaction	0.28

The values were subjected to square root transformation and transformed values are given in parenthesis

4.2.3.2 Effect of desiccation on embryo germination

Of the different desiccation treatments tried, slow drying was found to be beneficial for embryo germination. On the contrary, rapid drying resulted in drying of embryos and no germination could be observed. Slow drying in closed petridishes for 48 hrs could enhance embryo germination to

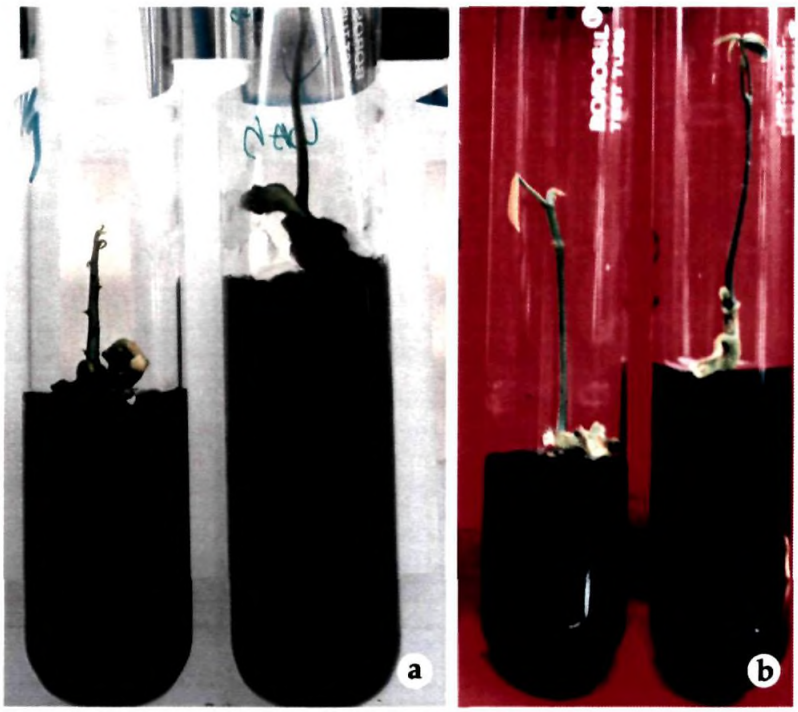
44 % (Table 4.8). However, after the initial boost up in germination; further growth was found to be retarded. The leaf expansion and plantlet development was found arrested (Fig. 4.4.a).

Table 4.8 Effect of desiccation treatment on embryo germination

Desiccation treatments (Hrs)	Embryo germination Percentage
T1 (6)	20.75
T2 (12)	23.75
T3 (24)	29.50
T4 (48)	**44.75**
T5 (72)	35.00
CD (0.05)	1.92

4.2.3.3 Effect of suspension cultures on embryo germination

When the embryos were cultured in liquid medium for a short period, it was found to be beneficial for the germination of the embryos. By visual scoring, it was seen that keeping the embryos in hormone free liquid medium for three days, and further transfer to germination medium fortified with growth regulators, had a beneficial effect on embryo germination. The cotyledons turned green quickly and shoot emergence was faster (Fig.4.4.b). However, the high risk of contamination prevented the use of the technique.



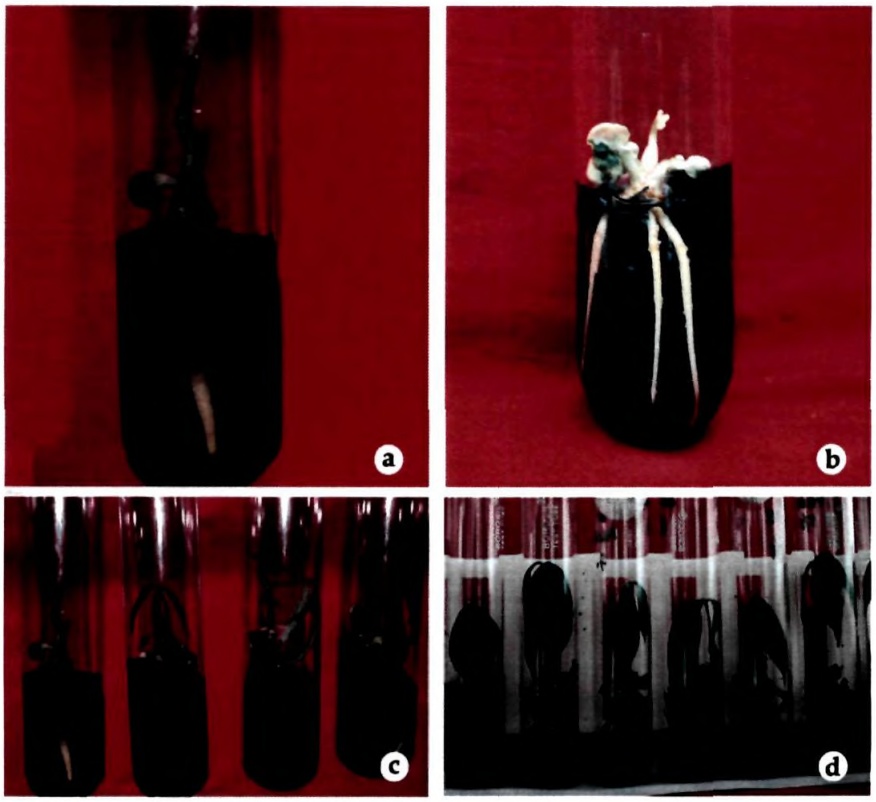
a. Germination after desiccation treatment
b. Germination after suspension culture

Fig. 4.4 Germination of transgenic embryos

4.2.4 Plant regeneration

The germinated embryos were transferred carefully into the plant regeneration medium so that the root tips are not damaged. During the plant regeneration phase, repeated subculturing of the regenerated plants resulted in much damage of the root and shoots leading to senescence of the plantlets. In order to overcome this, culture tubes were prepared with adequate medium enough to last for about two months. For plant regeneration, IBA was tried along with BA, in presence of 1.5 mg l^{-1} GA_3 (Table 4.9). Results showed that IBA along with BA had a positive influence on plant regeneration (Fig.4.5 a-d.). About 23 % plant conversion frequency could be

obtained in a combination of 3 mg l^{-1} BA and 1.5 mg l^{-1} IBA. Further increase in both the growth regulators reduced the percentage of plant conversion.



a & c. Germination and plant let development from transgenic embryo from anther derived callus
b & d. Germination and plant let development from transgenic embryo from zygote derived callus

Fig. 4.5 Germination and plantlet development of transgenic embryos

Table 4.9 Effect of IBA and BA on development of transgenic plantlets

BA (mg l^{-1}) → IBA ↓	1	2	3	4	5
0.5	4.0 (2.16)	8.0 (2.97)	7 (2.79)	7 (2.79)	10 (3.28)
1	8 (2.90)	9 (3.10)	10 (3.28)	13 (3.73)	8 (2.97)
1.5	10 (3.28)	12 (3.59)	23 (4.89)	19 (4.45)	15 (3.98)
2	10 (3.28)	9 (3.10)	15 (3.98)	12.00 (3.53)	14.0 (3.86)
2.5	9 (3.14)	8 (2.97)	8 (2.97)	7 (2.79)	8 (2.93)
				CD (0.05)	0.66

Values were subjected to square root transformation and transformed values are given in parenthesis.

4.2.5 Effect of sugars and sugar alcohols on different stages of plant regeneration

In order to improve the plant regeneration pathway, different sugars and sugar alcohols were incorporated in the already developed media, for different stages of plant regeneration. Results are summarized in Table.4.10. It was observed that in all stages of plant regeneration, mannitol had a definite role. Maximum frequency of embryo induction (67%), maturation (63%), germination (27%) and plant conversion (23%), was obtained in a medium supplemented with mannitol. The optimum concentration of mannitol was found to be 5 g l^{-1} except for embryo maturation. For maturation, a higher level of mannitol (10 g l^{-1}) was found to be ideal. All these observations clearly illustrates that mannitol played a major role in maximizing the results in different stages of plant regeneration from transgenic cell lines of *Hevea* integrated with osmotin gene. For the other

sugars and sugar alcohols tested, the values were on par or less than sucrose supplemented medium. Except for plant conversion, addition of mannitol was beneficial compared to the medium supplemented with sucrose alone.

Table 4.10 Effect of sugars and sugar alcohols on various stages of transgenic plant regeneration

Sugars/sugar alcohols(g l^{-1})		Induction	maturation (%)	Germination	Plant conversion (%)
Glucose	20	33.00	39.00	12 (3.46)	4 (2.04)
	50	48.75	44.00	10.8 (3.27)	9 (3.14)
	75	51.25	47.75	12.8 (3.58)	13 (3.73)
	100	51.50	49.50	9.2 (3.03)	11 (3.45)
	CD	*4.92*	*3.19*	*0.31*	*0.79*
Maltose	20	35.25	33.75	15.6 (3.95)	7(2.61)
	50	40.25	37.50	16.4 (4.05)	11 (3.30)
	75	42.75	40.25	17.2 (4.15)	13 (3.59)
	100	47.00	43.00	15.6 (3.95)	11 (3.26)
	CD	*2.6*	*2.29*	*0.26*	*0.66*
Sorbitol	2	39.5	47.5	13.2 (3.63)	4 (2.16)
	5	45.25	51.25	14.4 (3.79)	6 (2.62)
	10	43.25	47	13.6 (3.68)	11 (3.42)
	20	46.25	40	16.8(4.10)	9 (3.11)
	CD	*3.12*	*1.77*	*0.25*	*0.79*
Mannitol	2	51.5	47.00	22.4(4.73)	17 (4.23)
	5	**67.5**	52.50	**27.6 (5.25)**	**23(4.89)**
	10	54.25	**63.50**	16 (4.00)	7 (2.80)
	20	42.5	59.00	14(3.74)	4 (2.16)
	CD	*1.87*	*2.09*	*0.24*	*0.61*

The values were subjected to square root transformation and transformed values are given in Parenthesis

4.3. Acclimatization

Well-developed plants with 2-3 mature leaves were transferred for hardening. Transgenic plants integrated with osmotin gene could be

acclimatized successfully, when the plantlets were kept in environment controlled growth chamber with gradual reduction in humidity. Eight plants derived from the zygotic source were acclimatized and transferred to polybags. A few plants derived from anther derived transgenic cell lines are in the process of hardening (Fig. 4.6a).

4.4 Comparison of clonal and zygotic transgenic cell lines on different stages of plant regeneration

Plant regeneration was achieved from transgenic cell lines belonging to the two different explant sources viz. embryogenic calli derived from anther and embryogenic calii derived from zygote, and are shown in Fig. 4.5 & Fig. 4.6.

Table.4.11 Efficiencies of transgenic cell lines derived from clonal and zygotic sources on different stages of the regeneration pathway

No	Embryo induction (%)	Embryo maturation (%)	Embryo germination (%)	Plant conversion (%)	Hardening (%)
Clonal	67	63	27	23	10
Zygotic	72	65	44	40	50

It was observed that the initial source of target tissue had a strong influence on plant regeneration from transgenic cell lines (Fig. 4.5.a-d). During the course of plant development, the highest response was registered for zygotic embryo derived callus, compared to immature anther derived callus which is clonal in origin. For embryo induction and maturation, no significant difference was noticed between the cell lines. However, the embryos were larger and with well developed cotyledons in the case of

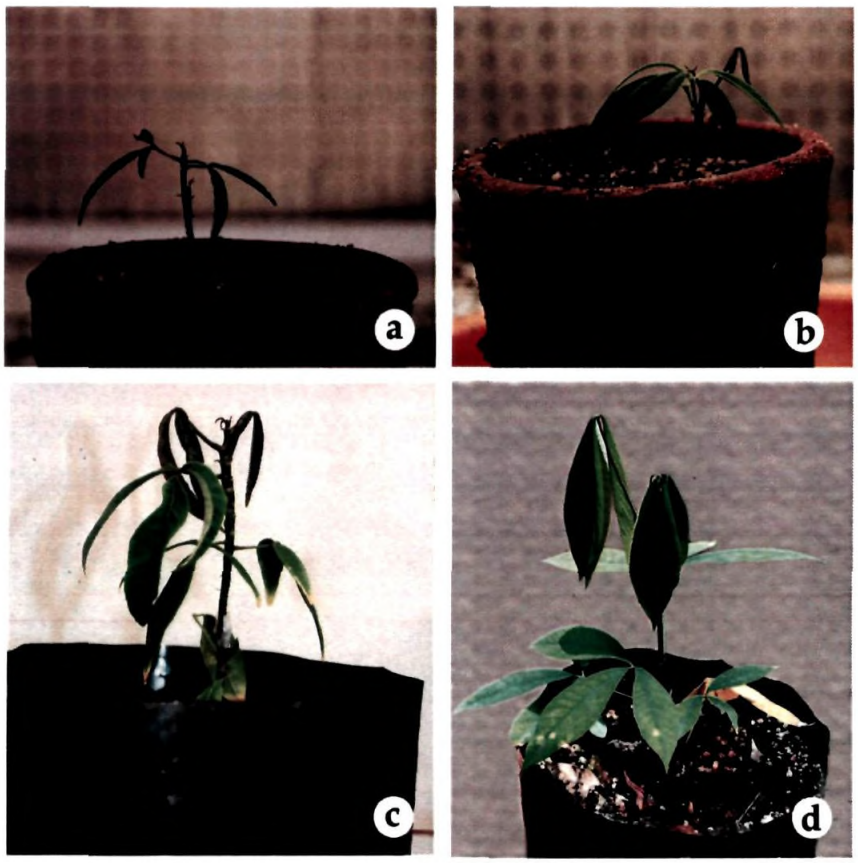
zygotic embryo derived transgenic callus (Fig. 4.5-b & Fig. 4.5.d). Also, plant conversion frequency for zygotic embryo derived transgenic embryos was much higher (almost double) compared to embryos of clonal origin. The plant conversion frequency of 40 per cent was obtained from zygotic embryo derived transgenic cell lines, whereas anther derived transgenic cell lines showed only around 23 per cent conversion frequency (Table 4.11).

It was also observed that the plants derived from zygotic source responded favorably to acclimatization. They are healthier and had more number of lateral roots compared to anther derived plants (Fig.4.6 b& d).

Thirty-five transgenic plants could be regenerated from the clonal material. However, upon acclimatization, the mortality rate was high leaving behind only 10 plants at the end of initial hardening in the growth chamber and they were transferred to big polybags. Later on, after 2 months two surviving plants could be transferred to the net house. On the contrary, out of the 16 transgenic plants raised from the zygotic source, eight plants could be transferred to the net house.

4.5 Micrografting

Since the acclimatization process was found to be a major hurdle in developing transgenic plants, attempts were also made for grafting of the transgenic shoots on to stock seedlings raised both *in vitro* and *in vivo*. Out of the ten transgenic shoots grafted to seedlings, raised *in vitro*, from immature embryos, one graft was successful and new flushes were developed from the transgenic shoot. The graft was transferred to polybags (Fig. 4.6.c). However, after a period of six months it dried off.



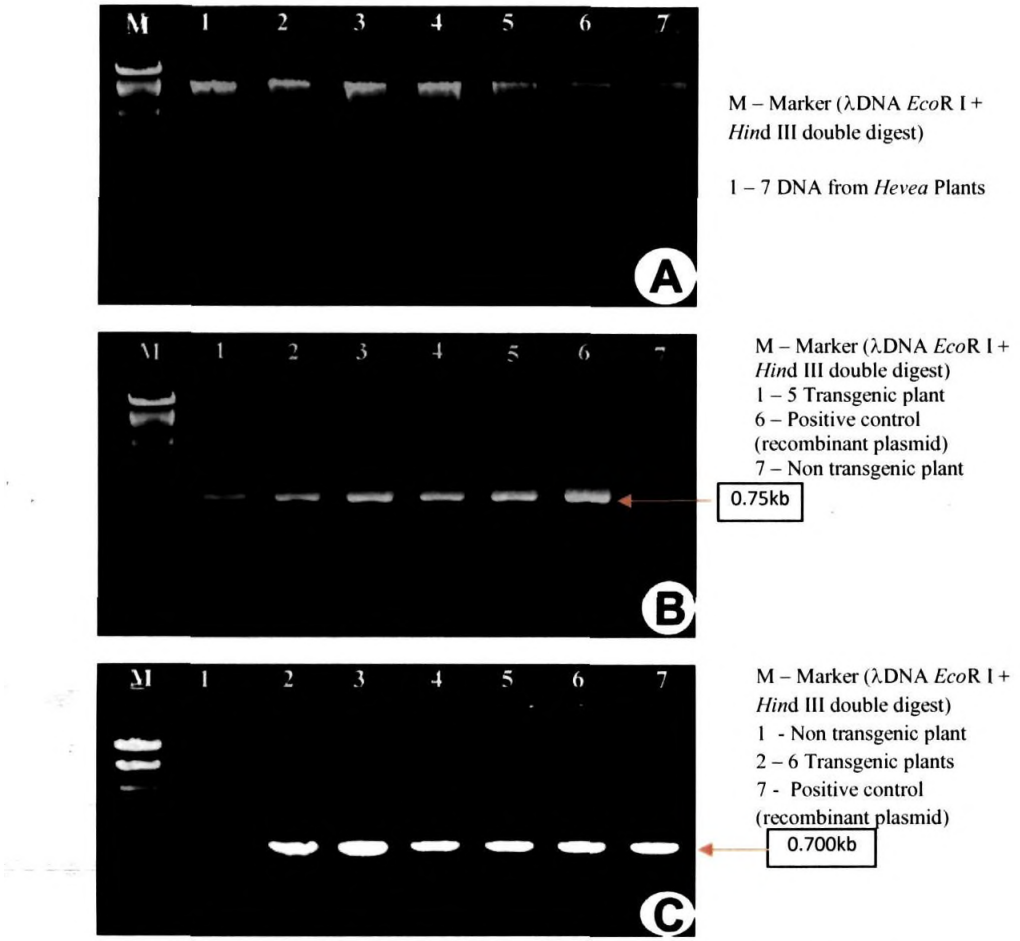
a. Transgenic plant derived from clonal source in the acclimatization process
c. Performance of transgenic shoot two months after micrografting
b.&d. Acclimatization of plantlet derived from zygotic source

Fig. 4.6 Acclimatisation of transgenic plant from different sources

4.6 Molecular confirmation of gene integration and expression in transgenic plants

4.6.1 PCR analysis

PCR analysis using gene specific primers could successfully amplify the 0.75 kb gene insert coding for osmotin protein and 0.7kb *nptII* gene (Fig. 4.7 B & C). The osmotin amplicon was cloned and sequenced and the sequence showed 100% similarity with the inserted gene, confirming the transgene integration.



(A) Quality of DNA isolated (B) Amplification of osmotin gene (750bp) (C) Amplification of npt II (700 bp) gene

Fig.4.7 Validation of transgenic plants for the presence of marker and trait specific genes

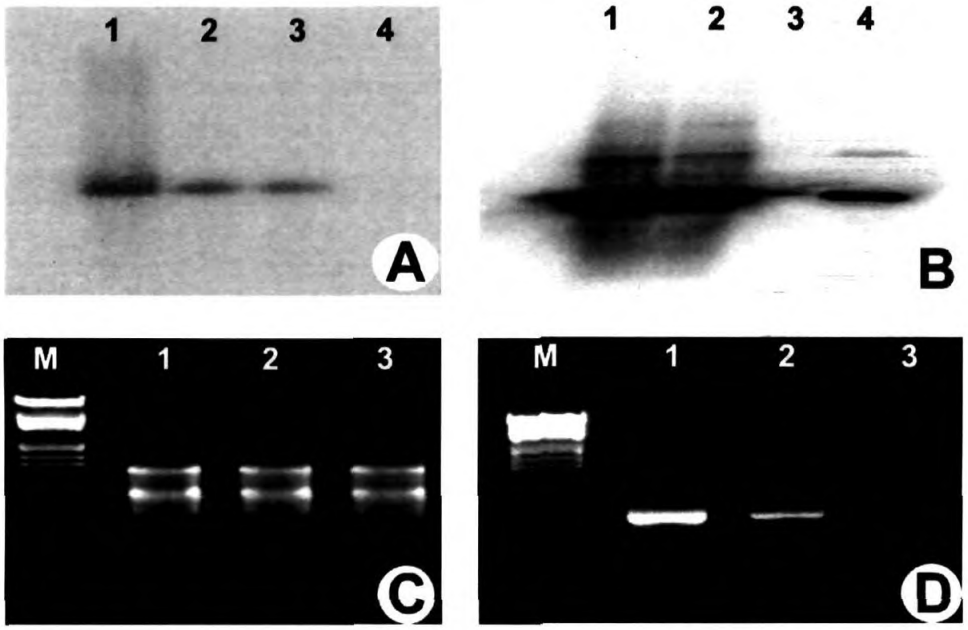
4.6.2 Southern blot analysis

Southern hybridization could prove the successful integration of the transgene in to *Hevea* genome. Results are presented in the Fig.4.8 A & B. Figures show the hybridization of the probe with the positive control, where the PCR amplified product of DNA was used as the template and positive signal was obtained in the lane corresponding to 0.75 kb in the marker. First lane is the amplified product from plasmid, second and third lanes showing

the same from DNA isolated from transgenic plants. Figure 4.8.B shows the blot with *Eco*R I digestion, positive signals were obtained at different lanes indicating the random integration of gene insert. Three bands were obtained in the first two transgenic plants and 2 bands were obtained for the 4th transgenic plant. In the 3rd lane, signals were absent.

4.6.3 RT-PCR-analysis

The 0.75 kb osmotin gene insert could be amplified from the cDNA prepared from the RNA isolated from the leaves of transgenic plants, which in turn confirms the transgene expression (Fig. 4.8 C & D).



(A) Positive signals obtained in the southern blot of transgenics (PCR product)
1 – Plasmid, 2 – 3 PCR amplicon of osmotin from transgenic plants
(B) Positive signals obtained in the southern blot of transgenics (*Eco*R I digest)
1 – 4 DNA from transgenic plants
(C) Quality of RNA isolated from *Hevea*
(D) Amplification of cDNA isolated from transgenic plants.
M-marker, lane1-plasmid, lane 2-amplification of osmotin gene from cDNA

Fig. 4.8 Confirmation of gene integration and expression by Southern and RT-PCR analysis

4.7 Evaluation of stress tolerance in transgenic cell lines

4.7.1 Evaluation of drought tolerance

The estimation of free proline content under unstressed condition revealed that transgenic cell lines show a slightly higher level of proline content compared to control. Under stressed conditions, the proline estimation data showed that there is an increase in the proline content with increase in water stress for both transgenic and non-transgenic cell lines. However the percentage increase in proline content was much higher for transgenic cell lines when compared to non-transgenic calli (Fig 4.9). Almost two-fold increase in the free proline content was observed for the transgenic cell line compared to control.

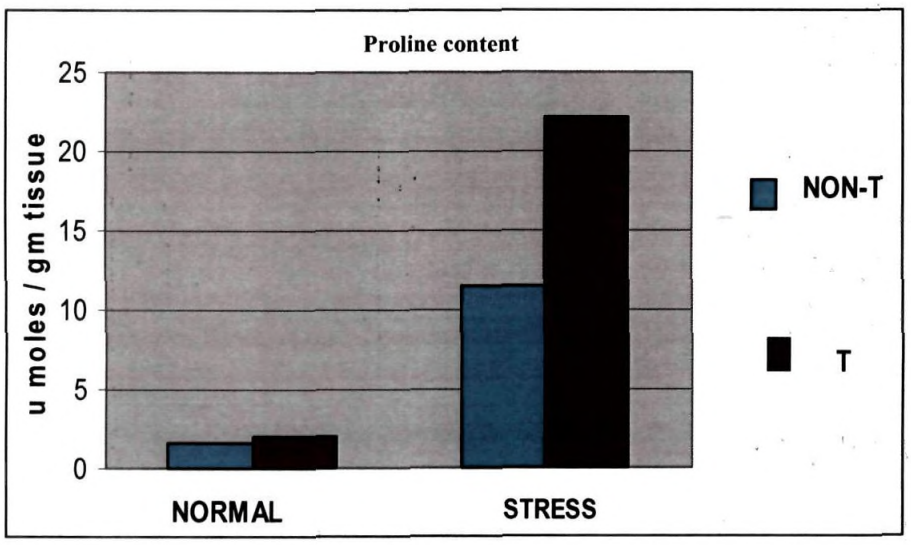


Fig.4.9.Proline estimation from transgenic and non- transgenic calli

4.7.2 Salt tolerance of transgenic cell lines

When transgenic cell lines were subjected to different levels of salt concentrations (Table 4.12), it was observed that the transgenic calli can tolerate up to 150 mM of NaCl concentrations while growth retardation was

observed in non-transgenic calli even in lower concentrations (50mM) of NaCl (Fig.4.10). About 70% of the transgenic calli could survive at high NaCl concentrations (150 mM) whereas non-transgenic calli could not tolerate even100 mM NaCl (Table 4.12). Upon subculture to medium without salt, the transgenic calli from all the treatments except 200mM, retained the growth and proliferated. The calli treated with 200 mM NaCl showed poor proliferation.

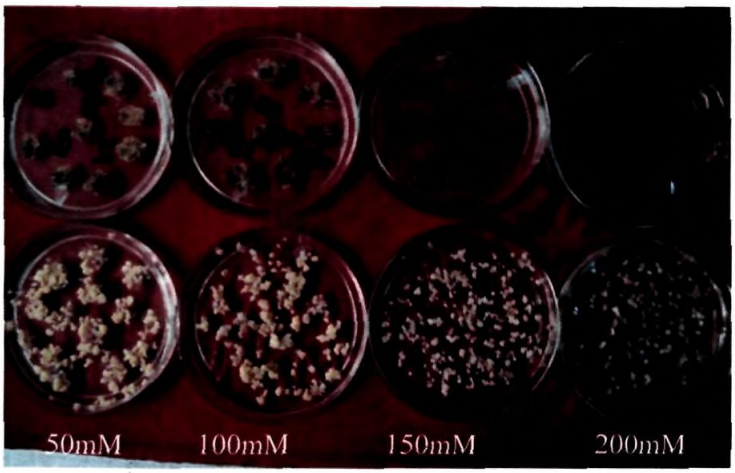


Fig.4.10 Salt tolerance of transgenic cell lines

Table 4.12 Effect of salt on transgenic and non transgenic calli

	50 mM	100 mM	150 mM	200 mM
Transgenic	100 (100)	88.8 (70.56)	72.8 (58.63)	46.4 (42.96)
Non-Transgenic	23.8 (4.98)	13 (3.74)	3.2 (2.04)	0.8 (1.31)
			CD	0.28

The value were subjected to arcsine transformation and the transformed values are given in parenthesis

4.8 *In silico* analysis of transgene (osmotin)

In the general blast, the inserted osmotin cDNA sequence showed high similarity with the osmotin sequences present in tobacco, tomato etc, where as no similarity was shown with *Hevea* (Table 4.13). When discontigious megablast was performed using contig assembly specific to *Hevea brasiliensis*, only 67 % identity was observed confirming the uniqueness of gene construct used for transformation (Table 4.14). Hence the gene expression observed can be solely due to the transgene.

Table.4.13 *In silico* analysis of osmotin gene with non-redundant nucleotide collection (NCBI)

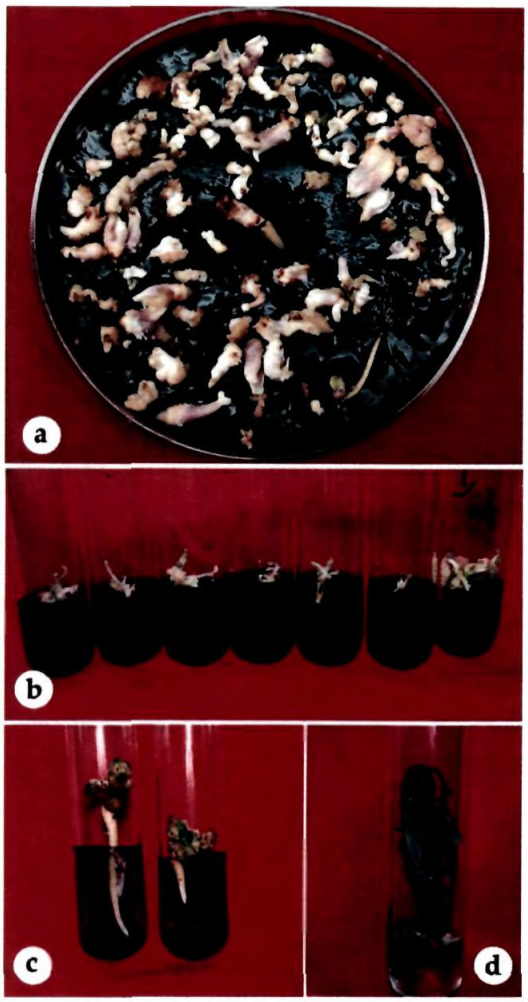
Description	Max score	Total score	Query cover	E value	Max ident	Accession
N.tabacum mRNA for osmotin	1363	1363	100%	0.0	100%	X61679.1
N.tabacum mRNA ap24	1338	1338	100%	0.0	99%	X65700.1
abscisic acid-activated [*Nicotiana tabacum* L.=tobacco, leaf, Genomic, 3033 nt]	1338	1338	100%	0.0	99%	S40046.1
Solanum phureja osmotin-like protein A13 mRNA, complete cds	837	837	99%	0.0	87%	AY743928.1
S.commersonii mRNA for osmotin-like protein	832	832	99%	0.0	87%	X67121.1
Petunia hybrida osmotin (OSM) mRNA, complete cds	806	806	100%	0.0	86%	AF376058.1

Table 4.14 ***In silico*** **analysis of osmotin gene with contig assembly specific to *Hevea* (discontiguos megablast)**

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Hevea brasiliensis	176	176	86%	7e-42	67%	AJJZ010960352.1
Hevea brasiliensis	143	143	80%	4e-32	67%	AJJZ010983111.1
Hevea brasiliensis	141	141	83%	1e-31	66%	AJJZ010962066.1
Hevea brasiliensis	113	113	83%	7e-23	66%	AJJZZ010893267.1

4.9 Constraints in the development of transgenics in *Hevea*

The process of development of transgenic *Hevea* plant encountered several constraints. The bacterial overgrowth during transformation, difficulty in proliferation of the callus, abnormal embryos (Fig.4.11a & b) and impaired germination (Fig.4.11 c & d) were some of them. Hardening was the most difficult step in the development of transgenic plant in *Hevea.*

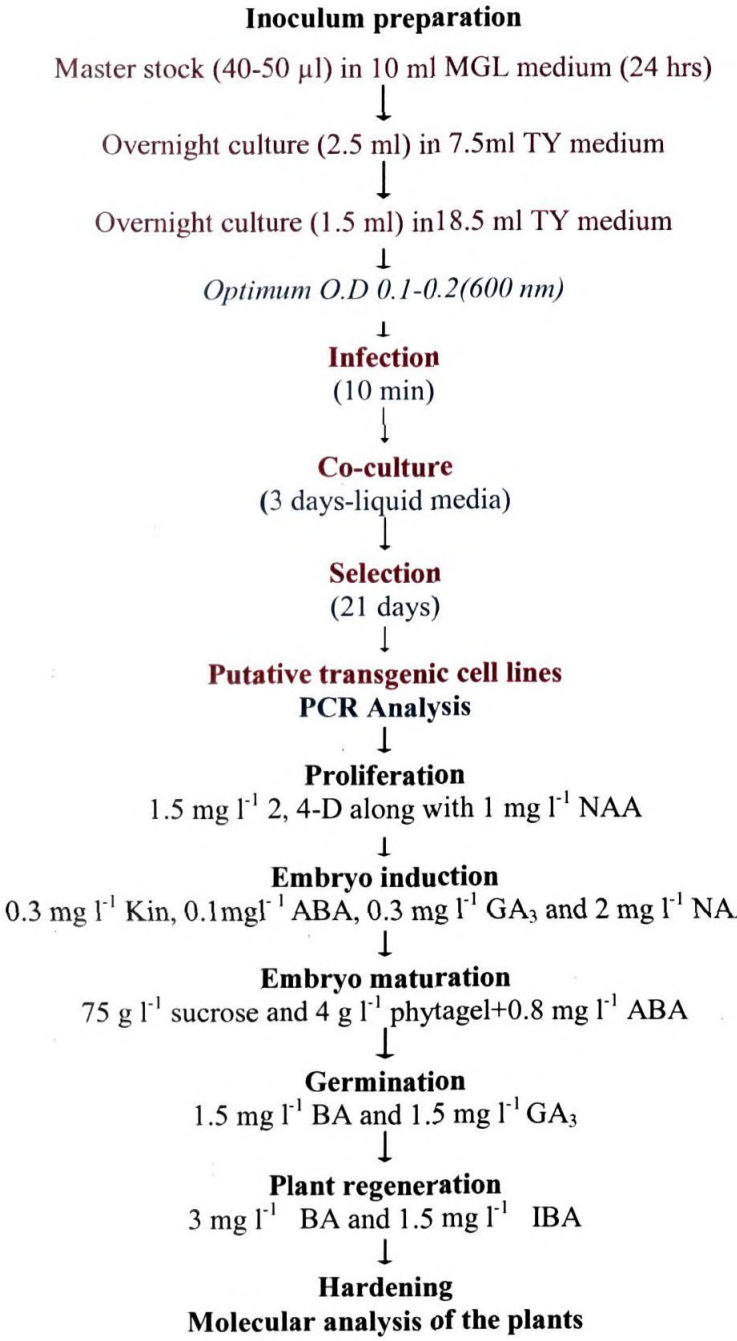


(a) & (b) Abnormal embryos (c) Germination of embryos without shoot
(d) Germination of embryos without root

Fig. 4.11 Abnormalities observed during the transformation and regeneration process

A schematic representation summarizing the development of transgenics in *Hevea*, integrated with osmotin gene is presented in Fig 4.12.

Fig.4.12. Schematic representation of development of transgenic plants in *Hevea*, integrated with osmotin gene



(a) Emergence of transgenic cell lines (b) Embryo induction (c) Germination
(d) Plant regeneration (e) Acclimatized transgenic plant in the polybags

Fig 4.13 Process of transgenic plant development

Fig.4.14 Transgenic plants in *Hevea* (6-12 months old)

In the present study, transgenic plants integrated with tobacco osmotin gene could be developed successfully *via Agrobacterium* mediated genetic transformation (Fig 4.13). The integration and expression of the transgene was confirmed using molecular techniques. Stress tolerance studies on transgenic callus showed positive indications towards drought and salinity tolerance. The regenerated plants are also expected to show these traits so that they can perform under adverse climatic conditions (Fig 4.14). This is the first report of developing transgenic plants of *Hevea,* integrated with a functional transgene and its expression.

CHAPTER 5

DISCUSSION

5. DISCUSSION

5.1. Development of transgenic cell lines.

5.1.1 Influence of target tissue on transformation efficiency

Explant selection is a critical parameter while performing transformation and regeneration experiments. The availability of totipotent cells as targets for transformation is today the limiting factor in genetic transformation of recalcitrant woody species (Birch, 1997). Seven different explants were evaluated in this study for their susceptibility to *Agrobacterium* infection. Wide variation in the transformation frequency was observed with different explants (Fig.5.1). Among the clonal explants, maximum number of transgenic lines could be obtained from embryogenic calli derived from anther (48%). A higher transformation frequency of 76 per cent could be obtained for embryogenic calli derived from zygotic embryos. A few transgenic cell lines were obtained from 2 month old fresh callus derived from anther where as 2 month old calli from ovule failed to give any positive results on transformation. This indicates that the initial source of the callus has a definite role in the frequency of transformation. The varying potential for different explants on transformation, somatic embryogenesis and organogenesis is well established (Piqueras *et al.,* 2010). Juvenility of the explant, friability and texture of the calli are the factors influencing transformation frequency. In Citrus, it has been reported that juvenile tissues showed higher sensitivity to *Agrobacterium* than the mature one (Cervera *et al.,* 2004). In cassava, *in vitro* induced shoots and germinating somatic embryos were used for transformation and highly variable plant regeneration efficiency (5–70%) was observed (Siritunga *et al.,* 2004; Puonti-Kaerlas *et al.,* 1997). Type of explant influenced transformation efficiency in monocots

like wheat also (Lengliz *et al.*, 2009). Sarker and Biswas (2002) tested four different explants for their transformation ability in wheat and obtained maximum transformation from immature embryo derived calli. It is generally observed that the texture of the callus and friability vary with initial explants and this will be reflected in the transformation and regeneration frequencies. The high transformation efficiency observed in the present study may be attributed to the juvenility, proliferation potential and fine texture of the callus derived from zygotic embryos. Similarly, embryogenic calli are more friable and have higher proliferation potential compared to two month old primary calli. Embryogenic calli has been used as a potential target tissue for genetic transformation in *Hevea* with different gene constructs (Rekha *et al.*, 2006; Kala *et al.*, 2006; Montoro *et al.*, 2003; Blanc *et al.*, 2006) and high frequency transformation has been reported. The low transformation frequency observed in the present study for the two month old primary calli may be due to the lack of friability of the callus. The absence of dedifferentiation and lack of penetration of the *Agrobacterium* may be the reasons for the failure of intact explants to give any positive results. From the present study, it can be inferred that embryogenic calli derived from anther as well as embryogenic calli from zygotic embryo are the potential target tissues for obtaining successful transformation in *Hevea.* Use of embryogenic calli as the target tissue has another advantage that it can considerably reduce the time span for transgenic plant regeneration, since it can bypass one crucial and most difficult step of the somatic embryogenesis pathway of *Hevea* namely induction of embryogenic calli. In the case of zygotic embryo derived explants, more regeneration potential also is expected. Embryos and embryogenic calli derived from embryos are being used in many crops for somatic embryogenesis (Gupta and Grob 1995;

Klimaszewska and Cyr, 2002; Von Arnold *et al.,* 2002). However, after regenerating plants from the transgenic cell lines, a conclusion on explant selection can be arrived at.

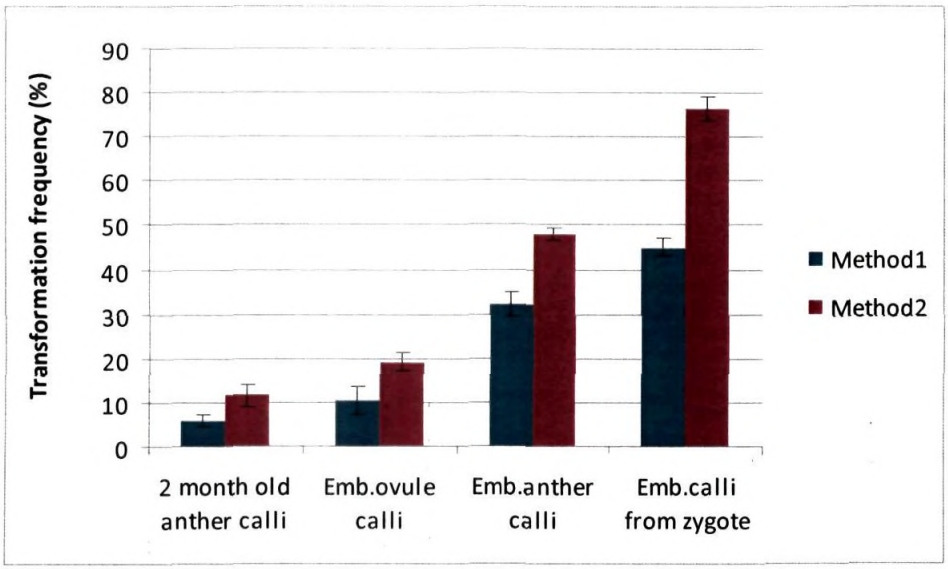


Fig.5.1 Transformation frequency with different explants

5.1.2 Genetic transformation protocol

The *Agrobacterium* infection process includes two stages; first one is a short period, typically a few minutes of inoculation by immersion of the target tissue in an *Agrobacterium* suspension followed by co-culture of the explants. *Agrobacterium* strain, density, co cultivation temperature, period of co-culture, method and medium have been experimented by different workers and found to influence transformation frequency in different ways (Dandekar and Fisk, 2004). In the present work, the protocol already reported for *Hevea* transformation and a modified protocol were compared for their efficiencies of transformation. Results from our experiments proved that the modified method was more efficient than the earlier one in terms of transformation frequency. In the method II, alterations were attempted during

inoculum preparation and co-cultivation. The efficiency of DNA delivery depends mainly on the virulence of the bacterium. The most direct effect on virulence induction is mediated by the presence of phenolic compounds such as acetosyringone (Kado, 1991). *Agrobacterium* responds to chemo-attractants released from wounded tissue and binds to them by a polar attachment mechanism (Tzfira and Citovsky, 2002; Winans, 1992). During attachment, coordinated expression from a group of genetic operons (vir regulons) critical to the gene transfer process also begins. Gene product from this vir operon perform functions that are critical to the transfer of a DNA fragment called T-DNA from the tumor inducing (Ti) plasmid localized in the bacteria into plant cells. In the modified method, the culturing of bacterial cells in acetosyringone medium for three consecutive days might have increased the virulence of the bacterium and resulted in the high transformation frequency obtained. The higher concentration of acetosyringone (40 mg l^{-1}) also might have contributed to this improvement.

Environmental factors such as pH, temperature, and osmotic conditions strongly influence the expression and induction of virulence gene (Dandekar and Fisk, 2004). Adoption of low temperature (23°C) for growing bacteria again helped in increasing the virulence of the bacteria. The influence of temperature on T-pilus (transfer pilus) biogenesis in *Agrobacterium* (Park *et al.*, 1997), tumor formation (Braun, 1947; Brown, 1942), and on the action of VirA and VirD proteins (Jin *et al.*, 1993) were studied extensively and the positive influence of low temperature (20-23°C) have been reported. In our studies, the growth of the bacteria at 23°C, also may have contributed towards the higher transformation frequency (76%). Co-cultivation temperature is another important factor which influenced the transformation frequency. An increase in GUS activity as a result of reduction in co culture temperature was

reported in *Hevea* transformation experiments earlier (Blanc *et al.,* 2006). Furthermore, during liquid co-culture, the phenolic compounds, which are supposed to trigger the action of *vir* genes in the *Agrobacterium,* may be available more easily which in turn lead to the high transformation frequency. Improvements in transformation frequency *via* liquid co-culture have been reported in tobacco (Uranbey *et al.,* 2005) and banana (Huang *et al.,* 2007). Suitable modifications in the transformation protocol could result in high transformation frequency (48 and 76 per cent for anther derived and zygotic embryo derived respectively) in *H. brasiliensis.*

5.1.3. Selection of putative transgenic cell lines

After co-culture, only a small proportion of target cells typically receive the DNA and only a small proportion of these cells survive the treatment and stably integrate the introduced DNA (Franks and Birch, 1991). It is therefore generally essential to detect or select the transformed cells among a large excess of untransformed cells (Birch and Bower, 1994). There are different systems available for the screening of transformants, which permits the preferential growth of transformed cells. Selectable marker genes based on negative selection such as genes encoding resistance to specific antibiotics or herbicides have proved particularly effective for rapid identification of transformed cells, and are commonly employed for this purpose. In the present experiment, the selectable marker used was kanamycin (negative selection), which has been proven effective in previous gene transfer experiments of *Hevea* with other gene constructs (Jayashree *et al.,* 2003; Rekha *et al.,* 2006; Kala *et al.,* 2006). Kanamycin is an amino glycoside antibiotic which kills cells by inhibiting protein translation. The *nptII* gene isolated from *Eschericia coli,* and coding for *neomycin phosphotransferase* inactivates the antibiotic by phosphorylation, thus

allowing preferential growth of the transformed cells. Kanamycin at a concentration of 300 mgl^{-1} reported earlier (Jayashree *et al.* 2003, Rekha *et al.*, 2006, Kala *et al.,* 2006), was used for screening the transformants. Removal of *Agrobacterium* from the cultures is highly essential in order to prevent the bacterial overgrowth which leads to the total loss of the cultures. Hence, it is necessary to include antibiotics along with the selection agent to eliminate or arrest the growth of *A.tumefaciens*. Generally, antibiotics that do not affect plant cells such as Cefotaxime 250-500 mg l^{-1} or Carbenecillin 500 mg l^{-1}) are used to serve the purpose. In our experiment, callus proliferation medium for the infected callus was supplemented with the antibiotic kanamycin (300 mg l^{-1}) as the selection agent and (Cefotaxime 500 mg l^{-1}) as the killing agent. Putative transgenic cell lines were selected and proliferated. After the first screening, a second level of screening was also given with the same media in order to avoid false positives. Later, the proliferated putative transgenic lines were tested for the presence of transgene by PCR analysis.

5.1.4 Proliferation of transgenic cell lines

Callus proliferation was a difficult step in the development of transgenic cell lines. Growth was very slow in already reported media standardized for untransformed tissue (Jayasree *et al.*, 1999). The integration of transgene might have made some genetic change in the cell lines leading to poor response noticed in the media standardized for the proliferation of normal callus. The experiments on callus proliferation with different growth regulators revealed that, for the proliferation of transgenic calli, 1.5 mg l^{-1} 2, 4-D with 1 mg l^{-1} NAA was the ideal one. Positive influence of 2, 4-D on callus induction and proliferation has been well documented in many crops including *Hevea.* The promotive effect of 2, 4-D on callus induction from different explants such as anther (Wang *et al.,* 1980; Chen., 1984; Jayasree

et al., 1999; Asokan *et al.,* 2002) and leaf (Kala *et al.,* 2005) has been reported. The synergistic effect of NAA with 2, 4-D in presence of kinetin on callus induction and proliferation from immature inflorescence of *Hevea* was reported by Sushamakumari *et al.,* (2000). For callus induction from root explants of transgenic plants, a combination of 2, 4- D and NAA was found to be ideal (Sobha *et al.,* 2009). Present study reveals that a combination of two auxins was superior to a single auxin in promoting callus induction and proliferation. Higher concentration of 2, 4, D reduced the callus friability. A reduction in callus friability with higher concentrations of 2, 4 D was observed by Jayasree *et al.*, (1999). The present result also is in conformity with this observation.

5.2 Developing transgenic plants

Once a plant cell has incorporated the introduced DNA in a stable manner, the next step is the regeneration of plants from the transformed cells. According to Birch (1997), because of the greater complexity and lesser understanding of the biological interaction preceding the gene transfer event from *Agrobacterium,* there is no guarantee that a transformable plant cell type will prove regenerable, even in the hands of the most successful tissue culturist. Plant regeneration from the proliferated callus involves a multistep process starting with embryo induction, maturation, germination and plantlet development. Hormonal combinations and culture requirements vary with each step. The experiments carried out for the optimization in each step are discussed below.

5.2.1 Embryo induction

Induction of the embryogenic capacity is influenced by several factors, including basal medium components, plant growth regulators, and

culture conditions. Somatic embryogenesis has been studied extensively in *Hevea* by many workers (Carron *et al.*, 1995; Montoro *et al.*, 2003; Jayasree *et al.*, 1999; Etienne *et al.*, 1993; Sushamakumari *et al.*, 2000). The culture conditions and basal media have already been standardized. However, the insertion of a foreign gene can alter the genetic makeup of the cells and change the regeneration capacity. Each transgenic line can be considered to be originated from one single event. Hence optimization of conditions for establishing somatic embryogenesis is highly essential for the transgenic cell lines separately. The present study has been carried out accordingly and experiments were done for optimizing the growth regulator combinations. A combination of NAA and kinetin worked well for the induction of somatic embryos from the transgenic cell lines of *Hevea* integrated with osmotin gene and an embryo induction frequency of 43.5 % was obtained. Jayasree *et al.*, (1999) reported a profound influence of NAA on somatic embryogenesis in *Hevea.* NAA at 0.2 mg l^{-1} promoted embryo induction whereas higher concentrations beyond 0.3 mg l^{-1} resulted in low embryo induction frequency. However in the present study, the maximum percentage of embryo induction was observed at a higher concentration of NAA (2 mg l^{-1}). NAA beyond 3 mg l^{-1} resulted in reduction in the embryo induction frequency. It was reported that auxins and cytokinins are necessary for inducing and sustaining embryogenesis except in a few cases (Lelu *et al.*, 1999). In *Centella asiatica* it was reported that NAA, and kinetin, were superior in the induction of somatic embryogenesis to 2, 4-D and kinetin (Martin, 2004). Callus developed on NAA and kinetin supplemented medium favored induction and maturation of embryos in a better way compared to that on 2, 4-D and kinetin.

Embryo induction frequency could be enhanced to 50% when GA_3 and ABA were supplied along with NAA and kinetin. ABA being a growth retardant prevents the excess proliferation of the callus and enhances embryo induction. Also the stress induced by ABA may trigger the induction of embryos. A combination of GA_3 and ABA along with NAA and kinetin helped synchronized development of embryos. Embryogenesis and further development mainly depend on the combined effect of different growth regulators at definite proportions rather than individual effects.

The role of ABA in embryo induction and maturation is well established. Cailloux *et al.,* (1996) reported that a higher concentration of ABA (2.64 mg l^{-1}) promoted long term somatic embryogenesis as well as embryo maturation in *Hevea.* A combination of ABA along with kinetin and GA_3 positively influenced embryo induction and development from transgenic calli of *Hevea* integrated with MnSOD gene (Jayashree *et al.*, 2003). Promoting effect of ABA at lower concentrations and inhibitory effect at higher concentrations on embryo induction has been reported in *Hevea* by Sushamakumari *et al.,* (2000), for the calli derived from immature inflorescence. Similarly Linossier *et al.,* (1997) reported enhancement of somatic embryogenesis in the presence of ABA along with high PEG concentrations. ABA was proved to be an essential component for embryo induction from endosperm derived calli in *Hevea* (Rekha *et al.*, 2007). Abscisic acid was identified as an important media component in conifers (Hakman & Arnold 1985; 1988, Durzan &Gupta, 1987; Boulay ,1988; Dunstan *et al.,* 1988). Enhancement in the frequency of normal embryos with the addition of ABA has been reported in many other species also (Ammirato1977, Attree *et al.*, 1991). In the present study, ABA when supplied along with NAA, kinetin and GA_3 enhanced embryo induction

frequency considerably. MS basal with a growth regulator combination of 0.3 mg l^{-1} kinetin, 0.3 mg l^{-1} GA_3, 0.1 mg l^{-1} ABA and 2 mg $^{-1}$ NAA was selected as the ideal growth regulator combination for embryo induction.

5.2.2 Embryo maturation

Embryo maturation is a process in which the embryos accumulate enough reserve material for germination and is a key phase between embryo development and germination (Quatrano, 1987). According to Ammirato (1983), poor quality and incomplete maturation of the somatic embryos are considered to be the main factor limiting the conversion of embryos into plants and the culture conditions must be changed in a sequential manner during somatic embryo development and germination. Embryo maturation is effected by various physical factors, depending on the requirement of the species. In *Hevea*, a study of the zygotic model has revealed the need to induce a maturation phase prior to embryo germination, primarily for the completion of cauline meristem formation, accumulation of starch / protein and desiccation (Carron *et al.*, 1995). In the present study, different concentrations of ABA along with varying levels of phytagel were tried initially for embryo maturation. Embryo maturation occurred at a higher level of phytagel (0.5%) along with 0.8 mg l^{-1} ABA. In the second experiment, when different levels of phytagel and sucrose were tried in presence of 0.8 mg l^{-1} ABA, high level of sucrose (75%) and slightly lower level of phytagel (0.4%) were found to be ideal. The influence of phytagel in embryo maturation was reported earlier in *Hevea*, for the maturation of endosperm derived embryos (Rekha *et al.*, 2007) and transgenic embryos (Sobha, *et al.*, 2003). The use of high concentrations of phytagel as gelling agent reduces water availability. Rinne and Adams (1980), suggested that water relations between the embryo and its environment, *in vivo* or *in vitro*, play an important role in embryo

development, particularly during the maturation phase. The importance of water relations in controlling embryo maturation has been supported by evidence from both zygotic and somatic embryo culture experiments (Xu *et al.,* 1990). High concentrations of phytagel have been used for reducing the vitrification of coconut somatic embryos (Perera *et al.,* 2011). The positive influence of ABA for cotyledon development, organization of meristem, procambial bundles, the epidermis and the latex bearing vessels, protein reserve accumulation (Etienne *et al.,* 1993) and for preventing the precocious germination in *Hevea* is reported earlier. However, in contrast to the present study Sushamakumari *et al.,* (2000) reported that ABA had no significant effect on embryo maturation. ABA along with activated charcoal and high sucrose for improving the maturation and germination of somatic embryos of *H. brasiliensis* has been reported earlier (Carron *et al.,* 1995). Etienne *et al.,* (1993) reported that high levels of ABA along with sucrose developed desiccation tolerance in *Hevea.* Beneficial effect of sucrose, ABA and water stress on increasing the storage proteins has been reported in other crops like *Theobroma cacao* (Pence,1992) and *Pinus strobus* (Bornmann,1993). Although high osmolarity level have been demonstrated to be beneficial for embryo maturation, their role has not been made clear. Dodeman *et al.,* (1997) indicated that ABA and water stress may be inducing gene expression responsible for the synthesis and accumulation of storage and late embryogenesis abundant (LEA) proteins. According to Morris *et al.,* (1988) water stress does not necessarily cause an increase in endogenous ABA; rather, both factors may operate in a complementary manner (Benech-Arnold *et al.,* 1991). ABA has also been shown to regulate the amount of storage lipids in zygotic (Finkelstein and Crouch, 1986) as well as somatic (Kim and Janick, 1991) embryos. The synergic effect of ABA, sucrose and phytagel

might have helped the maturation of transgenic embryos integrated with osmotin gene in the present study.

5.2.3 Germination and plant regeneration

Germination is the process in which the embryo is awakened, is necessary for getting complete plants and it requires specific physical conditions. Plant regeneration is the process of full plant development from the germinated embryos. In our experiments maximum germination occurred in half strength MS medium supplemented with 1.5 mg l^{-1} BA and 1.5 mg l^{-1} GA_3. Beneficial effect of GA_3 on germination is already reported in *Hevea* (Carron *et al.*, 1995, Jayasree and Thulaseedharan, 2002). A combination of BA and GA_3 induced 67 percent germination in somatic embryos developed from root explants in *Hevea* (Sushamakumari *et al.*, 2000). Similarly BA (0.3 mg l^{-1}) and GA_3 (0.3 mg l^{-1}) was used for the germination of rescued zygotic embryos in the immature stage in *Hevea* (Rekha *et al.*, 2006). Incorporation of GA_3 in combination with BA and IBA in the germination medium favoured bipolar differentiation and improved germination and plant regeneration in transgenic embryos of *Hevea* integrated with MnSOD (Sobha *et al.*, 2003). Eventhough the exact mechanism of the beneficial role of GA_3 on embryo germination is not clear, ultrastructural studies carried out by Choi *et al.*, (1999) showed that somatic embryos developed *in vitro* could be dormant after maturation and a breakage of the dormancy is required. During *in vitro* culture, many factors other than growth regulators were found to affect somatic embryo germination. In the present study, sucrose was reduced to 20 g l^{-1} for germination and plant regeneration. In *Hevea,* use of reduced levels of sucrose in the germination as well as in the plant regeneration medium has been reported earlier (Carron *et al.*, 1995, Kala *et al.*, 2008). The germinated embryos after root and shoot elongation, were

transferred to plant regeneration medium. In the present study, among the different combinations of BA and IBA tried, 3 mg l^{-1} BA and 1.5 mg l^{-1} IBA was found to be ideal for plant regeneration in presence of GA_3. IBA is a known component for root development and BA for shoot development.

In order to improve the germination and plant regeneration frequencies, different desiccation treatments were applied to the transgenic embryos. Slow desiccation for 48hrs was found to increase germination and the frequency could be improved from 23 to 44%. Desiccation seems to be beneficial in many crops for successful germination of somatic embryos. It was reported that desiccation switches the pattern of gene expression from a maturation programme to the programmes required for germination (Kermode & Bewley 1985) and playing a role in breaking dormancy and in the regulatory mechanism that controls post germination growth (Gray *et al.*, 1987). The positive effects of desiccation have been observed in many species, e.g. walnut (Preece *et al.*, 1995), ground nut (Durham and Parrott, 1992) cotton (Chaudhary *et al.*, 2003) etc. Desiccation is a characteristic of zygotic embryogenesis *in planta*, and is considered as a plausible treatment for terminating developmental process and triggering the germination and seedling developmental programmes. It is possible that the changes in turgor pressure caused by desiccation initiate a set of biochemical events affecting membrane permeability and levels of endogenous ABA which in turn induce expression of specific genes involved in the plant development (Oishi and Bewley, 1990; Striver and Mundy, 1990). In cotton, it is reported that slow physical desiccation was helpful in developing transgenic plantlets from transformed somatic embryos. In our experiments, even though germination was improved by slow desiccation of the embryos, after the initial boost up, further growth was found to be retarded. This may be due to some adverse

physiological changes happened during desiccation or may be due to the lack of absorption of the nutrients. However, this aspect needs further investigation.

- Improvement of transgenic embryo germination and conversion is a long-term effort, as many factors contribute to germination and conversion capacity especially in a perennial crop like *Hevea.*

5.3 Effect of sugars and sugar alcohols on transgenic plant development

Along with sucrose, other sugars and sugar alcohols are used in many crops for improving various stages of plant regeneration. Sugars like maltose and glucose and sugar alcohols like mannitol and sorbitol were supplied in the already developed media for improving the pathway and to obtain better regeneration of transgenic plants. Except for plant conversion, addition of mannitol was beneficial compared to the medium supplemented with sucrose alone, in the present study. All the other treatments were on par or inferior to sucrose supplemented medium. In carnations addition of mannitol along with sucrose was found to be beneficial in somatic embryogenesis (Deljou *et al.,* 2007). Incorporation of mannitol to the semi-solid MS medium was tested at different concentrations for its ability to induce somatic embryogenesis of tea (*Camellia sinensis* L.) derived from cotyledon slices. In *Hevea,* embryogenic callus was induced on the medium supplemented with 500 or 1000 mg l^{-1} mannitol within 8 weeks of culture initiation. The induction response was further enhanced in presence of ABA (7.5 mg l^{-1}) (Ozudogru, *et al.,* 2006). Usually plants respond to stress by trying to complete its life cycle as fast as possible. The mechanism(s) whereby stress affects embryogenesis and organogenesis has not been fully elucidated, although there is evidence that multiple cellular responses are triggered. Krishnaraj and Thorpe (1996)

showed that salt stress might be required for altering cellular energy status and reducing power which increase the rate of the pentose phosphate pathway. Activation of this pathway has been associated with *in vitro* morphogenesis. Stress has also been implicated in the production of arabinogalactan proteins, which have been found to promote somatic embryogenesis. In a study in Carrot (Mccabe *et al.*, 1997), it was shown how nonembryogenic cultures can acquire embryogenic competence if exposed to arabinogalactan proteins produced from embryogenic cells. The stress induced by mannitol may be forcing the callus/embryos to pass to the next development stage.

5.4 Acclimatization

Acclimatization of the plantlets is a major problem in woody plant species. In the present study, also the difficulty was observed for plants derived from clonal explants. However for zygotic embryo derived plants, better acclimatization was observed and about 50% hardening was obtained in this case. Explant juvenility is the major factor behind this. The more juvenile the tissue, response also will be more. The high mortality observed for the somatic plants developed from clonal explants is an established fact in recalcitrant woody perennials. In the present study, an initial success of 10% was obtained for the plantlets derived from clonal material and the plants survived up to 3 months. However, gradual retardation in growth was observed and the final success was meager. A great number of *in vitro* produced plants do not survive the transfer from the *in vitro* to the *ex vitro* environment under greenhouse or field conditions. Due to their anatomical and physiological characteristics, these kinds of plants need a gradual adaptation or acclimatization to *ex vitro* environments in order to survive and be productive. The greenhouse and field have substantially lower relative

humidity, higher light level and septic environment compared to *in vitro* conditions that are stressful to micropropagated plants (Hazarika, 2003, Hazarika & Bora, 2010). According to Gutiérrez-Mora *et al.,* (2012), during acclimatization process, the *in vitro* developed plants should develop cuticle, epicuticular waxes, and should establish an effective stomatal regulation of transpiration, leading to stabilization of water status. Hypertrophy of cortical and pith parenchyma, large intercellular spaces, hypo lignifications of the vascular system, reduced and/or abnormal vascular system, disorganized cortex, epidermal holes, epidermal discontinuity, collapsed cells, are reported to be the characteristic of i*n vitro* developed plantlets (Gutiérrez-Mora *et al.,* 2012). All these can be attributed to the low survival rate of transgenic *Hevea* plants. Extensive optimization need to be done in this line.

5.5 Comparison of clonal and zygotic transgenic cell lines on different stages of plant regeneration

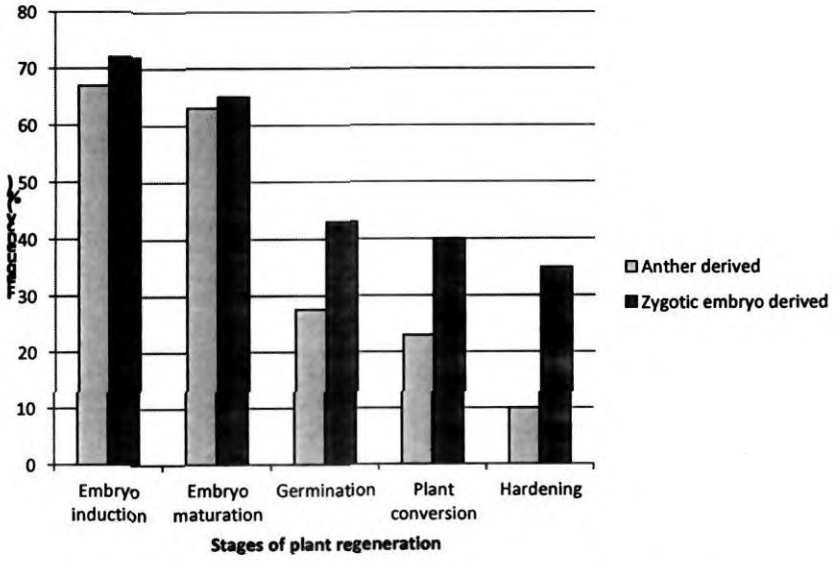


Fig.5.2. Influence of explant source on different sages of plant regeneration

In the present study, plant regeneration could be achieved from 2 different explants raised from two different sources *viz.* Anther and zygotic embryo. A notable difference in the frequencies of embryo induction, maturation germination and plant regeneration was observed in the presently developed media (Fig.5.2). The frequencies of germination, plant regeneration and hardening are very low for clonal explants compared to zygotic embryo derived transgenic lines. As a general rule, juvenile tissues, such as zygotic embryos, have better potential and competence to produce embryos and organs compared to more differentiated and mature tissues. Zygotic embryo has been used as explant for somatic embryogenesis and for developing transgenic plants in different crop species (Elhiti and Stasolla 2011). The increased efficiency of embryogenic callus formation and regeneration of plantlets was observed in many crops including wheat by using polyembryos derived from the zygote as the explant. The secondary somatic embryos derived from the cotyledonary explants of zygotic embryos were successfully utilized for the development of transgenic plants in Walnut (Dandekar *et.al.,* 1989). In clonally propagated woody plants, the explant source is an important consideration in maintaining clonal integrity. Use of zygote derived explants implies a passage through sexual stage and therefore a drastic reshuffling of the genome which results in a subsequent alteration of clonal properties. Eventhough zygotic explants are less desirable for maintaining clonal integrity; this can be used as a model system for functional validation of inserted gene especially in recalcitrant woody tree crops. Recalcitrance of clonal explants prevents the production of transgenic plants on a commercial scale. Maturation and aging seem to be responsible for the decline in regenerative potential in woody plant species. Hence, use of zygotic material is a viable option for transgenic plant development. The

majority of structural and physiological features needed for inducing somatic embryogenesis and organogenesis in culture are present in zygotic embryos. Zygotic embryo cells already express the "embryogenic potential" with many of the genes required for the induction process already expressed. Therefore, their fate is already committed and does not need to be redirected towards a new developmental path. This is why in many species embryogenic tissue can be readily obtained using immature or mature zygotic embryos. Degree of response in culture is also related to the developmental stage of the zygotic embryos. As a general rule, immature (early cotyledonary) embryos are more responsive than their fully mature counterparts. In the case of transgenics which is more difficult to regenerate, all these advantages of zygotic embryos could be well exploited. Regeneration of plants from more events is possible since the system allows easy and rapid plant regeneration and hardening. This in turn enables more effective evaluation and identification of the superior events among the transformants. In *Hevea* there is an added advantage of using these plants as stress tolerant root stocks and thus can bypass the biosafety concerns. However, embryogenic competence is often restricted to a short, yearly time window and therefore, identification of the stage of zygotic embryo to initiate an embryogenic line is of paramount importance. Over the last few years, the number of species regenerated in culture using somatic embryogenesis or organogenesis from zygotic embryos has increased.

5.6 Micrografting

Micrografting is a technique which can be used for rescuing the transgenic shoots developed from clonal explants. This technique has been successfully used in many horticultural plants to develop virus free clones (Murashigae *et al.,* 1972, Roistacher and Kitto, 1977) and fruit trees like

cherry (Ozambak and Schmidt, 1991), kiwifruit (Ke *et al.,* 1993) and apple (Richardson *et al.*, 1996). Since 1953, attempts were made for micrografting in *Hevea* and a few cases of success were reported (Muzik and Cruzada, 1958, Perrin *et al.,* 1994; Kala *et al.,* 2002). Since the scion is grafted on to a seedling with good root system, absorption of nutrients and water are not affected adversely. This finally results in better growth. In the present study, micrografting was attempted and one successful graft was obtained and developed with new flushes. However, the graft dried due to unknown reasons at a later stage. The vascular connection may be lost due to callose deposit or may be due to the incompatibility of the scion and stock. It may also be due to the low photosynthetic efficiency of the scion, which eventually leads to the senescence of the plant. Extensive experiments need to be conducted in this line to further optimize the conditions.

5.7 Molecular confirmation of gene integration and expression of transgenic plants.

The recovered plants after transformation are typically analyzed on a number of different levels to determine that they are transgenics. Once the plant grows large enough to spare some leaves without compromising health, they may be analyzed for the presence of transgene and its expression. The development of efficient crop transformation system has necessitated the development of efficient methods for detailed molecular characterization of putative transgenic events.

5.7.1 PCR analysis

Traditionally, standard polymerase chain reaction (PCR) methods have been utilized to detect the presence of recombinant DNA in the transformed plants. Since the gene construct does not have a reporter gene to detect the transformants easily, the only way of reassuring the transformation

after Kanamycin screening is *via* PCR analysis. In the present study, the putative transgenic lines emerged in the selection plates were subjected to PCR analysis and all the lines tested were PCR positive which is an indication of the successful integration of gene insert. PCR technique is routinely used in molecular biology for different purposes including the detection of transgene. In the final stage, the plants were also subjected to PCR analysis in order to ensure stable transformation. The PCR product was cloned and sequenced and 100% similarity was observed with the inserted sequence. All this confirm the presence of transgene.

5.7.2 Southern blot analysis

Southern blotting is the first blotting techniques developed which made analysis and recording of DNA easy. Identification of sequences, correlating DNA restriction fragments to RNA and protein mapping were facilitated by this techniques and which has become most popular component of all molecular biology studies. In our experiment, we have got positive signals in 2 blots with the DNA isolated from different transgenic plants. One with the positive control, ie.PCR product of the DNA isolated from transgenic plants, positive signal was obtained in the lane corresponding to 0.75 kb in the marker. In the other blot with *Eco*R1 digestion, positive signals were obtained for three transgenic plants .Signals were absent in non transgenic as well as one of the putative transgenic plant. From the results obtained, we can infer that the transgene is stably integrated in *Hevea* genome.

5.7.3 RT- PCR analysis

The study of transgene expression is of vital importance whenever transgenic plants are produced. RT-PCR enables researchers to quickly

identify plants that are expressing transgenes. The availability of kits has made RT-PCR a fast and reliable test for transgene expression analysis, circumventing the need for Northern analysis. It is identical to conventional PCR, except that the template DNA is created by reverse transcription of RNA. The reverse transcription PCR utilizes the ability of the enzyme reverse transcriptase to synthesise DNA from RNA template. The positive or negative PCR results represent the presence or absence of RNA transcription in the original RNA sample and therefore, are an indicator of specific gene expression. In the present study, positive PCR amplification was obtained from the DNA synthesized from RNA isolated from putative transformants. This indicates the expression of transgene. The amplification was absent in the negative control.

5.8 Evaluation of stress tolerance

5.8.1 Evaluation of drought tolerance

The proline estimation data showed slightly higher values for proline content for transgenic tissues under unstressed conditions. When subjected to water stress by incorporating PEG (6 %) in the culture medium, a higher proline accumulation was noticed in the transgenic calli compared to non transgenic calli. Osmotin induced proline accumulation under stress have been reported in transgenic plants of different crops like strawberry, wheat, cotton, tomato, chilli pepper, mulberry and soybean (Husaini and Abdin 2008; Noori and Sokhansanj, 2008; Parkhi *et al.*,2009; Goel *et al.*, 2010; Subramanyam *et al.*, 2012; Das *et al.*, 2011). Results of the present study are in conformity with these reports. Among the different compatible solutes, proline is an important quaternary amino acid derivative that accumulates during salt stress, drought, and low temperature. Proline accumulation under various abiotic stresses (heat, cold, drought, moisture and salinity) in

important crop plants is considered as a tolerance mechanism. It is suggested to act as an osmolytes as well as a source of nitrogen during recovery from stress. A large body of data suggests a positive correlation between proline accumulation and plant stress tolerance (Barthakur *et al.*, 2001; Gao *et al.*, 2009). Recent reports indicate enhanced stress tolerance when proline is supplied exogenously at low concentrations (Hayat and Hayat, 2012). The free proline plays important roles in osmotic adjustment, protecting cellular macromolecules and scavenging hydroxyl radicals in salinity-stressed plants (Chen *et al.*, 2011). In the present study, the proline accumulation as induced by osmotin in the transgenic tissue is a positive indication of stress tolerance. Constitutive over expression of osmotin gene modulating the transcript abundance of other stress responsive genes was also reported in transgenic tomato recently (Patade *et al.*, 2013).

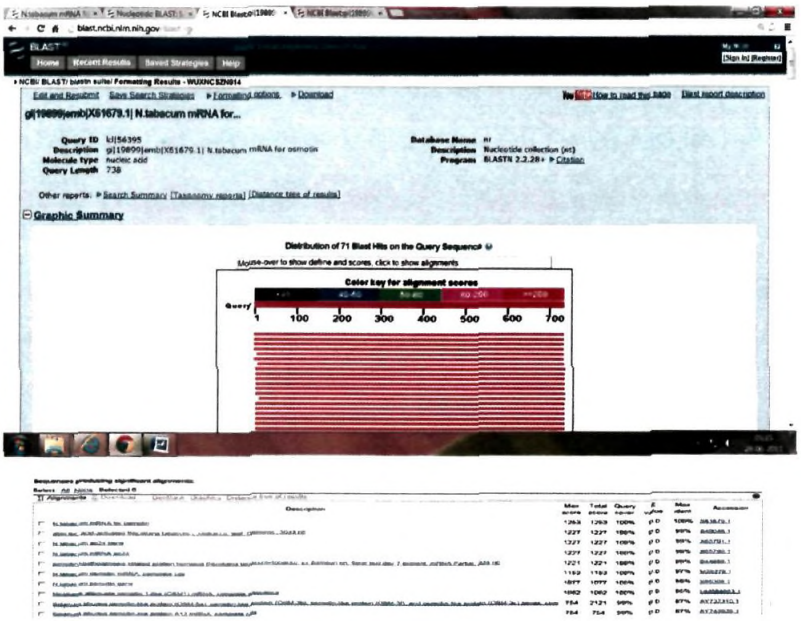
5.8.2 Evaluation of salt tolerance

When transgenic cell lines were exposed to different concentrations of salt, it was observed that the transgenic calli can tolerate up to 150 mM of NaCl concentrations while growth retardation was observed in non-transgenic calli even at lower concentrations (50 mM). It was hypothesized that osmotin might be involved in the modulation of plant responses to salinity stress either directly by acting as transcriptional regulator for the genes encoding key enzymes responsible for salinity tolerance or as signaling molecule acting through intracellular receptors (Abdin *et al.* 2011). Previous reports also proved that the expression of *Tbosm* in tobacco (Barthakur *et al.*, 2001), tomato (Sarad *et al.*, 2004; Goel *et al.* 2010), strawberry (Husaini and Abdin 2008), and chilli pepper (Subramanyam *et al.*,2011) improved the salinity tolerance. One of the main consequences of the salinity stress is the loss of intracellular water which causes osmotic

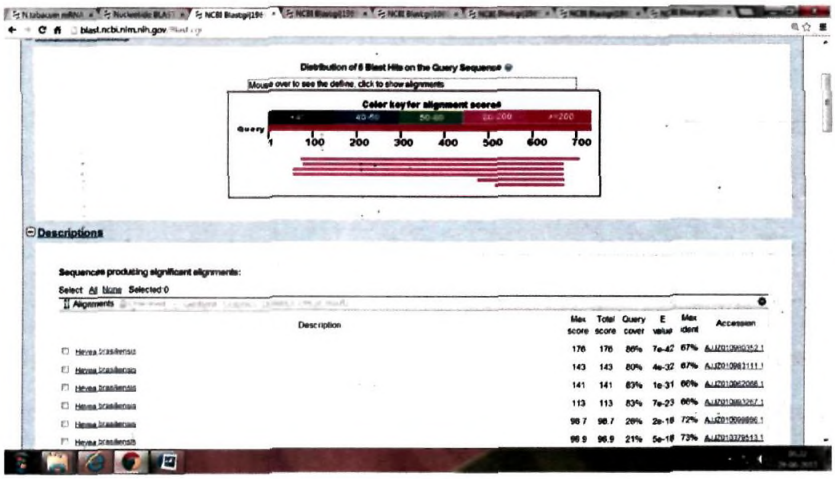
pressure. Plants accumulate compatible solutes in the cytoplasm to increase their hyper osmotic tolerance (Turkan and Demiral, 2009). In the present investigation, under the salt stress conditions, the transgenic calli expressing tobacco osmotin gene might have accumulated more proline than their non-transgenic counter parts. It indicates that the expression of Tbosm might activate the key enzymes of proline biosynthetic pathway, which in turn enhance the tolerance to salinity. The correlation between salinity tolerance and proline accumulation is reported in many crops like tobacco, wheat (Gao *et al.,* 2009), *Lycium barbarum* (Chen *et al.,* 2009), Chrysanthemum (Chen *et al.,* 2011) and in mulberry (Checker *et al.,* 2011). The salt tolerance exhibited by the transgenic calli is another positive indication for stress tolerance.

5.9 In silico analysis of transgene (osmotin)

In the general blast, the inserted osmotin cDNA sequence showed high similarity with the osmotin sequences present in tobacco, tomato, solanum etc. whereas no similarity was shown with *Hevea.* When blast analysis was done with the recently published *Hevea* genome sequence, no similarity was reported. When the blast was repeated with contig assembly specific to *H. brasiliensis*, only 67 % identity was observed confirming the uniqueness of gene construct used for transformation (Fig.5.3). Hence the gene expression observed as indicated by increased levels of proline etc. is due to the transgene only.



a. Mega blast with NCBI data base



b. Discontigious megablast with contig assembly specific to *Hevea*

Fig.5.3 *In silico* analysis of the transgene (osmotin)

5.10 Bottlenecks encountered in developing *Hevea* transgenics

The process of development of transgenics in a perennial tree crop is cumbersome. Starting with the generation of the explant, until the

development of transgenics, there are several constraints. In the present study, the explant used was of floral/fruit origin and the seasonal flowering nature prevented the continuous availability of the explant. Use of embryogenic callus could solve this problem to a greater extent since the proliferation of the callus is much easier after the attainment of embryogenic competence. However, this cannot be used indefinitely because of the decline of regeneration potential due to repeated sub culturing. Hence, it becomes inevitable to raise the explant every year. Moreover, the seasonal and year to year variation in explant response prevents the repeatability of the results. Developing a protocol for cryopreservation may help to preserve the callus with good regeneration capacity.

During the process of transformation, overgrowth of *Agrobacterium* resulted in loss of cultures frequently. Proper drying of the explants after co-cultivation could minimise this problem. Increasing the agar concentrations from 0.2% to 0.4% in the selection medium helped to reduce the overgrowth considerably. However, complete control over the problem was not possible. Since we got high transformation frequency we could develop a number of transgenic lines. Low proliferation potential of the transgenic cell lines was another problem. Even though numerous lines emerge initially from the infected cultures, further proliferation seemed to be very slow. However, media manipulations could solve this problem to a larger extent.

During plant regeneration, embryo induction was observed to be less difficult, but many of the embryos were found to be abnormal. Embryos of some transgenic cell lines remained white and with light pink colour under light incubation and, instead of producing normal shoot, showed abnormal development. There is no control over the site of integration of transgene in the whole genome. Hence, random integration of the insert inside a useful

functional gene can result in such developmental abnormalities since the foreign gene integration alters the genetic makeup of the cells. Only way to overcome these problems is the development of as many transgenic events as possible, so that we can select the most responsive events among them.

Hardening was the most difficult step encountered. Even normal, healthy plantlets derived from clonal explants showed difficulty in hardening. During the initial phase of acclimatization, plants survived in the growth chamber and new leaves were developed. However, after transferring from the growth chamber growth retardation followed by dieback of plants was observed. The plants derived from zygotic embryo as the initial source could survive the hardening process better. Hardening problem in recalcitrant woody species is well established. The probable solution is to develop more number of plants so that at least a few of them will acclimatize. In the case of rubber, once a single plant is acclimatized it can be multiplied easily by bud grafting. Also, the transgenics derived from zygotic source can be a good option as rootstock with stress tolerance. Moreover, it can also bypass biosafety concerns and environmental biodiversity issues due to gene transfer through out-crossing.

To summarise, transgenic plants of *Hevea* integrated with tobacco osmotin gene was developed successfully and the gene integration and expression were confirmed by molecular analysis. The plants are expected to perform better under stressful environment. This study is the first report of development of transgenic plants of *Hevea,* integrated with a functional alien gene and its expression.

CHAPTER 6

SUMMARY & CONCLUSION

6. SUMMARY AND CONCLUSION

Agrobacterium mediated gene transfer was attempted in *Hevea* with the gene coding for osmotin protein with a view to impart abiotic stress tolerance, so as to increase productivity. Osmotin gene construct with CaMV 35S promoter and kanamycin as selectable marker, obtained from Dr. K. C. Bansal, Director, NBPGR, New Delhi on material transfer agreement was used for the purpose. Among the different types of target explants used, transformation frequency of about 76 per cent was obtained for the embryogenic calli derived from immature zygotic embryo, followed by embryogenic calli derived from anther (48%). Among the two methods of transformation attempted, the liquid co-cultivation was found to be better and the transformation frequencies were significantly higher. Transgenic cell lines could be proliferated in MS medium with the growth regulators 2, 4- D (1.5 mg l^{-1}) and NAA (1.0 mg l^{-1}). A proliferation frequency of 60 per cent could be achieved.

From the different experiments carried out for embryo induction, it was clearly observed that growth regulators play a pivotal role in embryogenesis from the osmotin transgenic cell lines. When NAA and Kinetin combinations were tried, an embryo induction frequency of 43% could be obtained with 2.0 mg l^{-1} NAA and 0.3 mg l^{-1} kinetin. This could be enhanced when ABA and GA_3 were supplemented along with NAA and kinetin. The highest embryo induction frequency of 50% could be obtained in a growth regulator combination of 0.1mgl^{-1}ABA, 0.3 mg l^{-1} kinetin, 2.0 mg l^{-1} NAA and 0.3 mg l^{-1} GA_3.

For embryo maturation, effect of ABA in combination with different levels of phytagel was studied initially in hormone free medium. It was

observed that higher levels of phytagel, as well as ABA could improve embryo maturation considerably. Maximum number of mature embryos could be obtained in a combination of 5 per cent phytagel and 0.8 mg l^{-1} ABA. Since the influence of sucrose on embryo maturation was already reported in *Hevea,* different levels of sucrose and phytagel were tried along with 0.8 mg l^{-1} ABA. A combination of 75 g l^{-1} sucrose and 4 g l^{-1} phytagel favoured embryo maturation and a high maturation frequency of 51 per cent could be obtained.

The mature embryos with well developed cotyledons were transferred to germination medium. Among the different levels of growth regulators tried, maximum embryo germination (23.6 %) was obtained in a combination of 1.5 mg l^{1-} BA and 1.5 mg l^{-1} GA_3. Among the desiccation treatments, slow drying of embryos in closed petri dishes for 48 hrs could enhance embryo germination to 44 per cent. However, after the initial boost up in germination, further growth was found to be retarded. For plant regeneration, IBA was tried along with BA, in presence of 1.5mg $l^{-1}GA_3$. In this study enhanced plant conversion frequency (23%) could be obtained with a combination of 3 mg l^{-1}BA and 1.5 mg l^{-1} IBA.

Mannitol has been proved to have a strong influence in improving the efficiency of the regeneration pathway at all stages *viz.* embryo induction, maturation and germination. Percentage of embryo induction, maturation and germination could be enhanced to 67, 63, and 27 per cent respectively when mannitol was supplemented along with sucrose.

Transgenic plants integrated with osmotin gene could be developed from both clonal and zygotic sources. Plants were acclimatized in growth chamber, under controlled conditions. It was observed that the survival rate

of plants derived from zygotic source was higher compared to the plants derived from clonal material. Plantlets were healthier and had more number of lateral roots.

Attempts made for micrografting of the transgenic shoots on to stock seedlings raised *in vitro* showed only initial success with the development of a few new flushes.

The genetic transformation, gene integration and expression were validated through different assays. When PCR was carried out using gene specific primers, it could successfully amplify the 0.75 kb gene insert coding for osmotin protein confirming transgene integration. In the Southern hybridization, the labeled probe hybridized with the integrated gene insert in the DNA isolated from transgenic plants, indicating successful integration of the transgene. On RT-PCR analysis, 0.75 kb osmotin gene insert could be amplified from the cDNA prepared from the RNA isolated from the leaves of transgenic plants, which in turn confirms the transgene expression.

The stress tolerance studies conducted using transgenic calli, showed better expression for stress tolerance. The proline estimation data showed that there is an increase in the proline content with increase in water stress for both transgenic and non-transgenic calli. The percentage increase in proline content was much higher for transgenic cell lines when compared to non-transgenic calli. Similarly, the transgenic calli could survive and proliferate in culture media containing 150 mM NaCl, whereas the non-transgenic calli showed retarded growth even in the presence of 50 mM NaCl.

. Transgenic plants of *Hevea* were developed and hardened. Transgene integration and expression were confirmed by molecular analysis. Stress tolerance studies on transgenic callus showed positive indications towards

drought and salinity. The *in silico* analysis of the transgene could prove the uniqueness of the inserted sequence over the native osmotin gene. The regenerated plants are expected to show resistance to abiotic stress such as drought, salinity and cold. Once established in the field, these transgenic *Hevea* plants have immense potential to perform well under adverse climatic conditions, especially in the non-traditional areas of rubber cultivation. The transgenics derived from zygotic source can be a good option as stress tolerant root stock and can bypass the biosafety concerns and environmental biodiversity issues due to gene transfer through out-crossing. This is the first report of developing transgenic plants of *Hevea* integrated with a functional alien gene for abiotic stress tolerance.

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ANNEXURES

ANNEXURE-1

Modified MS Medium

Major

NH_4NO_3	:	1000mg l^{-1}
KNO_3	:	1900mg l^{-1}
$CaCl_2$	:	440 mg l^{-1}
$MgSO_4$	:	370 mg l^{-1}
KH_2PO_4	:	170 mg l^{-1}

Minor

H_3BO_3	:	6.20 mg l^{-1}
$MnSO_4.6H_2O$	:	22.3 mg l^{-1}
$ZnSO_4.7H_2O$	:	8.6 mg l^{-1}
KI	:	0.083 mg l^{-1}
$Na_2MoO_4.2H_2O$	:	0.25 mg l^{-1}
$CuSO_4.5H_2O$	:	0.025 mg l^{-1}
$CoCl_2.6H_2O$	:	0.025 mg l^{-1}
Iron Sodium EDTA	:	37.3 mg l^{-1}
B5 vitamins		
Myoinositol	:	100 mg l^{-1}
Glutamate	:	150 mg l^{-1}
Casein hydrolysate	:	400 mg l^{-1}
Adenine sulphate	:	50 mg l^{-1}

ANNEXURE-II

Nitsch Medium

Major

NH_4NO_3	:	720mg l^{-1}
KNO_3	:	950mg l^{-1}
$MgSO_4$	:	185 mg l^{-1}
KH_2PO_4	:	68 mg l^{-1}

Minor

$MnSO_4.4H_2O$	:	25 mg l^{-1}
$ZnSO_4.7H_2O$	:	10 mg l^{-1}
$Na_2MoO_4.2H_2O$	:	0.25 mg l^{-1}
$CuSO_4.5H_2O$	:	0.025 mg l^{-1}
$CoCl_2.6H_2O$	:	0.025 mg l^{-1}
Iron Sodium EDTA	:	27.8 mg l^{-1}
Myoinositol	:	100 mg l^{-1}
Thiamine Hcl	:	0.50 mg l^{-1}
pyridoxine Hcl	:	0.5 mg l^{-1}
Nicotinic acid	:	5 mg l^{-1}
Glycine	:	2 mg l^{-1}
Folic acid	:	0.5 mg l^{-1}
Biotin	:	0.05 mg l^{-1}

ANNEXURE III

EI Medium

Major

NH_4NO_3	:	500 mg l^{-1}
KNO_3	:	800 mg l^{-1}
$CaCl_2$	:	90 mg l^{-1}
$MgSO_4$	:	90 mg l^{-1}
KH_2PO_4	:	85 mg l^{-1}

Minor

H_3BO_3	:	0.620 g l^{-1}
$MnSO_4.6H_2O$	:	1.69 g l^{-1}
$ZnSO_4.7H_2O$	:	0.86 g l^{-1}
KI	:	0.083 g l^{-1}
$Na_2MoO_4.2H_2O$	:	0.025mg l^{-1}
$CuSO_4.5H_2O$	:	0.0025mg l^{-1}
$CoCl_2.6H_2O$	:	0.0025mg l^{-1}
Iron Sodium EDTA	:	36 mg l^{-1}
Myoinositol	:	100 mg l^{-1}
Glutamate	:	150 mg l^{-1}
Casein hydrolysate	:	400 mg l^{-1}
Adenine sulphate	:	50 mg l^{-1}
Proline	:	100 mg l^{-1}
Arginine	:	37 mg l^{-1}
Sucrose	:	50 g l^{-1}
Coconut water	:	10%
pH	:	5.6-5.8
Phytagel	:	5 g l^{-1}

ANNEXURE IV

AELB MEDIUM

Tryptone	:	5 g l^{-1}
Yeast Extract	:	2.5 gl^{-1}

MGL MEDIUM

Tryptone	:	5 g l^{-1}
Yeast Extract	:	2.5 g l^{-1}
NaCl	:	5 g l^{-1}
Glutamic acid	:	1.16 g l^{-1}
KH_2PO_4	:	250 mg l^{-1}l
$MgSO_4.7H_2O$	:	100 mg l^{-1}
Biotin	:	1 mg l^{-1}
pH	:	7.0

TY MEDIUM

Tryptone	:	1.25g l^{-1}
Yeast Extract	:	0.75g l^{-1}
Acetosyringone	:	10m g l^{-1}
pH	:	5.5

ANNEXURE V

CO-CULTURE MEDIUM

ECII Major	:	50ml
MS Minor	:	5ml
Iron Sodium EDTA	:	37.5 mg l^{-1}
Myoinositol	:	100 mg l^{-1}
Sucrose	:	50 g l^{-1}
Glutamic Acid	:	150 mg l^{-1}
Arginine	:	37 mg l^{-1}
pH	:	5.6-5.8

To be filter sterilized

Acetosyringone	:	20 mg l^{-1}
Glycine Betaine HCl	:	153.6 mg l^{-1}
Proline	:	115.5 mg l^{-1}
Hormones:		
NAA	:	0.2 mg l^{-1}
Kin	:	0.3 mg l^{-1}
GA	:	0.5 mg l^{-1}
BA	:	0.2 mg l^{-1}
2, 4-D	:	0.1 mg l^{-1}