Molecular Characterization and Expression of Somatic Embryogenesis Receptor Kinase (SERK) Gene from *Hevea brasiliensis* (Muell. Arg.)

Project report submitted to

MAHATMA GANDHI UNIVERSITY

In partial fulfillment of the requirements for the award of the degree of

MASTER OF SCIENCE

in

BIOTECHNOLOGY

Submitted by

SOYA MARY JOSE
Reg. No: 030910

School of Biosciences
Mahatma Gandhi University
Kottayam, Kerala
2009 – 2011

Undertaken at
Biotechnology Division
Rubber Research Institute of India (RRII),
Kottayam
September 2011

MAHATMA GANDHI UNIVERSITY

SCHOOL OF BIOSCIENCES

Priyadarshini Hills P. O.
KOTTAYAM - 686 560
Kerala, South India
Phone: (0481) 2731035

Prof. (Dr.) JYOTHIS MATHEW
Director

No. BMS/101/AMS/23/11-12

CERTIFICATE

This is to certify that the dissertation entitled, "**Molecular Characterisation and Expression of Somatic Embryogenesis Receptor Kinase (SERK) Gene in *Hevea Brasiliensis* (Muell.Arg)** " is an authentic record of the project work done by Ms.Soya Mary Jose at Rubber Research Institute of India, Kottayam, under the guidance of Dr.P.Kumari Jayasree, Scientist C, Biotechnology, Rubber Research Institute of India, Kottayam , in partial fulfillment of the requirement for the award of the Degree of Master of Science in Biotechnology at the School of Biosciences, Mahatma Gandhi University, Kottayam and this dissertation has not formed the basis for the award of any other degree or diploma earlier.

August 2011

DIRECTOR

The Rubber Research Institute of India
Rubber Board, Ministry of Commerce and Industry, Govt. of India
Kottayam – 686 009, Kerala, INDIA

Dr. A. Thulaseedharan
Deputy Director (Biotechnology)

September 21, 2011

CERTIFICATE

This is to certify that the dissertation entitled **"Molecular Characterization and Expression of Somatic Embryogenesis Receptor Kinase (SERK) Gene in *Hevea brasiliensis* (Muell. Arg.)"** submitted by **Soya Mary Jose**, School of Biosciences, Mahatma Gandhi University, Kottayam, was carried out at Biotechnology Division, Rubber Research Institute of India, under the supervision of Dr. P. Kumari Jayasree, Scientist C (Biotechnology), during the period April – June 2011. It is also certified that this work has not been presented for any other degree or diploma.

(Dr. A. Thulaseedharan)

Tele Phone: 91- 481- 2353311 to 2353320 Fax: 91- 481- 2353327
E-mail thulaseedharan@rubberboard.org.in

The Rubber Research Institute of India
Rubber Board, Ministry of Commerce and Industry, Govt. of India
Kottayam – 686 009, Kerala, INDIA

Dr. P. Kumari Jayasree
Scientist C (Biotechnology)

September 20, 2011

CERTIFICATE

This is to certify that the dissertation entitled **"Molecular Characterization and Expression of Somatic Embryogenesis Receptor Kinase (SERK) Gene in *Hevea brasiliensis* (Muell. Arg.)"** submitted by **Soya Mary Jose**, School of Biosciences, Mahatma Gandhi University, Kottayam, was carried out at Biotechnology Division, Rubber Research Institute of India, under my supervision during the period April – June 2011. It is also certified that this work has not been presented for any other degree or diploma.

(Dr. P. Kumari Jayasree)

Tele Phone: 91- 481- 2353311 to 2353320 Fax: 91- 481- 2353327
E-mail thulaseedharan@rubberboard.org.in

DECLARATION

I, Soya Mary Jose, hereby declare that the project report entitled **"Molecular** cular **Characterization and Expression of Somatic Embryogenesis Receptor Kinase (SERK) Gene in *Hevea brasiliensis* (Muell. Arg.)"** is an authentic record of the work done by me under the guidance of Dr. P. Kumari Jayasree, Scientist C, Biotechnology Division, Rubber Research Institute of India, Kottayam; in partial fulfillment of the requirements for the award of the Degree of Master of Science in Biotechnology from Mahatma Gandhi University; and that, no part of this work has been presented earlier for the award of any other degree or diploma.

Soya M.J.

Priyadarshini Hills,
September 2011

Soya Mary Jose

ACKNOWLEDGEMENT

I would like express my deep reverence to God Almighty for His benevolence and for blessing me with a loving and supportive family, for answering my prayers and for giving me the strength to complete this work.

I express my sincere thanks to Dr. James Jacob, Director (Research) of Rubber Research Institute of India (RRII), Kottayam; for validating me permission for conducting this study at the Biotechnology Division of RRII.

I express my heartfelt gratitude to Dr. A. Thulaseedharan, Deputy Director, Biotechnology Division, Rubber Research Institute of India, Kottayam; for his valuable suggestions and for granting me permission to carry out this work in the Biotechnology Division laboratory without any restriction to its resources.

With high esteem and gratification, I express my indebtedness to Dr. P. Kumari Jayasree, Scientist C, Biotechnology Division, Rubber Research Institute of India, Kottayam; my Guide, for giving me superb guidance and constant support, which have helped me a lot in my work. Her passion for science has been a constant source of encouragement and I have benefited vastly from my association with her. I am indebted to her.

I express my sincere regards to Dr. Jyothis Mathew, Director of School of Biosciences, for granting me permission to join and work at RRII. No words can express my respect towards my teachers Dr. K. Jayachandran, Dr. Linu Mathew and Dr. Prakash Kumar for their invaluable suggestions and for igniting a passion for science in me during my tenure as a post – graduate student at the School of Biosciences.

I record my deep sense of gratitude to Mrs. Supriya R, Senior Research Fellow, Biotechnology Division, RRII, for her constant support and encouragement throughout the program. I am proud to record that I had several opportunities to work with such an exceptionally experienced researcher like her. I express my heartfelt regards to Dr. Babitha S. and Miss Suni Anie Mathew for their constant support and warm companion ship extended to me in the lab, which gave me the confidence that I needed.

With great respect, I record my sense of gratitude to Dr. S. Sushamakumari (Senior Scientist), Dr. S. Sobha, Mrs. R.G. Kala, Mrs. K. Rekha, & Mrs. R. Jayashree

(Scientists), and Mrs. Leda Pavithran (Scientific Assistant),, Biotechnology Division, RRII for their timely help and advices. I also thank all the staff, research scholars and trainees at RRII for their help and support.

To all my teachers at the School of Biosciences, I bow my head before you. You have come so far along the path that I have only started to tread. To the technical staff at the School of Biosciences, thank you for your cooperation.

I consider myself immeasurably fortunate to have been blessed with a ring of friends. Thank you for laughing, sharing, and holding me up through everything good & bad. Thank you for all those innumerable, unforgettable memories...

Something that has always puzzled me all my life is why, when I am in special need of help, the good deed is usually done by somebody on whom I have no claim.

~William Feather

Soya

ABBREVIATIONS

2,4-D	2,4-dichloro phenoxyacetic acid
ABA	Abscisic acid
BA	6-Benzyl amino purine / Benzyl adenine
BAK1	Brassinosteroid Insensitive1 Associated kinase
BR1	Brassinosteroid Insensitive1
CTAB	Cetyl trimethyl ammonium bromide
EDTA	Ethylene diamine tetra acetic acid
H.brasiliensis	*Hevea brasiliensis*
IBA	Indole-3-butyric acid
IPTG	Isopropyl-ß-D-1-thiogalactopyranoside
Kn	Kinetin
LB	Luria Bertani
LEA	Late embryogenesis abundant
Leu	Leucine
LRR	Leucine Rich Repeat
MS	Murashige & Skoog
MW	Molecular weight
NAA	a-Naphthalene acetic acid
PEG	Poly ethylene glycol
PEM	Proembryonic mass
PGR	Plant Growth Regulator
RLK	Receptor – like kinase
Ser/Thr kinase	Serine/Threonine kinase
SERK gene	Somatic Embryogenesis Receptor Kinase gene

SPP motif	Serine – Proline – Proline motif
TBE	Tris borate EDTA
TDZ	Thidiazuron
TE	Tris EDTA
WPM	Woody Plant Medium
X – Gal	5-bromo-4-chloroindoyl-ß-D-galactoside
ZEA	Zeatin

INDEX

INTRODUCTION

INTRODUCTION

Hevea brasiliensis (Muell. Arg.), the rubber tree, originated in Brazil and is now widely grown in tropical regions of Asia. This tree crop belonging to the botanical family *Euphorbiaceae,* is the only species commercially cultivated as a source of natural rubber. In plant kingdom, among the over 12,500 plant species producing latex, natural rubber is found only in the latex of over 2000 species. Since several centuries, natural rubber has been an important industrial commodity used in the manufacture of a wide range of products essential for mankind. The ever growing demand for natural rubber is mainly in the tyre industry and the rising prices due to this demand was the main impetus for the rapid expansion of rubber cultivation worldwide. In India, rubber cultivation started in 1902. In 2009-10, the production of natural rubber in India was 8.3lakh tonnes. Presently, India occupies first position in terms of productivity and fourth in production of natural rubber. Thailand, Indonesia and Malaysia are the top three producers of rubber in the world (Indian Rubber Statistics, 2010). In India, more than 98% of rubber production is from the traditional rubber growing tracts of Kerala, Tamil Nadu and Karnataka (fig: 1).

More than 50 years ago, using the original Wickham gene pool, *Hevea* breeding attempts were initiated in India, and several outstanding clones with a substantial enhancement in productivity were released. Conventional breeding is one of the effective breeding tools for genetic improvements in this tree crop. However, the efficiency of conventional breeding techniques for specific desired traits become rather slow and quite difficult due to the very narrow genetic base, seasonal nature of flowering and low fruit set and the lack of reliable early selection methods. Moreover, the heterozygosity of the tree as well as long immaturity period makes *Hevea* breeding very complicated. Molecular breeding by genetic transformation represents an attractive alternative approach to conventional breeding. However, an essential pre-requisite for this genetic manipulation studies is the availability of an efficient *in vitro* regeneration system. Plant regeneration through somatic embryogenesis is considered as a powerful tool for micropropagation as well as an essential component for genetic transformation because of single cell origin of somatic embryos (Merkle *et al.*, 1995).

Figure 1: Healthy rubber plantation with a clear view of tapping panel in the inset

Somatic embryogenesis is the process by which somatic cells, under induction conditions generate embryogenic cells, which go through a series of morphological and biochemical changes that result in the formation of somatic embryos (Zimmerman, 1993). This unique phenomenon has been widely reported for almost all plant species including perennial tree crops like *Hevea*. In this plant species, plant regeneration based on somatic embryogenesis can provide a rapid *in vitro* method for mass propagation as well as for

opening up new avenues for crop improvement. In *Hevea*, somatic embryogenesis has been successfully induced from different explants; however, somatic embryo formation is limited to only a few genotypes of *Hevea* (Kumari Jayasree *et al.*, 1999). As far as Indian rubber clones are concerned, successful embryo induction and plant regeneration was reported from the clone RRII 105 and different explants like immature anther (Kumari Jayasree *et al.,* 1999), inflorescence (Sushamakumari *et al.*, 2001) and leaf (Kala *et al.*, 2005) very well responded for this phenomenon. However, only limited success has been reported on transgenic rubber plant development with functional gene of interest. One underlying factor for this obstacle is the prolonged transformation procedure causing a decreased regeneration potential and difficulties in embryogenic calli formation. Induction of embryogenic calli and potency of embryogenesis are crucial aspects that need to be improved during somatic embryogenesis. Generally, auxin is used for the induction of embryogenic cells and its subsequent withdrawal from the medium leads to embryo formation. In plant tissue culture system, competent cells give rise to embryogenic cells following the application of auxin that later develops into somatic embryos (Albertini *et al.*, 2005).

During somatic embryogenesis, biochemical and morphological changes occur throughout the development of induced tissues (Sharp *et al.*, 1980) which is strongly related to alteration in gene expression pattern (Zimmerman, 1993). Therefore some genes are differentially expressed during somatic embryogenesis induction, while others are expressed during differentiation from embryo maturation upto full plant development (Zimmerman, 1993). Some research groups conducted light and electron microscopy to study structural and cellular changes and these research reports provided detailed descriptions of the morphological and ultrastructural changes of embryogenic competent calli and embryoid initiation (Namasivayam, 2007). Based on these observations on various plant systems, including pearl millet and cork oak, the embryogenic cells that form somatic embryos are characterized generally as small, isodiametric in shape, have large and densely staining nuclei and nucleoli and are densely cytoplasmic (Namasivayam, 2007). Some of the early attempts to find indicators for embryogenic competence relied on biochemical markers. Several biochemical variables like proteins and isozymes have been shown to discriminate between embryogenic and non embryogenic cells (Blanc *et al.,* 1997; Alves *et al.,* 1994; Asokan *et al.,* 2001). However, the molecular mechanism underlying this phenomenon is not well understood.

Understanding the molecular mechanism of somatic embryogenesis would explain and help to overcome frequently encountered difficulties such as genotype dependence of embryogenic response, initiation of embryogenic calli and regeneration of plants. During the past decade, molecular biologists focused in plant development have resulted in the identification of different genes involved in the regulation of this process.

In somatic embryogenesis, the transition of somatic cells to embryogenic cells involves the genetic reprogramming of cells by the differential expression of a set of genes; which in turn elicits diverse cellular and physiological responses that confer the ability of embryogenic competence. Among the genes involved with the induction of somatic embryogenesis, the SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) gene is claimed to have an important role. SERK gene belongs to the plant receptor kinase superfamily (Ito *et al.*, 2005). The plant receptor kinases, often termed Receptor – like Kinases (RLKs), are characterized by an extracellular domain, a single transmembrane domain and a cytosolic kinase domain and they play a role in controlling a broad range of developmental processes in plants (Cock *et al.*, 2002). SERK gene has been characterized for its Leu-rich repeat receptor-like kinase (LRR-RLK) activity. This gene has been linked to somatic embryogenesis in numerous species.

Carrot (*Daucus carota*) DcSERK was the first SERK gene isolated from the suspension culture cells that developed further to somatic embryos (Schmidt *et al.*, 1997). DcSERK gene expression was detectable for 7 days following incubation in medium containing auxin and the expression was extended to globular zygotic embryo stage, suggesting a correlation between SERK gene expression and somatic embryogenesis (Schmidt *et al.*, 1997). In *Arabidopsis thaliana*, the function of this gene has been extensively studied and a small group of SERK proteins with five members involved in different signalling pathways have been identified. Among these, AtSERK1 is the most extensively characterized and have been shown to be involved in acquisition of embryogenic competence. This gene is expressed in embryogenic structures, while it is not detectable in non embryogenic cells (Hecht *et al.*, 2001). Since then, several SERK genes have been isolated from other plant species including *Helianthus annuus* (Thomas *et al.*, 2004), *Citrus unshiu* (Shimada *et al.*, 2005), *Triticum aestivum* (Singla *et al.*, 2008), *Cocos nucifera* (L.) (Perez- Nunez *et al.*, 2009) etc.

Since the frequency of somatic embryogenesis is low in some species and is also genotype dependent, the expression of SERK genes has been used as a marker for identifying competent cells that subsequently develop into somatic embryos. Like many woody tree crops, *Hevea* is considered as a recalcitrant tree crop for tissue culture and somatic embryogenesis induction is strongly genotype specific. In *Hevea*, no genes related to somatic embryogenesis have been reported so far. Thus it is highly significant to detect the presence of SERK gene in *Hevea* and to investigate the SERK gene expression as a potential marker of this process. Keeping these points in mind, the present study was undertaken with the following objectives.

OBJECTIVES

1. To isolate SERK gene from genomic DNA of *Hevea* clone RRII 105
2. To study the expression of SERK gene in embryogenic calli
3. To study the possible application of SERK gene as a molecular marker for screening embryogenic calli in *Hevea*

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Hevea brasiliensis (Muell. Arg.), the principal source of natural rubber, is an open-pollinated tree crop belonging to *Euphorbiaceae* family. Of the 10 species reported to produce natural rubber (Wycherly, 1992), *Hevea brasiliensis* is the source of virtually all the world's rubber production. Other major species include *H. guianensis, H. benthamiana, H. nitida, H. camargoana, H. rigidifolia, H. microphylla, H. camporum, H. pauciflora* & *H. spruceana* (Schultz,1990); and minor sources are *Parthenium argentatum, Manihot glaziovii, Ficus elastica, Funtumia elastica, Castilla elastica and Cryptostegia grandiflora.*

Hevea is a woody deciduous tree with annual leaf fall. It has been established that the chromosome complement of rubber tree in somatic cells is 2n=36 (Ramer, 1935; Saraswathyama *et al.*, 1984). The leaves are compound trifoliate alternate with a pair of nectarean glands. Small monoecious light-yellow flowers are found in clusters at young branch apices. Male flowers are smaller and numerous compared to the female flowers. Natural rubber is synthesized in highly specialized cells called latex vessels or laticifers which are present in every part of the plant except in the heart wood. The laticifers originate from the cambium and they are articulated & anastomosing (Hebant, 1981). *H.brasiliensis* is a tropical tree and it grows best at temperatures of 20-28°C with an average rainfall of 1800-2000 mm. It grows satisfactorily on moist soils with adequate drainage.

Latex is "tapped" or harvested by making shallow oblique cuts on the bark, exposing the cambium. It is then coagulated and processed to yield natural rubber. Chemically, natural rubber is a polymer of cis-1,4-isoprene units, with 200,000 - 8000,000 MW. It has high viscosity & elasticity. Natural rubber in this native form cannot be utilized by most industries. Instead, it is vulcanized with sulphur under high temperature and utilized. Properties like elasticity, heat dispersion, abrasion & impact resistance, malleability at cold temperatures and insolubility in alcohol, water, dilute acids & alkalis (Cornish, 2000) make vulcanized natural rubber the principle raw material for many large industries like automobile industry. More than 50,000 rubber-based products like gloves, footwear, toys etc are manufactured wordwide. It is also useful in surfacing roads, stabilizing soil etc.

The wood from this tree, refered to as 'Para wood' or rubber wood, is used in the manufacture of high-end furniture. It is valued for its dense grain, minimal shrinkage, attractive collar and acceptance of different finishes. It is also prized as an "environmentally friendly" wood, as it is made out of trees that have been cut down at the end of their latex producing cycle. Rubber wood has become a second product of rubber cropping.

The genetic base of rubber is very narrow as it originated from about 10 mother trees grown from the 70,000 odd seeds collected by Wickham in 1876 on the banks of the Tapajo in Para, Brazil (Schultes, 1977). This narrow genetic base along with its long breeding cycle and high juvenility period, which extends up to seven years, makes the conventional crop improvement programmes rather difficult in rubber. Rubber plantations were originally established with unselected seedlings which resulted in considerable heterogeneity. From 1920 onwards, research was carried out along several lines to improve the quality of plant material.

Hevea has very low rhizogenic potential, which is often fugacious and exhausted easily (Muzik and Cruzado, 1958). For a very long time, "selected" seeds were preferred to grafts because the resulting trees were more vigorous and displayed better resistance to diseases. Rubber was introduced in India as "rooted cuttings" or directly as seeds. Budding and grafting was not popular in the early days. However, between 1940-1950, almost all plantations had been established with grafted clones where the trees were comparatively homogeneous and also because of the difficulty in obtaining selected seeds in sufficient quantities for development programmes (Nayanakantha and Seneviratne, 2007). Conventional breeding methods include introduction of exotic clones, selection and hybridization. In *Hevea* cultivation, seedlings obtained by pollination, and clones obtained as a result of budding can be used as planting materials. It is currently propagated vegetatively in nurseries through budgrafting (budding). At present, seeds are used mainly for the production of rootstocks. And polyclonal seeds are used for propagation directly.

In India, more than 50 years ago, *Hevea* breeding programmes were initiated using the Wickham gene pool. Improvement of established high-perfomance clones cannot be done feasibly using traditional methods alone. Tissue culture provides an alternative approach by which clones can be propagated effectively with reduced time.

Plant tissue culture

Haberlandt started his first attempt in plant tissue culture in 1902. However, due to technical problems, he did not succeed. Later, many scientists put their effort in this line and by exploiting the phenomenon of totipotency of plant cells, much progress was carried out in plant tissue culture. When researching the history of plant tissue culture, significant achievement was made in the cultivation of free cells and cell groups derived from angiosperms in a chemically defined medium. Subsequently, by changing the hormonal balance of the medium, regeneration potential of the plant cells was induced. A type of regeneration frequently observed is the development of embryo – like structures in the culture. Since the embryos are derived from somatic cells of plants, this phenomenon is referred to as "somatic embryogenesis".

Somatic embryogenesis

Plants are unique in their ability to produce somatic embryos (SE) (Jayashankar *et al.*, 2001). In plant tissue culture, somatic embryogenesis is the process of embryo initiation and development from vegetative or non-gametic cells (Bhojwani and Razdan, 2004). Somatic embryos resemble zygotic embryos morphologically; they are bipolar and bear typical embryonic organs. According to Fehér (2006), this phenomenon demonstrates two important aspects of plant embryogenesis:

i. the fertilization trigger can be substituted by endogenous mechanisms,
ii. in addition to the fertilized egg, somatic cells in higher plants can maintain or regain the capability for embryogenic development.

Fehér *et al.* (2003) considered this to be one of the most extreme examples of flexibility in plant development. However, the greatest interest of somatic embryos is centered in its practical application for large-scale vegetative propagation, particularly because of the possibility to scale up the propagation by using bioreactors (Fuentes *et al.*, 2000). Somatic embryogenic cultures are also an attractive target for gene transformation.

Since the first observation of somatic embryo formation in *Daucus carota* cell suspensions by Steward *et al.* (1958) and Reinert (1958), the potential for somatic embryogenesis has been shown to be characteristic of a wide range of tissue culture systems in plants. During the past 40 years, somatic embryogenesis has been described in

a large number of plant species. Somatic embryos are used as a model system in embryological studies. In addition, in most cases, somatic embryos or embryogenic cultures can be cryopreserved, which makes it possible to establish gene banks. Also, large scale production and propagation of artificial seeds is another important application of somatic embryogenesis.

Changes during somatic embryogenesis

In tissue explants, the first response is often noted to be the rapid replacement of the vacuole with cytoplasm, followed by the first division. In *D.carota*, following an increase in cytoplasmic content, cell division was resumed in provascular cells, but not in cortical or in epidermal cells (Guzzo et al., 1994). Broadly, there is active cell wall synthesis, along with increased activity of genes in cell cycle regulation. Continued cell divisions then result in the formation of masses of small isodiametric cells. These cells then enlarge and finally detach into the culture medium to form the developing embryogenic cell culture. Somatic embryos go through four distinct stages of growth: globular stage, triangle stage, heart – shaped stage and finally torpedo stage; by which the developing embryo has distinct root & shoot primordial.

Plant regeneration via somatic embryogenesis includes five steps (Arnold *et al.*, 2002):

i. Initiation of embryogenic cultures by culturing the primary explant on medium supplemented with plant growth regulators (PGRs), mainly auxin (2,4-D) but often also cytokinin.
ii. Proliferation of embryogenic cultures on solidified medium or in liquid medium supplemented with PGRs, similar as under initiation.
iii. Prematuration of somatic embryos in medium lacking PGRs; this inhibits proliferation and stimulates somatic embryo formation and early development.
iv. Maturation of somatic embryos by culturing on medium supplemented with ABA and/or reduced osmotic potential.
v. Development of plants on medium lacking PGRs.

i. Initiation of embryogenic cultures:

The induction of somatic embryogenesis must consist of the termination of a current gene expression pattern in the explant tissue, and its replacement with an

embryogenic gene expression programme. One possible mechanism for downregulation of current gene expression is DNA methylation, which is influenced by auxins (LoSchiavo *et al.*, 1989). PGRs and stress play a central role in mediating the signal transduction cascade leading to the reprogramming of gene expression. This results in a series of cell divisions that induce either unorganised callus growth or polarized growth leading to somatic embryogenesis (Dudits *et al.*, 1995).

Two mechanisms appear to be important for *in vitro* formation of embryogenic cells:

- asymmetric cell division and
- control of cell elongation (De Jong *et al.*, 1993; Emons, 1994).

Asymmetric cell division is promoted by PGRs that alter cell polarity by interference with the pH gradient or the electrical field around cells (Smith and Krikorian, 1990). The ability to control cell expansion is associated with polysaccharides of the cell wall and corresponding hydrolytic enzymes (De Jong *et al.*, 1993; Emons, 1994; Fry, 1995). The capacity for somatic embryogenesis is genetically determined. *Daucus carota* and *Medicago sativa* are capable of developing into Somatic embryos, irrespective of the explant that is used. However, initiation of somatic embryogenesis in other plants depend strongly on the nature of explant used; and embryonal or highly juvenile tissues are preferred.

ii. Proliferation of embryogenic cultures:

Once embryogenic cells have been formed, they continue to proliferate, forming PEMs or proembryogenic masses. Low pH of around 5.8 to 4 is essential for maintaining cultures in the proliferation phase (Smith and Krikorian, 1990). The composition of the culture medium remains similar to that of the induction medium and can be solid / semi – solid. For large scale proliferation and better synchronization of growth, suspension cultures are preferred (Arnold *et al.*, 2002). However, the occurrence of somaclonal variation increases with repeated subculturing, and in most crops embryogenic potential decreases or may be lost.

iii. Prematuration of somatic embryos:

The PEMs need to reach an appropriate developmental stage before it can subjected to maturation treatments. If this stage is not reached, most of the PEMs fail to give rise to well – developed somatic embryos. Therefore, in order to stimulate further growth of the somatic embryos it is necessary to transfer the embryogenic cultures to

medium lacking auxin. With the depletion of auxin, the block on the expression of those genes required for the transition to the heart stage is removed (Zimmerman, 1993).

iv. Maturation of somatic embryos:

During the maturation stage the somatic embryos undergo various morphological and biochemical changes. The synthesis and deposition of storage and late embryogenesis abundant (LEA) proteins during somatic and zygotic embryogenesis are usually regulated through ABA and water stress induced gene expression (Dodeman *et al.*, 1997). Thus, application of exogenous ABA in amounts of 10 – 50μM in maturation medium prevents secondary embryogenesis and precocious germination. In conifers, ABA treatment triggers embryo maturation. Also, inducing water stress by adding PEG with MW>4000 promotes dessication; which mimics normal zygotic embryo development (Rains, 1989). Many other factors such as ethylene, osmotic stress, pH and photoperiod have been reported to influence somatic embryo maturation in different species.

v. Development of plants:

Only mature embryos with a normal morphology and which have accumulated enough storage materials and acquired desiccation tolerance at the end of maturation develop into normal plants. They are grown in media lacking PGRs. Mature SEs develop into plantlets just like normal seedlings, but are often much smaller. After attaining suitable size, they can be transferred as potted plantlets into greenhouses, and later onto fields.

Tissue culture in *Hevea*

The common method for propagating elite clones of *Hevea*, a predominantly cross pollinated species, is budgrafting. However, due to stock scion interactions, a certain amount of heterogeneity is observed in a population propagated by budgrafting. Micropropagation through tissue culture presents a suitable solution for this problem. Most of the *in vitro* culture work in *Hevea* is directed towards shoot tip culture, nodal culture and somatic embryogenesis.

The first successful work on *Hevea* micropropagation was done by Enjalric and Carron (1982), using shoots of young plants grown in greenhouses. Culturing of mature explants was done in RRII by Sobhana *et al.* (1986) and Asokan *et al.* (1988). *In vitro*

multiplication was later done using seedling explants (Gunatilleke and Samaranayake, 1988; Seneviratne and Flegmann, 1996). Sterilization techniques for preventing fungal contamination in cultures were studied and perfected by Asokan *et al.* (1988) and Enjalric *et al.* (1987). Soaking of shoot explants in benzyl adenine (BA) induced better bud sprouting and elongation (Enjalric and Carron, 1982). Rooting of such sprouted shoots can be induced by soaking the base of shoots in Indole – 3 – butyric acid (IBA). However, shoot tip and nodal explants are highly recalcitrant in *Hevea*. Several studies suggest that this problem can be solved to an extent by using juvenile explants (Perrin *et al.*, 1994; Seneviratne and Wijisekare, 1997). Presently, majority of tissue culturing of *Hevea* is done through micropropagation and somatic embryogenesis.

Application of biotechnology in *Hevea* improvement

Elite clones of *Hevea* are susceptible to tapping panel dryness and suffer from many diseases like abnormal leaf fall caused by *Phytophthora*. Hence, introduction of resistant genes into the clones is in need.

Also, these elite clones are propagated in the field by grafting their buds onto seedling rootstocks. But these root stocks are highly heterozygous and the field performances of these clones are often not upto par because of emergence of intraclonal variations due to stock-scion interactions (Combe, 1975; Seneviratne and Flegmann, 1996). In this backdrop, development of uniform, self rooted plantlets offers an effective approach. Somatic embryogenesis can be employed here, because embryogenic calli consist of a large population of totipotent cells and the embryos are unicellular in origin, thus reducing the likelihood of chimeras. Further, the potentially large number of rooted plants which can be produced using somatic embryogenesis provides an alternative system for mass propagation of *Hevea*, and genetic engineering of such embryogenic calli can be done *in vitro*.

Somatic embryogenesis in *Hevea*

Somatic embryogenesis is one of the most powerful tissue culture techniques for mass propagation of elite *Hevea* clones as well as for development of genetically modified rubber plants. Molecular farming in *Hevea* through genetic engineering is another future application of somatic embryogenesis. In *Hevea,* somatic embryogenesis was initiated simultaneously by China and Malaysia, using anther wall as explants. Calli

derived from anther wall was first developed into embryoids and subsequently into shoots (Paranjothy, 1974; Paranjothy and Ghandimathi 1975; Paranjothy and Rohani 1978). It was Wang *et al.* (1980, 1984) and Wan *et al.* (1982) who succeeded in developing plantlets via somatic embryogenesis from anther wall derived calli. Later, Wang and Chen (1995) developed plantlets from stamen cultures. At CIRAD, somatic embryos were developed from the inner integument of immature seeds through four successive phases: (1) callogenesis (2) differentiation of embryos (3) multiplication of embryos and (4) germination of embryos and development into plantlets (Carron and Enjalric, 1982). In 1985, Carron and Enjalric succeded in plantlet development derived from somatic embryos of integumental tissues and further establishment in soil.

El Hadrami *et al.* (1989, 1991 & 1993), Michaux-Ferrier and Carron (1989), Auboiron *et al.* (1990) and Etienne *et al.* (1991) studied the effect of various factors influencing somatic embryogenesis in detail. Veisseire *et al.* (1994a, 1994b), Etienne *et al.* (1993a, 1993b) and Montoro *et al.* (1993, 1995) reported that a high level of sucrose supplemented with 1mM ABA or 12mM $CaCl_2$ caused maturation of somatic embryos, which strongly improved germinability and conversion to plantlets. A qualitative and quantitative comparison of somatic embryogenesis in four different *Hevea* clones (PB 260, PR 107, RRIM 600 and PB 235) was made by Carron *et al.* (1995a). In 1996, Cailloux *et al.* became successful in establishing a long term embryogenic line with recurrent embryogenesis. Etienne *et al.* (1977a, 1977b) developed and standardized a technique for pulsed air temporary immersion in high concentration solution of $CaCl_2$, that enhanced embryogenesis in calli. It was Engelmann *et al.* (1997) who reported rapid regrowth as well as production of somatic embryos with cryopreserved callus using freezing protocols. Most recently, Lardet *et al.* (2007) reported that calli precultured on 1mM $CaCl_2$ displayed better regeneration ability than those precultured on medium lacking $CaCl_2$; and that non regenerant lines became regenerant after cryopreservation. The team also demonstrated that embryos derived from cryopreserved tissues led to plant recovery with no phenotypic differences between the cryopreserved and non cryopreserved calli. Wang *et al.,*() identified suitable temperature requirements for callus & embryo induction and plant regeneration. The role of carbohydrates in somatic embryogenesis was investigated by Blanc *et al.* (1999); and they reported that maltose is significantly more effective. Charbit *et al.* (2004) was the first to identify 28 differentially expressed cDNAs during induction of embryogenic lines in *Hevea*.

Although plant regeneration via somatic embryogenesis was successful in *Hevea*, this regeneration system is still a fleeting phenomenon because of low germination percentage, fugacious embryo formation and low plant conversion (Carron *et al.*, 1989; Cailloux *et al.*, 1996; Linnossier *et al.*, 1997; Kumari Jayasree *et al.*, 1999). Substantial progress has been achieved in this direction (Thulaseedharan *et al.*, 2000). Somatic embryogenesis remains difficult due to low frequency plant regeneration and reliable embryogenesis is restricted to a few genotypes like PB 260, PR 107, PB 235, RRII 105, RRIM 600). The molecular mechanisms involved in embryogenic callus formation and induction and differentiation of embryos were poorly understood (Etienne *et al.*, 1993a). By the advent of genetic transformation using rDNA technology, there has been renewed interest in the development of plantlets through somatic embryogenesis (Kumari Jayasree *et al.*, 1999).

Research in *in vitro* plant regeneration via somatic embryogenesis for Indian *Hevea* clones was initiated at the Rubber Research Institute of India (RRII). The immature anthers and inflorescence were identified to be ideal explants for somatic embryogenesis (Kumari Jayasree *et al.*, 1999; Sushamakumari *et al.*, 2000b) of the high yielding Indian *Hevea* clone RRII 105. Kumari Jayasree *et al.* (1999) carried out in depth studies on the hormonal, nutritional and environmental conditions for maximum callus induction, embryogenesis and plantlet regeneration. With immature anther, optimum callus was induced on modified MS medium supplemented with 2.0mg/l 2,4-D and 0.5mg/l Kinetin. Subsequent culturing onto modified MS medium supplemented with 0.7mg/l Kinetin and 0.2mg/l NAA induced embryogenesis; while plantlet regeneration was achieved in hormone free medium. Cytological analyses confirmed the diploid nature of the regenerated plantlets. These plantlets were then successfully established in fields at the RRII. Further, plant development was enhanced by induction of multiple shoots from germinative embryos (Sushamakumari *et al.*, 1999b). It was Sushamakumari *et al.* (2000b) who conducted studies on the role of sucrose and ABA in induction and development of callus and embryo, and subsequent plant regeneration. Such somatic embryogenesis derived plantlets were then established in field.

Later, Kumari Jayasree *et al.* (2001) conducted detailed studies on enhancing embryo induction and plant regeneration from immature anthers. From the studty, it was concluded that an initial pre culturing of anthers in liquid medium ten days prior to culturing onto solid medium enhanced callus induction. Polyamines had no significant effect, except with spermidine (0.5mg/l). Addition of 150 – 200mg/l glutamine and

400mg/l casein hydrolysate enhanced embryo induction. Also, incorporation of 2.0mg/l GA_3 increases embryo induction and germination percentage (Kumari Jayasree and Thulaseedharan, 2001). Study of isozymes at different developmental stages of somatic embryos revealed a marked difference between embryogenic and non embryogenic calli (Asokan *et al.*, 2001). Repetitive embryogenesis from primary somatic embryos of integumental tissue was achieved by Asokan *et al.* (2002). Kumari Jayasree and Thulaseedharan (2004) reported that the embryonic potential of calli can be retained for upto three years. For high frequency plant regeneration of upto 80% activated charcoal was found to be essential. Inclusion of 100mg/l proline increased the regeneration potential. In 2005, Kumari Jayasree and Thulaseedharan concluded that out of the four cytokinins: [6-Benzylaminopurine (BA), Zeatin (ZEA), Kinetin (Kn) and Thidiazuron (TDZ)] tested for germination; TDZ at 0.25mg/l is superior for somatic embryo germination, followed by 0.25mg/l BA.

Kala *et al.* (2005, 2006), and Sushamakumari *et al.* (2006), made attempts to induce somatic embryos from *in vitro* derived leaf and root explants respectively. Friable embryogenic calli derived from root explants differentiated into somatic embryos on culturing in Woody Plant Medium (WPM) (Lloyd & McCown, 1980) supplemented with 4.35µM GA_3 and 8.84µM BA. Transfer of regenerated plantlets into small poly bags filled with autoclaved soil rite in controlled – environment glass houses was successful. Subsequently, acclimatized plantlets were transferred to larger poly bags and successfully Plantlet regeneration and transfer of plantlets into soil was also successful (Sushamakumari *et al.*, 2006). Yellow, friable embryogenic calli were induced from leaf explants in modified MS medium containing 2,4-D, NAA and BA. Callus proliferation was obtained in the same medium with reduced auxin and increased sucrose with incubation in dark. They later on differentiated into embryos. Embryogenesis was achieved in modified MS medium containing 270mg/l KH_2PO_4 and 360mg/l $CaNO_3$. Good embryo induction of more than 60% was obtained in a medium containing the amino acids, and reduced hormone levels. Maturation of the embryos was achieved in WPM medium containing vitamins, phytohormones and organic supplements like 10% CW, 100mg/l malt extract and 400mg/l casein hydrolysate. Fully developed plantlets were obtained in 3 to 4 weeks of culture (Kala *et al.*, 2005).

Somatic Embryogenesis Receptor Kinase gene/SERK gene

Somatic embryogenesis is in fact, the basis of totipotency in higher plants. Here, differentiated somatic cells revert to embryogenic pathway by developmental restructuring. This developmental switching involves differential gene expression; which confers embryogenic potential to the somatic cells. And this differential gene expression is brought about by various signal transduction pathways that activate/repress specific gene sets. Addition of auxin in high concentrations to the medium induces the callus into globular stage of somatic embryos. Several studies report that the exposure to auxin in high concentration serve as a trigger for cell-cycle related *cdk* and cyclin genes' expression, resulting in the formation of somatic embryos (Dudits *et al.*, 1991, 1995; Mahalakshmi, 1994; de Klerk, 1997).

When cDNAs isolated from *Daucus carota* suspension cultures were analysed, one of them was found to exhibit homology with animal and plant receptor protein kinases. Since it was expressed in somatic embryos, it was called *'somatic embryogenesis receptor kinase'* gene or *'SERK'* gene. It was established later that expression of SERK gene was specific to embryogenic cells of *D.carota* calli; and that SERK gene can be used as a potential molecular marker for screening calli capable of somatic embryogenesis (Schimdt *et al.*, 1997).

Structure and function of SERK protein

The SERK protein has an N-terminal domain with five leucine-rich repeats (LRRs) that functions as the receptor binding to signal proteins. A proline-rich domain is present between the extracellullar domain and the transmembrane region which contains a Ser – Pro – Pro motif, or the SPP motif. This is unique to SERK. Also, this is a conserved feature of plant cell wall proteins known as 'extensins'. One of the intracellular domains and the LRR motif contain N-glycosylation sites. The intracellular domain of SERK contains eleven sub-domains, characteristic of the catalytic core of protein kinases; with the domains VI and V being of serine/threonine kinase activity. The second half of the C-terminal motif may be mediating protein – protein interaction, a prerequisite for transmission of an intracellular phosphorylation cascade. SERK protein, like many other protein kinases, has autophosphorylation activity as demonstrated by bacterially expressed SERK fusion protein (Schimdt *et al.*, 1997). Thus, the SERK gene family is identified by:

- Firstly; the presence of 11 exons with conserved splicing boundaries and the tendency for each exon to encode a specific protein domain.
- Secondly; the SERK amino acid sequence contains a particular order of domains from N to C-terminal: Signal peptide (SP), leucine zipper (ZIP), 5 LRRs, a proline-rich domain (SPP), transmembrane, kinase and C-terminal domains.

Phylogenetic studies have confirmed that SERK is an RLK protein; ie, a Receptor – like Kinase. It belongs to the 14 member subfamily II of LRR kinases. SERK protein shares the canonical structure of LRR kinases; but has limited number of LRR motifs. Like all plant RLKs, it has Ser/Thr kinase specificity. And all RLKs (which include SERK) have a monophyletic origin within the superfamily of plant kinases (Shiu and Bleecker, 2001).

The function of this gene has been linked to somatic embryogenesis in numerous species, including *Daucus carota* (Schmidt *et al.,* 1997), *Arabidopsis thaliana* (Hecht *et al.*, 2001), *Medicago truncatula* (Nolan *et al.*, 2003), *Zea mays* (Baudino *et al.*, 2001), *Helianthus annuus* (Thomas *et al.*, 2004), *Ocotea catharinensis* (Santa-Catarina *et al.*, 2004), *Poa pratensis* (Albertini *et al.*, 2005), *Citrus unshiu* (Shimada *et al.*, 2005), *Oryza sativa* (Hu *et al.*, 2005), *Triticum aestivum* (Singla *et al.*, 2008), *Vitis vinifera* (Maillot *et al.*, 2009), and *Musa acuminata* (Huang *et al.*, 2009).

SERK gene in different plants

The first SERK genes identified were linked to competence of cultured cells to form somatic embryos in carrot (*Daucus carota*), orchard grass (*Dactylis glomerata*) and *Arabidopsis thaliana* species (Schmidt *et al.,* 1997; Somleva *et al.*, 2000; Hecht *et al.*, 2001). The carrot SERK or DcSERK does not contain a signal peptide, but rather starts from the leucine zipper (exon 2 in other SERKs).

SERK gene has been most well studied in the model plant *Arabidosis thaliana.* In *Arabidopsis*, there are five members of the SERK family; AtSERK1, AtSERK2, AtSERK3, AtSERK4, and AtSERK5. A perfect leucine zipper (Leu-X6-Leu-X6-Leu-X6-Leu), is not present in AtSERKs 4 and 5 and the specific SPP motif of the SPP domain is also lacking in these sequences. The AtSERK3 gene was identified as the BRASSINOSTEROID INSENSITIVE1 (BRI1)-ASSOCIATED KINASE1 (BAK1) (Nam and Li, 2002). Two other members of the family, AtSERK1 (Karlova *et al.,* 2006)

and AtSERK4/BKK1 (He *et al.*, 2007), have also been reported to be involved in BR signaling. However, AtSERK4 has only overlapping functioning with AtSERK3 & AtSERK1 (Albrecht *et al.*, 2008). AtSERK1 and AtSERK2 proteins are functionally redundant and essential for tapetum specification and pollen development during male sporogenesis in Arabidopsis (Albrecht *et al.*, 2005; Colcombet *et al.*, 2005). Among these five SERK genes, AtSERK1 is the gene involved in somatic embryogenesis (Hecht *et al.*, 2001), and can be employed as a marker for embryogenic calli (Singla *et al.*, 2008; Hecht *et al.*, 2008).

A total of nine SERK or SERK-like genes have been identified in *M.truncatula.* The SERK1 gene of the model legume, *Medicago truncatula* functions in somatic and zygotic embryogenesis, and during many phases of plant development, including nodule and lateral root formation (Nolan *et al,* 2011). They identified eight putative SERK genes in *M. truncatula,* in addition to the already characterized MtSERK1. All of them contain both identifying characters of the SERK gene family. Phylogenetic analysis showed that five of these genes are SERKs, belonging to the SERK 3/4/5 subfamily. The other three do not fall into the SERK family as defined in *Arabidopsis*, but are SERK-like genes. MtSERKL1 and MtSERKL2 fall into the NIK family, which is highly similar to the SERK family. The third one, MtSERKL3 is also closely related but is not in the same clade as the SERK or NIK genes.

The SERK gene family in rice (*Oryza sativa)* comprises of two members, OsSERK1 and OsSERK2 (Hu *et al.*, 2005; Ito *et al.*, 2005); and the SERK-like (SERL) gene family comprises of nine members, designated as OsSERL 1 – 9 (Bhumica *et al.*, 2009). Gene structure and phylogenetic analysis of rice SERK & SERL genes by Bhumica *et al.* (2009) reported that a signal peptide sequence is present in all except OsSERL5; one cysteine pair is present in OsSERL5, OsSERL6, and OsSERL7; five LRRs are present in OsSERK1, OsSERK2, OsSERL2, OsSERL4, OsSERL6, and OsSERL7; transmembrane domain is present in all; SPP motif is present in only two, OsSERK1 and OsSERK2; and a protein kinase domain is present in all except OsSERL4. Among the 11 members, most have been annotated as BRI-associated receptor-like kinase. The overall identities among the OsSERK and OsSERL proteins range from 35% to 87% and from 25% to 87% with the Arabidopsis SERK proteins, respectively.

In *Zea mays*, two full-length cDNAs, ZmSERK1 and ZmSERK2, and one expressed sequence tag (EST) sequence, putative to ZmSERK3, have been isolated and characterized (Baudino *et al.*, 2001). ZmSERK1 is preferentially expressed only in male

and female reproductive tissues, with the strongest expression in microspores. In contrast, ZmSERK2 expression is ubiquitous in all tissue types. Both genes are expressed in embryogenic and non-embryogenic callui (Baudino *et al.*, 2001). The 1,872-bp full-length cDNA of ZmSERK3 was obtained (ACF87700) by Zhang *et al.* (2011). The protein encodes a predicted amino acid sequence containing 623 residues, with a calculated molecular mass of 68.624 KDa and a predicted pI of 5.85. At the amino acid level, the ZmSERK3 sequence showed a high percentage of identity, namely, 94% with ZmSERK2 (AJ277703.1), 91% with ZmSERK1 (AJ277702.1), 88% with OsSERK1 (AAU88198), 85% with MtSERK1 (AAN64293), 83% with DcSERK (AAB61708.1), 84% with AtSERK2 (AAK68073.1), and 82% with AtSERK1 (A67827.1). In addition, ZmSERK3 shared all of the characteristic features of SERK protein, including the five LRRs, the Pro-rich domain containing the Ser-Pro-Pro (SPP) motif, a signal peptide, and a kinase domain (Zhang *et al.*, 2011). The identification of SERK genes in maize, wheat, rice, and *D. glomerata* demonstrates that these genes mimic similar functions in wheat (Singla *et al.*, 2008), rice (Hu *et al.*, 2005), and *D. glomerata* (Somleva *et al.*, 2000).

MATERIALS AND METHODS

MATERIALS AND METHODS

1. Initiation of embryogenic calli

Embryogenic calli were induced from immature anthers of *Hevea*, clone RRII 105 (a high yielding Indian clone), according to the protocol described previously by Kumari Jayasree *et al.*, (1999, 2004).

Flower buds were first collected from field-grown trees of *Hevea* clone RRII 105. After thorough washing in tap water, floral buds were sterilized with 0.1% (w/v) mercuric chloride solution containing two drops of Tween-20. Immature anthers (i.e., anthers before microsporogenesis) were dissected out aseptically and transferred to callus induction medium consisting of modified MS salts (Table: 5); supplemented with 2.0mg/l 2,4-D and 0.5mg/l Kinetin (Kn). Cultures were kept in dark at 25 ± 2°C for 40 days. Resulting calli were transferred to embryo induction medium consisting of modified MS salts (Table: 6) supplemented with glutamine: 200 mg/l, casein hydrolysate: 400 mg/l, NAA: 0.2mg/l, Kn: 0.7mg/l and gibberellic acid (GA_3): 2.0mg/l. Cultures were again maintained under darkness at 25 ± 2°C for eight weeks.

Upon subculturing into embryo induction medium, yellow calli turned to brownish calli. Highly friable yellow embryogenic calli were induced from these brown calli clumps by subculturing into fresh embryogenic induction media. The embryogenic calli thus developed were maintained by monthly transfers to fresh embryogenic induction medium. These embryogenic callus cultures were used for further experiments.

2. Genomic DNA Isolation

Plant Material:

Plant material used in this study was obtained from the tissue culture laboratory in Biotechnology Division of Rubber Research Institute of India, Kottayam, Kerala. In the present study the *Hevea brasiliensis* clone RRII 105 had been selected. Highly friable embryogenic calli initiated in tissue culture media as described above were identified and collected. Selected calli were immediately kept in ice buckets.

DNA Extraction buffer:

2 % CTAB, 1.4 M NaCl, 20 mM EDTA, 0.1 M Tris-HCl (pH- 8.0), 1% Poly Vinyl Polypyrrolidone and 0.1% ß- mercaptoethanol.

(ß-mercaptoethanol was added freshly at the time of isolation).

All glasswares were soaked in detergent and washed in tap water, rinsed in distilled water, allowed to dry in hot air oven and autoclaved at a pressure of 15lbs and 121°C for 20 minutes. The samples were washed thoroughly in distilled water. Adhering water particles were removed using blotting sheets; then the leaves were wiped clean with alcohol using pre-sterilized cotton balls. DNA extraction was done following a modified protocol of Doyle *et al.* (1989):

i. Leaf sample weighing 2g was frozen with liquid nitrogen and ground to a very fine powder using a mortar & pestle.
ii. 20ml of 2X CTAB extraction buffer was added and transferred to a 50ml centrifuge tube.
iii. The tube was incubated at 55°C for 30 minutes with occasional swirling.
iv. The suspension was mixed gently at 10,000 rpm for 10 minutes to pellet the debris; the supernatant was transferred to a new tube.
v. Equal volume of Tris – saturated phenol: chloroform: isoamylalcohol (25:24:1) was added and mixed by gentle inversion.
vi. The sample was spun at 10,000 rpm for 10 minutes & aqueous phase was carefully transferred to a new centrifuge tube.
vii. RNA present in the sample was eliminated by treatment with *DNase* free *RNase* and solution was incubated at 37°C for 1 hour.
viii. *Proteinase K* was added to inactivate the *RNase* and to remove other residual proteins. Incubation was continued for another 1 hour.
ix. Equal volume of chloroform:isoamyl alcohol (24:1) was added and mixed gently and centrifuged at 10,000rpm for 10 minutes.
x. Aqueous phase was carefully transferred to a new centrifuge tube.
xi. Equal volume of chloroform was added and mixed gently and centrifuged at 10,000 rpm for 10 minutes.
xii. Aqueous phase was carefully transferred to a new centrifuge tube.

xiii. To the samples 0.6 volume of ice cold isopropyl alcohol was added to precipitate the DNA.

xiv. The tubes were kept in ice for 20 minutes and the DNA was pelleted by centrifuging at 8000 rpm for 10 minutes at 4°C.

xv. DNA was washed twice in 70% ethanol.

xvi. The pelleted DNA was suspended in TE buffer.

xvii. DNA was stored at -20°C.

Agarose Gel Electrophoresis:

The quality of isolated genomic DNA sample was checked by agarose gel electrophoresis. Agarose gel was prepared by suspending dry agarose powder at a concentration of 0.8% in 0.5 X TBE (pH 8) buffer in a conical flask, mixed by swirling, and boiled in a microwave oven until all the agarose had melted to form a clear solution. The melted agarose was cooled to 50°C and ethidium bromide was added to give a final concentration of 5µg/ml. This was poured into a suitable clean gel casting tray containing a comb of appropriate size to form wells, and allowed to cool to form a rigid gel. After the gel had set, the comb was carefully removed and the gel was placed into an electrophoresis tank filled with running buffer. 2µl of loading buffer (0.25% bromophenol blue and 40% sucrose in TE buffer) was added to 2µl of DNA. After thorough mixing, the samples were loaded into the wells. The gel was subjected to a constant electric field of 50V and electrophoresis was done until the bromophenol blue dye front had migrated to the bottom of the gel. The molecular marker used was ?-DNA double-digested with EcoRI and HindIII restriction enzymes. The gel was visualized using a UV transilluminator. DNA bands showed up in the gel as bands of fluorescence and photographed under UV light using Kodak EDAS 290 gel documentation system.

3. PCR Amplification of putative SERK gene

PCR can rapidly amplify a specific region of a DNA molecule *in vitro* to yield sufficient quantities that can be cloned, sequenced or analysed by restriction mapping. Based on the earlier reported sequences of SERK genes in *Arabidopsis thaliana, Camelia sinensis, Helianthus annus, Zea mays, Cocos nucifera,* and *Dimocarpus longan*; several gene specific primers were designed corresponding to their consensus sequences [forward

primers: F1, F2, F3 and reverse primers R1, R2, R3, R4]. These primers were screened in all possible combinations for potential specific amplification of *H.braziliensis* SERK gene(s). Primers had been synthesized and supplied by M/S Sigma Aldrich, Bangalore, India. After analyzing the result, the primer combination 'F3R4' was selected.

Forward primer: **5′ -GCT TGT ATA TCC CTA TAT GGC- 3′ - F3**

Reverse primer: **5′ -CGA GCA AGA TCA AAA GCC C- 3′ - R4**

PCR amplification was performed with 20ng of genomic DNA. PCR was carried out in 20µl reaction volumes with the following constituents.

Table 1. PCR Reaction components:

PCR Reagents	Quantity
Sterile water	11.84 µl
PCR buffer	2 µl
dNTPs mix (dATPs, dGTPs, dCTPs, dTTPs [100µM each])	2 µl
Taq polymerase (3units/µl)	0.16 µl
Template DNA	2 µl
Forward primer (250 nM)	1 µl
Reverse primer (250 nM)	1 µl
Total	**20 µl**

The PCR mixture was overlaid with a drop of mineral oil and amplification was carried out in Perkin Elmer Thermal Cycler 480.

Table 2. PCR profile:

Step 1	Initial denaturation	94°C	3 minutes
Step 2	Denaturation	92°C	1 minute
	Annealing	55°C	1 minute
	Extension	72°C	1 minute
Step 3	Repeat step 2	36 cycles	
Step 4	Final elongation	72°C	10 minutes

Agarose Gel Electrophoresis:

PCR products were analyzed by agarose gel electrophoresis. 1.5% agarose gel was prepared and PCR products were loaded into the respective wells. Gel was run at 50V until the bromophenol blue front had migrated to the bottom of the gel. The gel image was captured by Kodak EDAS 290 gel documentation system.

4. Purification of amplified bands from agarose gel

Agarose gel electrophoresis showed an amplification of one distinct band of approximately 0.6kb in size. The amplified gene fragment band was excised with a clean scalpel blade from agarose gel containing the DNA bands and transferred into a preweighed 1.5ml microcentrifuge tube. The tube containing the agarose gel slice was weighed and the weight of an empty tube subtracted from it, and this way the actual weight of the slice was determined. The PCR product was eluted and purified from the gel using the **GFX PCR DNA and Gel Band Purification Kit,** M/S Amersham Biosciences.

The purification was done according to the manufacturer's instructions, which are as follows:

i. The bands were sliced to the finest pieces and approximately 10µl of capture buffer was added for each 10mg gel in the tube.

ii. Closed the tube and vortexed vigorously. Incubated at 60°C for about 5-15 minutes until the agarose had completely dissolved.

iii. The samples were centrifuged at 10,000 rpm for 1 minute and transferred to the GFX column (glass fibre matrix) placed in a collection tube and incubated for 1 minute at room temperature.

iv. Centrifugation was repeated with 10,000 rpm for 1minute. Discarded the flow through by emptying the collection tube and placed the GFX column back into the collection tube.

v. 500µl of wash buffer was added to the column and the whole set-up was centrifuged at 10,000 rpm for 1 minute.

vi. Discarded the collection tube and transferred the GFX column into a fresh 1.5ml centrifuge tube.

vii. About 30µl of sterile double distilled water was added directly to the top of the glass fibre matrix and it was incubated at room temperature for 1 minute.

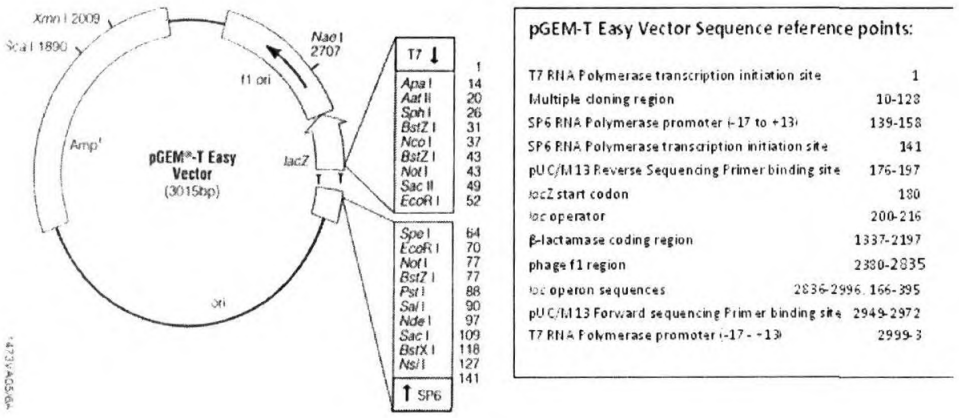
viii. Centrifuged at 10,000 rpm for 2 minutes to recover the purified DNA.

5. Molecular cloning and transformation

Cloning:

Cloning of the purified PCR product was carried out with **pGEM®-T Easy Vector System** (fig: 2) cloning kit (M/S Promega Corporation, USA), following the manufacturer's instructions.

Figure 2: pGEM®-T Easy Vector circle map



Preparation of competent cells of *E.coli*:

E.coli DH5a cells were streaked aseptically on to an LB Agar plate containing no antibiotics. The plate was then kept overnight in an incubator at 37°C. On the following day, a single colony from this plate was inoculated into 25ml of autoclaved LB broth in a conical flask. Incubated at 37°C in an incubator with shaking (220 rpm) for 16 hours. After incubation, 2ml from this culture was inoculated into another conical flask containing autoclaved 50ml LB broth, incubated at 37°C in an incubator with shaking at 220 rpm for 2 hours. The culture was then transferred to a fresh centrifuge tube and incubated in ice for 10 minutes. Then it was centrifuged at 2000 rpm for 3 minutes at 4°C. The supernatant was poured off carefully and the pellet was suspended in 5ml ice-cold $CaCl_2$ solution (60mM $CaCl_2$, 15% [v/v] glycerol and 10mM PIPES [(Piperazine – 1, 4 – Bis) (2 – ethane Sulphonic acid)] pH 7.0). Again centrifuged in cold (4°C) at 2000

rpm for 3 minutes. Poured off the supernatant, the pellet was again suspended in 5 ml ice-cold $CaCl_2$ solution and incubated in ice for 30 minutes. Centrifuged at 2000 rpm for 3 minutes in a cold centrifuge, the supernatant was poured off and suspended the pellet in 1ml $CaCl_2$ solution. 100µl each was then dispensed in to fresh 1.5ml micro centrifuge tubes and kept at -80°C for further use.

Transformation of *E.coli*

Transformation was done with *E.coli*-DH5a chemically competent cells prepared as above.

i. Added 4µl of the preformed pGEMT cloning reaction mixture into a vial of one shot chemically competent *E. coli- DH5a* cells and mixed gently.
ii. Incubated at room temperature for 2 hours.
iii. Heat shocked the cells for 1 minute at 42°C without shaking.
iv. The tubes were immediately transferred to ice and incubated in it for 2 minutes.
v. Added 250µl of room temperature S.O.C medium.
vi. The tube was tightly capped and kept the tube horizontally (220 rpm) at 37°C for 1.5 hours.

Preparation of SOC medium:

2.0g tryptone, 0.5g yeast extract, 1ml 1M NaCl and 0.25ml 1M KCl were added to 97ml distilled water and dissolved. This was autoclaved and allowed to cool to room temperature. Then, 1ml 2M Mg^{2+} stock (filtersterilized) and 1ml 2M glucose (filtersterilized) were added aseptically to a final concentration of 20mM and made up to 100ml with sterile distilled water. The pH of the medium was adjusted to 7. This completed medium was then filter sterilized through a 0.2µm filter unit, and stored sealed at 4°C.

Preparation of antibiotic selection medium:

Luria-Bertani (LB) Medium (Sambrook *et al.,* 1989) was prepared to spread-plate the transformed competent cells for screening. 2g LB powder and 1.5g agar were added to 100ml deionized water in a conical flask; dissolved, plugged and autoclaved for 15 minutes at 15 lbs. Under sterile conditions in a laminar air flow hood,

100µg/ml of Ampicillin was added into the autoclaved LB agar medium after cooling to 45°C and gently mixed to avoid any bubble formation. The medium was poured into plates and allowed to solidify. Then 40µl 2% X-gal and 100µl 100mM IPTG was added onto the plates and allowed to dry by incubating it right-side-up at 37°C for 30 minutes.

Liquid LB Medium:

1g LB powder was added and dissolved in 50ml deionized water. 3ml of this medium was poured into 50ml sterile test tubes and autoclaved for 15 minutes at 15lbs. 100µg/ml Ampicilin was added to these tubes just before inoculation.

Plating of transformed cells:

Plating of transformed cells was done under sterile conditions in the laminar air flow chamber. 75µl and 100µl of transformed cells were spread on pre-warmed selection plates. Incubated the plates right side up at 37°C. White transformed colonies appeared after 16 hours of incubation. Each transformed white colony was inoculated aseptically to 3ml liquid LB medium and incubated at 37° C for 16 hours in a shaker at 220 rpm.

6. Colony PCR

Colony PCR was carried out to identify positive colonies carrying the cloned genomic DNA (transformed colonies) with gene-specific forward and reverse primers.
PCR had been done in 20µl reaction volume; as described in section 3.
Agarose gel electrophoresis of PCR reaction product was done to identify positive amplification.

Table 3. Colony PCR profile:

Step 1	Initial denaturation	94°C	10 minutes
Step 2	Denaturation Annealing Extension	92°C 55°C 72°C	1 minute 1 minute 1 minute
Step 3	Repeat the step 2	28 cycles	
Step 4	Final elongation	72°C	10 minutes

7. Isolation of Plasmid

Plasmid DNA extraction from recombinant bacteria was done using the **Illustra plasmidPrep Mini Spin Kit,** M/S GE Healthcare.

i. 1.5ml of over night culture of transformed cells was transferred to a 1.5ml micro centrifuge tube and centrifuged at 12,000g for 1 minute.

ii. The supernatant was decanted without disturbing the pellet. Repeated the first step twice to get the maximum possible bacterial pellet.

iii. Added 175µl of lysis buffer (type 7) to the pellet and resuspended the pelleted bacteria in it by vortexing vigorously.

iv. Added 175µl of lysis buffer (type 8) to the resuspension and mixed by repeated gentle inversions of the tube to lyse the bacteria.

v. Added 350µl of lysis buffer (type 9) and immediately mixed thoroughly by gentle inversion of the tube several times to neutralize the bacterial lysate.

vi. Centrifuged the neutralized lysate at 12,000g for 4 minutes to pellet bacterial cell wall debris, precipitated protein and chromosomal DNA. The supernatant was transferred to a spin column in a collection tube.

vii. Centrifuged the spin column-collection tube assembly at 12,000g for 1 minute.

viii. Decanted the filtrate and placed the spin column into a fresh micro centrifuge tube.

ix. Added 50µl of the elution buffer to the spin column capped the tube and centrifuged at 12,000g for 1 minute, to recover the plasmid DNA as flowthrough in the micro centrifuge tube.

x. Purified plasmid was stored at -20° C

Agarose gel electrophoresis:

The quality of the isolated recombinant plasmid was checked by the 1% agarose gel electrophoresis as described earlier. Non-recombinant plasmid was used as the control.

8. Sequencing of recombinant plasmids

The sequencing of the isolated recombinant plasmids has been done at M/S Macrogen, South Korea with an automated sequencer. The sequencing was carried out with the universal M13 forward and reverse primers.

Sequence analysis:

Blastn homology search tool at NCBI (National Center for Biotechnology Information, USA) (http://www.ncbi.nlm.nih.gov/blast) was used to obtain the sequences producing significant alignments with the sequence of the gene of interest. The sequence of the gene from *Hevea* clone RRII 105 was aligned with the reported genomic sequence of SERK-3 from *Arabidopsis thaliana* using the multiple pair-wise sequence alignment software tool EMBOSS at EMBL-EBI (European Bioinformatics Institute) (http://www.ebi.ac.uk/Tools/psa/).

9. Isolation of RNA from leaf and callus samples

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

i. 1g of leaf tissue (washed with DEPC treated water) and 1g of regenerated callus was ground to a fine powder in liquid Nitrogen separately.
ii. Then 10ml of RNA 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.

RNA 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 was recovered.

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

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

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

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

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

xi. Pellet was air-dried and re-suspended in 200µl 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, and removed by *DNase* treatment.

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

Agarose gel electrophoresis:

The quality of the isolated RNA was determined by 1% agarose gel electrophoresis. *RNase* free water was used for preparing the gel and for washing the electrophoresis tank thoroughly. *RNase* free gel loading buffer was used for loading the RNA samples into wells. RNA bands were then visualized under UV light, and the gel was documented.

10. First strand cDNA synthesis

First strand cDNA was synthesised from the isolated embryogenic callus RNA & mature leaf RNA (*Dnase* treated) by reverse transcription reaction with oligo-(dT) primers, using the **Improm-II™ Reverse Transcription System** (M/S Promega, USA). The protocol followed is as follows:

i. Primer annealing to the target RNA and denaturation was performed in a 0.5 ml reaction tube in ice. 1µl of total RNA (1µg) was combined with 1µl oligo-(dT) primers (0.5µg). The reaction was made up to 5 µl by the addition of nuclease-free water.

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

iii. The tube was then spun down for 10 seconds in a micro-centrifuge to collect the condensate and maintain the original volume. This RNA-primer combination was kept on ice until the reverse transcription (RT) reaction mix was readied.

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

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

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

vii. Primer extension was carried out at 42°C for 1 hour in a heated block.

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

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

11. Reverse Transcription PCR of SERK gene

RT PCR of SERK gene was done using 100ng of the cDNA synthesized as template, with the primer combination F3R4 (The sequence of the primer is shown below). The constitutively expressing ß – actin gene was used as an endogenous control to differentiate the expression pattern of SERK gene in mature and embryogenically competent tissues, with constitutively expressing ß-actin as the endogenous control

Forward primer: **5′ -GCT TGT ATA TCC CTA TAT GGC- 3′ - F3**

Reverse primer: **5′ -CGA GCA AGA TCA AAA GCC C- 3′ - R4**

Table 4: RT PCR profile:

Step 1	Initial denaturation	94°C	3 minutes
Step 2	Denaturation	92°C	1 minute
	Annealing	55°C	1 minute
	Extension	72°C	1 minute
Step 3	Repeat step 2	32 cycles	
Step 4	Final elongation	72°C	10 minutes

Agarose gel electrophoresis:

The RT PCR products were subjected to gel electrophoresis in a 1.5% agarose gel. After electrophoresis for a required time, the gel was taken from the tank and visualized in a UV transilluminator and documented.

Table 5: Composition of callus induction medium

Ingredients	Concentrations (mg/l)
Modified MS major salts	
NH_4NO_3	1000.00
KNO_3	1900.00
$CaCl_2$ (anhydrous)	333.00
$MgSO_4$ (anhydrous)	181.00
KH_2PO_4	170.00
MS Minor salts	
KI	0.83
H_3BO_3	6.20
$MnSO_4.4H_2O$	6.20
$ZnSO_4.7H_2O$	22.30
$Na_2MoO_4.2H_2O$	8.60
$CuSO_4.5\ H_2O$	0.25
$CoCl_2$	0.025
$FeSO_4.7H_2O$	**27.85**
$Na_2EDTA.2H_2O$	**37.25**
B5 vitamins	
Myo-inositol	100.00
Coconut water	5%
Sucrose	**5%**
Growth regulators	
2,4-D	2.0
Kn	0.5
Gelrite	**0.2%**
pH	**5.6**

Table 6: Composition of embryo induction medium

Ingredients	Concentrations (mg/l)
Modified MS major salts	
NH_4NO_3	1000.00
KNO_3	1900.00
$CaCl_2$ (anhydrous)	333.00
$MgSO_4$ (anhydrous)	181.00
KH_2PO_4	170.00
MS Minor salts	
KI	0.83
H_3BO_3	6.20
$MnSO_4.4H_2O$	6.20
$ZnSO_4.7H_2O$	22.30
$Na_2MoO_4.2H_2O$	8.60
$CuSO_4.5\ H_2O$	0.25
$CoCl_2$	0.025
$FeSO_4.7H_2O$	**27.85**
$Na_2EDTA.2H_2O$	**37.25**
B5 vitamins	
Myo-inositol	100.00
Glutamine	200.00
Casein hydrolysate	400.00
Coconut water	5%
Sucrose	**7%**
NAA	0.2
Kn	0.7
GA_3	2.0
Gelrite	**0.2%**
Activated charcoal	**0.2%**
pH	**5.7**

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

Somatic embryogenesis is a remarkable illustration of plant totipotency. Conversion of somatic cells into somatic embryos involves genetic reprogramming by differential expression of genes in the cells. This in turn elicits diverse cellular and physiological responses that confer the ability of somatic embryogenesis to these cells. Though the intricate molecular mechanisms involved in somatic embryogenesis are still not fully understood, an array of genes that are activated during somatic embryogenesis have been isolated employing various molecular techniques. Among these genes, the SERK gene have been identified and confirmed in several plants as the 'trigger – gene' that catapults somatic cells into somatic embryos.

The present report describes PCR amplification, cloning and sequence characterization of the SERK gene from *Hevea* clone RRII 105; and the possible application of this gene as a molecular marker in *Hevea* for embryogenic calli capable of somatic embryogenesis. For the PCR amplification of the genomic sequence, the forward and the reverse primers were designed based on consensus sequences from previously reported sequences of SERK genes in *Dimocarpus longan* (NCBI accession no: HM773391), *Cocus nucifera* (NCBI accession no: AY791293), *Camelia sinensis* (NCBI accession no: GU968640), *Arabidopsis thaliana* (NCBI accession no: AF384970, AF384969), *Helianthus annus* (NCBI accession no: AF485387, AF485386, AF485385, AF485384), and *Zea mays* (NCBI accession no: NM_001111662).

1. Initiation of embryogenic calli

After collection, immature flower buds (fig: 3) were surface sterilized and anthers at the diploid stage were dissected out asceptically and inoculated for callus (fig: 4). At 40 days of incubation in darkness, yellow friable callus was induced from the explant These calli turned brown upon subculturing into embryo induction medium initially. Subsequently, yellow friable embryogenic calli emerged from the brown calli (fig: 5). These embryogenic calli were used for the present study.

Figure 3: Immature floral buds

Figure 4: Immature anther inoculated on callus induction medium

Figure 5: Yellow friable embryogenic callus bearing somatic embyos

2. DNA Isolation

The genomic DNA was isolated by the CTAB extraction procedure and the isolated DNA was in good concentration for PCR amplification. Although, RNA contamination was present, prolonged treatment with *RNase* degraded all the RNA and a single band of DNA with good quantity and quality was observed on agarose electrophoresis gel. No RNA was observed the lanes loaded with the isolated DNA (fig: 6).

In the present study, somatic embryogenic calli were frozen in liquid nitrogen before grinding, and then powdered using a mortar & pestle, followed by homogenization in CTAB extraction buffer. This mixture was treated with phenol:chloroform and RNase solution to eliminate all the contaminating molecules other than DNA. The extracted DNA was precipitated with ethanol, air dried and dissolved in TE buffer. The above treatment helped to get genomic DNA in good quality and concentration. The concentration of extracted DNA was analyzed and adjusted to 1µg/µl. For PCR, the concentration of DNA was adjusted to 10ng/µl.

3. Amplification of SERK gene

PCR of the isolated callus DNA was done using three possible primer combinations specific for SERK gene and several amplified bands were obtained in agarose gel on electrophoresing the PCR products. Out of them, the primer combination F3R4 produced a single amplified band of approximately 0.6 kb in size. (fig: 7). This amplified single gel band was then cut and DNA eluted from the gel using GFX PCR DNA & Gel Band Purification Kit (M/S Amersham Bioscience).

4. Cloning of SERK gene

After eluting the specific bands from agarose gel, cloning was carried out as per the manufacturer's instructions using the pGEM®- T easy cloning kit, for sequencing. In the antibiotic selective LB medium (Luria Bertani) with IPTG and X-Gal, colonies were developed after 16 hours of incubation which was further selected through blue white screening. White transformed colonies containing the inserts were confirmed through colony PCR, inoculated into 3ml of the liquid LB + Ampicillin (100µg/ml) broth and kept in a shaker at 37^0C & 220 rpm for 16 hours. After 16 hours, good growth of the bacteria was observed and plasmids were isolated from them in sufficient quantity.

5. Colony PCR

To confirm the transformation, colony PCR was carried out with gene specific primers in 20µl reaction volumes. SERK gene was amplified as a single prominent band of approximately 6 bp in size; from two colonies transformed with the gel-eluted DNA (fig: 8).

6. Plasmid Isolation

Plasmids were isolated in good concentration according to the manufacturer's instructions using Illustra plasmid Prep Mini Spin Kit (M/S GE Healthcare). Presence of recombinant plasmids was confirmed through 1% agarose gel electrophoresis (fig: 9). The isolated plasmids were sequenced at M/S Macrogen, South Korea.

7. Nucleotide sequence analysis

The nucleotide sequence data confirmed the presence of the SERK gene in the *Hevea* clone RRII 105. The gene sequence revealed a partial coding DNA sequence of *Hevea* SERK gene. The partial genomic DNA sequence is 612 base pairs in length enclosing three introns (fig: 13, 14) comprising of 44, 151 and 9 base pairs. The sequence homology search indicated that the obtained sequence of the SERK gene from *Hevea* is showing a maximum alignment with the SERK genes reported from *Ricinus comminus* which is from the Euphorbiaceae family itself (96% identity). It is also showing similarity with other crops such as *Dimocarpus longan*, *Cyclamen persicum* etc. (table: 7).

Comparison of the sequence with the earlier reported SERK genes:

On comparing previously reported sequences of SERK genes using Blastn tool, with the sequence of *Hevea* SERK from the clone RRII 105; sequences showing homology greater than 90% are: *D.longan* SERK, *C.persicum* SERK1 & SERK2, and *R.communis* BRASSINOSTEROID INSENSITIVE 1 – associated receptor kinase 1 precursor. The details of the sequence variation with the reported sequences are shown in the table 7. The obtained nucleotide sequence was paired – wise aligned and compared with *Arabidopsis thaliana* SERK gene - AtSERK3 (NCBI accession no: AF384970), using the EMBOSS online software (fig: 15). The cDNA sequence of the partial SERK gene was constructed by compairing with reported cDNAs of AtSERK3 gene from

Arabidopsis thaliana (fig: 14). Upon comparison with the cDNA sequences of the earlier reports, it was found that the open reading frame of the mature protein starts in continuation with the initial cytosine (C) of the obtained sequence; with 'CTT' coding for 'Leucine' amino acid. The protein sequence coded by this partial SERK gene sequence has 135 amino acids (fig: 15).

8. RNA isolation and cDNA synthesis

RNA was isolated from embryonic calli and mature leaves of *Hevea* clone RRII 105 according to a modified protocol of Venkatachalam *et al.* (1999) (fig: 10). The extracted RNA was treated with *DNase* to remove any contaminant DNA and the RNA thus obtained had good quality and quantity (fig: 10). First - strand cDNA was synthesized from these RNA by reverse transcription reaction with oligo-(dT) primers, using Improm-II^{TM} Reverse Transcription System (M/S Promega, USA).

9. Reverse Transcription PCR (RT PCR)

PCR was done with the synthesized mature leaf cDNA and embryonic callus cDNA, using F3R4 primer combination. On visualizing the agarose gel after electrophoresis, it was observed that there was no amplification in mature leaf cDNA; while embryonic callus cDNA showed a single amplified band with size of around 0.4kb (fig: 11). Here, ß-actin was used as an endogenous control, as it is constitutively expressing (fig: 12). This corresponds to the size of the predicted partial cDNA sequence (fig: 16). The partial cDNA sequence is predicted to code for an amino acid sequence of 135 amino acids (Fig: 17). On comparison with the other reports it was understood that the ORF starts from the 2nd nucleotide (C) of the isolated sequence.

From this observation, it can be concluded that SERK is preferentially expressed in juvenile tissues and calli that are capable of somatic embryogenesis. In short, it is proved that SERK gene in *Hevea* can be used as a molecular marker for screening embryogenic callus cultures. Further studies need to be conducted for fully characterising *Hevea* SERK gene, and to identify and characterize possible isoforms of SERK in *Hevea*.

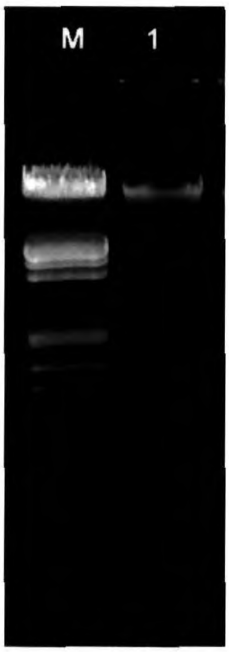


Figure 6: DNA isolation from embryogenic callus.
Lanes: M – Marker (λ DNA *Eco* R1, *Hind* III double digest)
1 – callus DNA

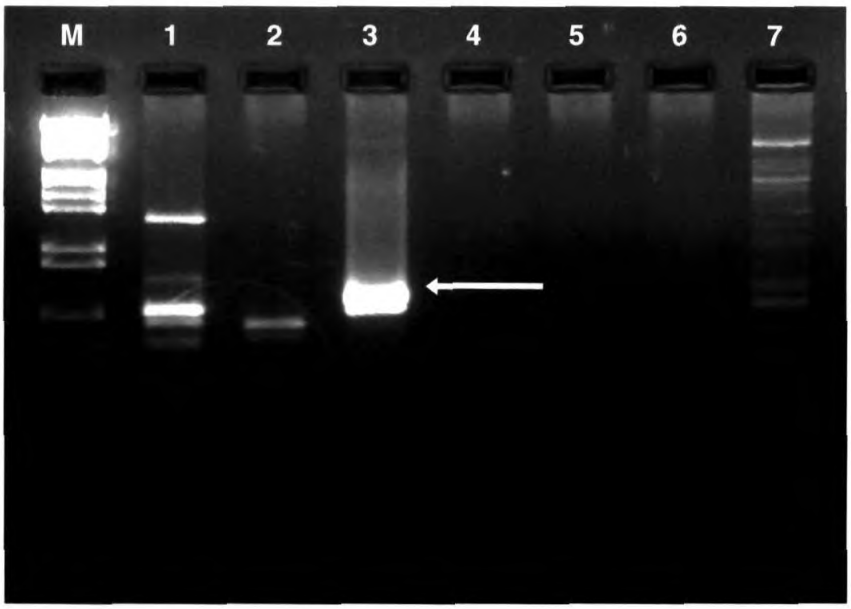


Figure 7: PCR amplification of callus DNA with different primers of SERK gene

Lanes: M – Marker (λ DNA double digest); 1 – F2R4; 2 – F3R3;
3 – F3R4; 4 – F2 alone; 5 – F3 alone; 6 – R3 alone;
7 – R4 alone

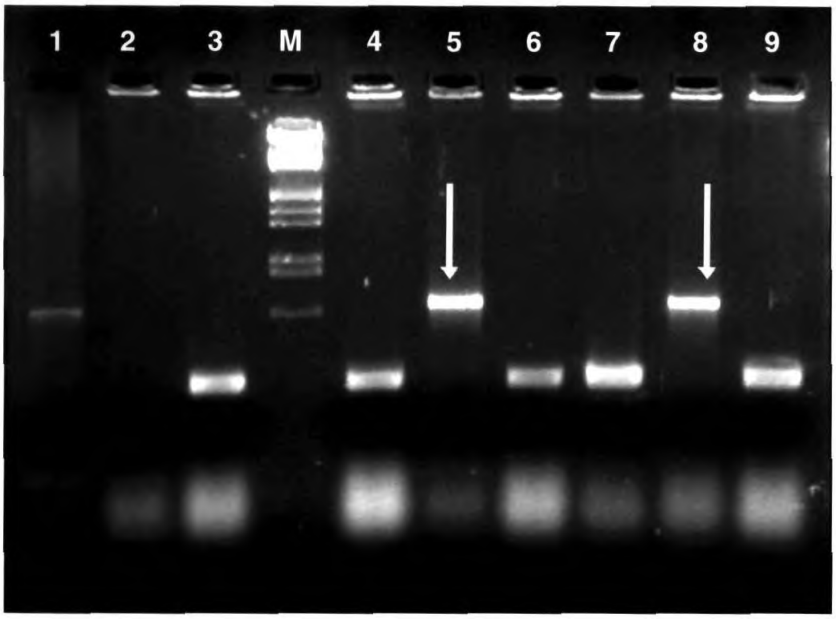


Figure 8: Colony PCR of transformed colonies obtained in antibiotic selection plate.

Lanes: 1 – callus DNA ; 2 – callus DNA (F3 alone);
3 – callus DNA (R4 alone); M – Marker (λ DNA double digest);
4, 6, 7 & 9 – untransformed colonies;
5 & 6 – transformed colonies

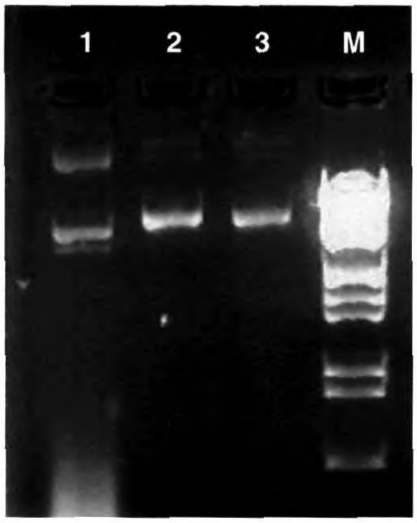


Figure 9: Recombinant plasmid isolation from positively transformed colonies.

Lanes: 1 – control plasmid;
2 & 3 – Recombinant plasmids with the insert;
M – Marker (λ DNA double digest)

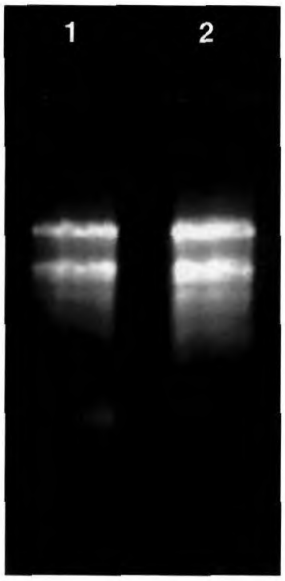


Figure 10: RNA isolation from leaf and callus.
Lanes: 1 – callus RNA (*DNase* treated);
2 – leaf RNA (*DNase* treated)

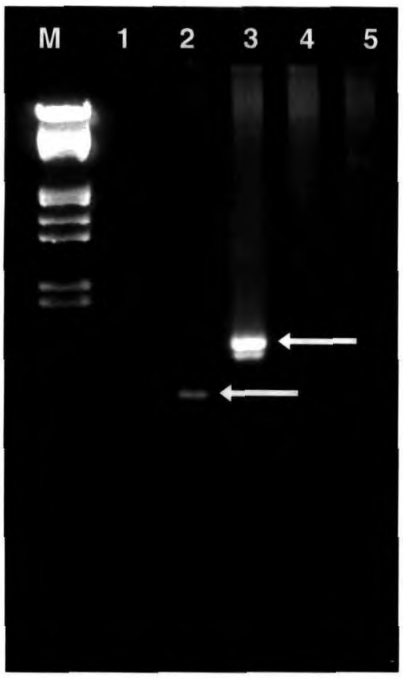


Figure 11: RT PCR of SERK gene using cDNA as template.
Lanes :M – Marker (λ DNA double digest) ;
1 – Mature leaf cDNA ;
2 – Embryogenic callus cDNA;
3 – Callus genomic DNA amplification;
4 – Callus genomic DNA (F3 alone);
5 – Callus genomic DNA(R4 alone)

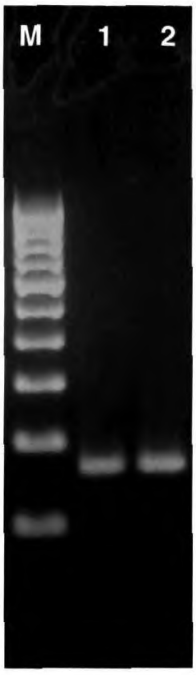


Figure: 12 . Amplification of β- actin gene (endogenous control)
from callus cDNA(lane 2) and leaf cDNA (lane 3)
M - 100 bp ladder

Table 7: BLAST Analysis table showing the sequence homology of the isolated *Hevea* SERK gene with other reported sequences

Accession	Description	Max indent
HM773391.1	Dimocarpus longan somatic embryogensis receptor kinase (SERK) gene, promoter region and complete cds	**96%**
JF511659.1	Cyclamen persicum somatic embryogenesis receptor kinase 1 (SERK1) gene, complete cds	**96%**
GU189408.1	Cyclamen persicum somatic embryogenesis receptor kinase 2 (SERK2) gene, complete cds	**94%**
FJ013227.3	Dimocarpus longan cultivar Honghezi somatic embryogenesis receptor kinase mRNA, complete cds	**96%**
XM_002534446.1	Ricinus communis BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 precursor, putative, mRNA	**96%**
EF672247.2	Cyclamen persicum somatic embryogenesis receptor kinase 1 (SERK1) mRNA, complete cds	**95%**
AB188249.1	Oryza sativa Japonica Group ORK1 gene for SERK family receptor-like protein kinase, partial cds	**86%**
AP003882.3	Oryza sativa Japonica Group genomic DNA, chromosome 8, BAC clone:OJ1134_B10	**86%**
AP004619.3	Oryza sativa Japonica Group genomic DNA, chromosome 8, PAC clone:P0583B06	**86%**
AM467416.2	Vitis vinifera contig VV78X254129.6, whole genome shotgun sequence	**90%**
GU968640.1	Camellia nitidissima somatic embryogenesis receptor-like kinase mRNA, partial cds	**90%**
XM_002262716.1	PREDICTED: Vitis vinifera similar to BAK1 (BRI1-ASSOCIATED RECEPTOR KINASE); kinase (LOC100243623), mRNA	**90%**

Figure 13: Partial genomic DNA sequence of the isolated *Hevea* SERK gene.

5'GCTTGTATATCCCTATATGGCAAATGGAAGTGTTGCATCATGTTTAAGA
GGTAAGCATGCTCTTGTTTTACACATTCATGGATATTTTAACTGCAACCT
CACATAGCATGCTCTTGTTTTACACATTCATGGATATTTTAACTGCAACC
TCACATATTGTTTTGATGATATGATTGGCTGATTGCATTTGTTTTTATCAT
TGACCTTCTGCTGCGTTTCATGGTTCTCATCAATCTCCTGATGGATGCTT
GTAGAGCGCCCGCCATCTCAACCTCCTCTTGATTGGCCAACACGGAAGC
GAATTGCACTGGGATCTGCTAGGGGTCTATCTTATTTGCATGATCATTGT
GACCCAAAGATTATTCATCGTGATGTTAAAGCTGCAAATATTTTATTGGA
TGAGGATTTTGAGGCTGTTGTTGGGGATTTTGGGTTGGCTAAGTTGATG
GACTACAAGGATACTCATGTCACTACTGCCGTCCGTGGTACAATAGGGC
ATATAGCTCCAGAGTACCTCTCTACTGGAAAATCATCGGAAAAAACTGAT
GTTTTTGGGTATGGGATCATGCTTCTGGAGCTAATTACTGGACAGAGGG
CTTTTGATCTTGCTCG3'

Intron 1 – 44 bp

Intron 2 – 151 bp

Intron 3 – 9 bp

Exon – 408 bp

Total: 612 bps

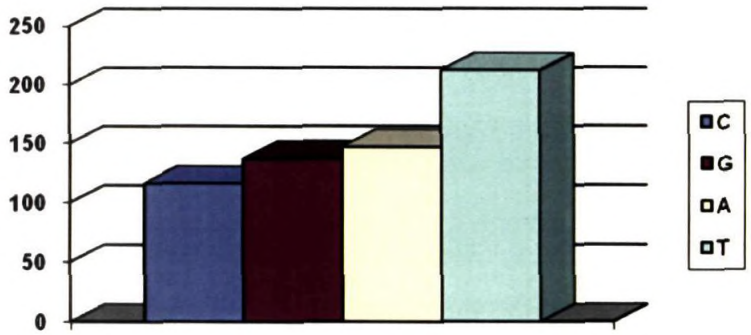


Figure 14: Frequency of nucleotides (A/T/G/C) in the 612 bps of the isolated *Hevea* SERK gene.

Figure 15: EMBOSS gene sequence alignment of the isolated partial SERK gene sequence of *Hevea* with that of the cDNA of AtSERK gene from *Arabidopsis thaliana* (NCBI accession no *:*. The gaps shows the position of the introns within the genomic sequence of the isolated *Hevea* SERK gene.

```
Hevea      1 GCTTGTATATCCCTATATGGCAAATGGAAGTGTTGCATCATGTTTAAGAG     50
             ||||||.||||||||.|||||.|||||||||||||||.||.||||||||||
arab       1 gcttgtttatccctacatggctaatggaagtgttgcctcctgtttaagag     50

Hevea     51 GTAAGCATGCTCTTGTTTTACACATTCATGGATATTTTAACTGCAACCTC    100
                                                       |||.||
arab      51 ----------------------------------------------aacgtc     56

Hevea    101 ACATAGCATGCTCTTGTTTTACACATTCATGGATATTTTAACTGCAACCT    150
             .|
arab      57 cc-------------------------------------------------     58

Hevea    151 CACATATTGTTTTGATGATATGATTGGCTGATTGCATTTGTTTTTATCAT    200

arab      59 -------------------------------------------------     58

Hevea    201 TGACCTTCTGCTGCGTTTCATGGTTCTCATCAATCTCCTGATGGATGCTT    250

arab      59 -------------------------------------------------     58

Hevea    251 GTAGAGCGCCCGCCATCTCAACCTCCTCTTGATTGGCCAACACGGA-AGC    299
                |||          ||.||.||.||.|||||||||||| |..|| |||
arab      59 ---gag---------tcccagccaccacttgattggccaa-agagacagc     95

Hevea    300 GAATTGCACTGGGATCTGCTAGGGGTCTATCTTATTTGCATGATCATTGT    349
             |.||||||.|||||||||.||.||.||..|.|||||.||||||||||.
arab      96 gtattgcgttgggatctgcaagagggcttgcgtatttacatgatcattgc    145

Hevea    350 GACCCAAAGATTATTCATCGTGATGTTAAAGCTGCAAATATTTTATTGGA    399
             ||||||||||||||||||.|||||.|||||||||||||||||.|||||
arab     146 gacccaaagattattcatcgagatgtgaaagctgcaaatattttgttgga    195

Hevea    400 TGAGGATTTTGAGGCTGTTGTTGGGGATTTTGGGTTGGCTAAGTTGATGG    449
             |||.||.|||||.||.||.|||||||||||||..|.||.||..|.||||
arab     196 tgaagagtttgaagccgtggttggggattttggacttgcaaaactcatgg    245

Hevea    450 ACTACAAGGATACTCATGTCACTACTGCCGTCCGTGGTACAATAGGGCAT    499
             |||||||.||.||.|||||.||.||.||.||.|||||.|||||.||.|||
arab     246 actacaaagacacacatgtgacaaccgcagtgcgtgggacaattggtcat    295

Hevea    500 ATAGCTCCAGAGTACCTCTCTACTGGAAAATCATCGGAAAAAACTGATGT    549
             |||||.||.||||||||.||.|||||||||||||.||.|||||.|||||
arab     296 atagcccctgagtacctttccactggaaaatcatcagagaaaaccgatgt    345

Hevea    550 TTTTGGGTATGGGATCATGCTTCTGGAGCTAATTACTGGACAGAGGGCTT    599
            .||||||||||..|||||||||.|||||.||.||||||||.|||||||
arab     346 ctttgggtatggagtcatgcttcttgagcttatcactggacaaagggctt    395

Hevea    600 TTGATCTTGCTCG------------------------------------    612
```

Figure 16: Predicted partial cdna sequence of the isolated Hevea SERK gene (obtained on comparison with the mRNA of the reported AtSERK gene from *Arabidopsis thaliana*)

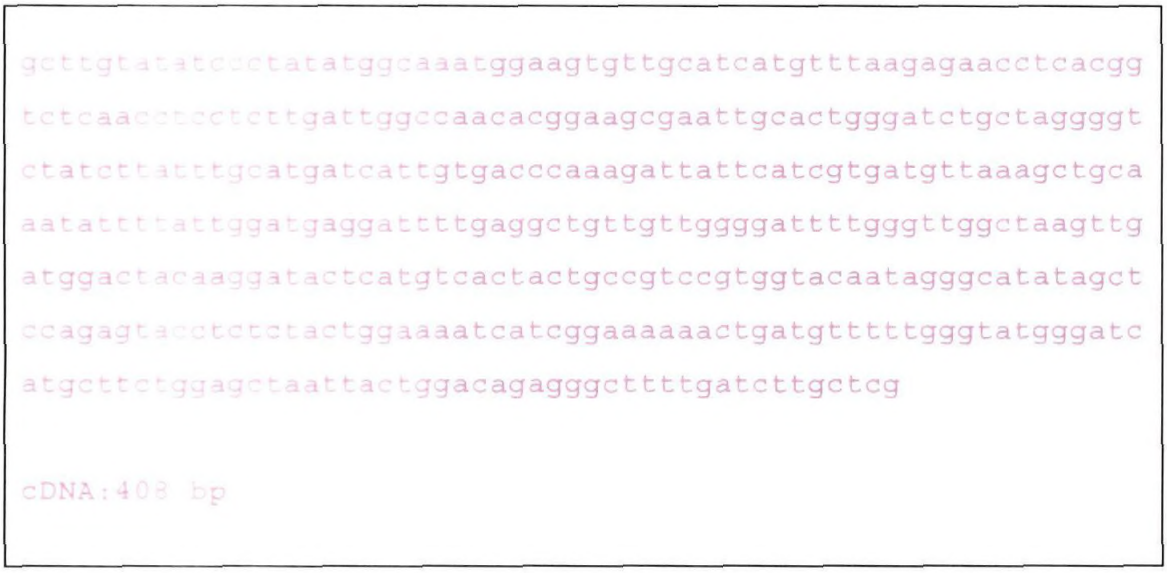
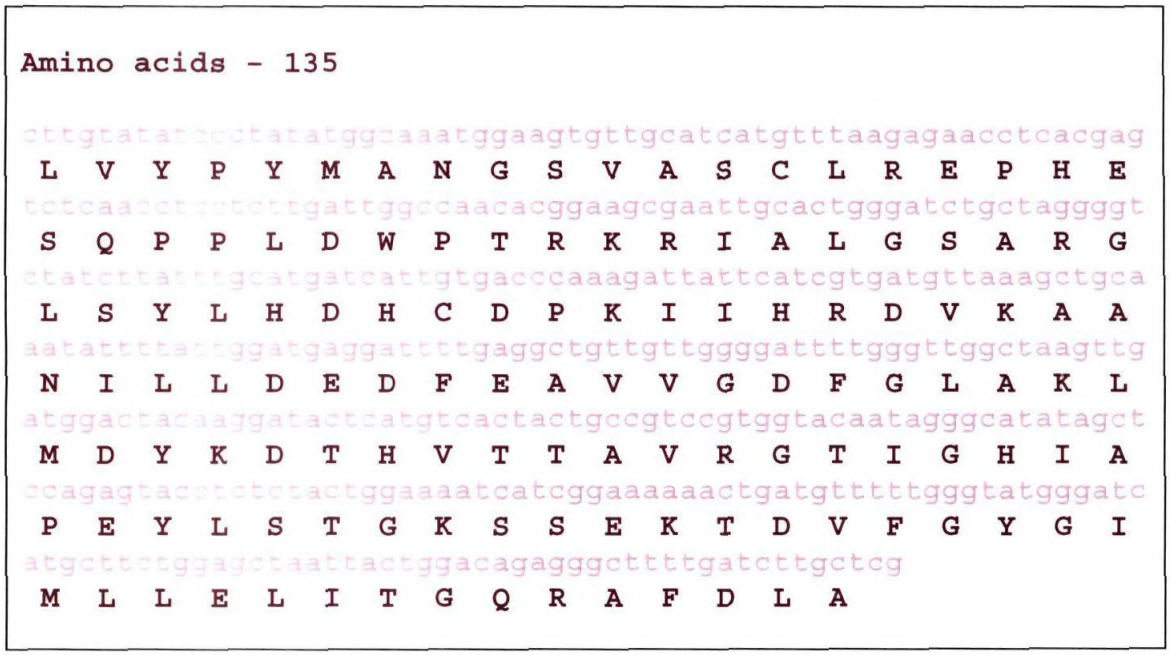


Figure 17: Predicted partial amino acid sequence of the isolated *Hevea* SERK gene.



SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

For a heterozygous woody crop like *Hevea*, the release of new cultivars with specific desired characters through conventional breeding is both time consuming and expensive. Genetic engineering could be an alternative, however, requires an *in vitro* plant regeneration system. Because of the high population of totipotent cells and unicellular origin of somatic embryos, induction of somatic embryogenesis is a viable approach for genetic improvement of this tree crop. For the effective employment of this system in breeding programmes, a high efficient embryogenesis system is required and so it is important to have a better understanding of this process. Several groups of genes are specially expressed during somatic embryogenesis. Among the genes involved with the induction of somatic embryogenesis, the somatic embryogenesis receptor kinase gene (SERK) is claimed to have an important role. The function of this gene has been linked to somatic embryogenesis in numerous species. However, in *Hevea*, no gene related to somatic embryogenesis has been reported so far.

In the present study a preliminary attempt has been made to isolate and characterize Somatic Embryogenesis Receptor Kinase gene from *Hevea brasiliensis* clone RRII 105. The starting material was the callus regenerated from immature anther of the *Hevea* clone RRII 105. Yellow friable embryogenic calli was obtained from these calli by culturing on the embryo induction medium. Genomic DNA isolated from the callus has been used as the template for the gene amplification. Forward and reverse primers were designed for the specific amplification of SERK gene from *Hevea.* The sequence of the primers has been selected based on the consensus sequence alignments reported from different plants like *Arabidopsis thaliana, Zea mays, Cocos nucifera* etc.. At optimum standardized conditions a single band amplification of 0.6 Kb has been obtained through PCR. The amplicon was later cloned in pGEM - T easy vector, and transformed to chemically competent E.*coli* DH5a cells. Positive colonies were picked up and recombinant plasmids were isolated for sequencing the insert. The sequencing of the insert revealed the presence of 0.6 kb fragment of SERK gene containing three introns on comparison with the other cDNA reports. The partial fragment codes for 135 amino acids. To study the differential expression of the gene in different tissues, RNA has been isolated from mature leaf and embryogenic calli, cDNA was synthesized and RT PCR has been performed. The RT PCR reult indicated that the newly isolated SERK gene is

showing high level expression in the callus where as no expression in the mature leaf. This result itself is indicating the role of SERK gene in conferring the embryogenic competence in the callus tissue. However the experiment is to be repeated with the callus tissues in different stages and also with non embryogenic calli in the future. This is the first report of the characterization of Somatic Embryogenesis Receptor Kinase (SERK) gene in *Hevea brasiliensis*.

REFERENCES

REFERENCES

Albertini E, Marconi G, Reale L, Barcaccia G, Porceddu A, Ferranti F, *et al.* (2005) SERK and APOSTART. Candidate genes for apomixis in Poa pratensis. *Plant Physiology* **138,** 2185-99

Albrecht C, Russinova E, Hecht V, Baaijens E, de Vries S (2005) The Arabidopsis thaliana SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASES1 and 2 control male sporogenesis. *Plant Cell* **17,** 3337-49.

Albrecht C, Russinova E, Kemmerling B, Kwaaitaal M, de Vries SC (2008) Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR KINASE proteins serve brassinosteroid-dependent and –independent signaling pathways. *Plant Physiology* **148,** 611-619

Arnold von S, Sabala I, Bozhkov P, Dyachok J, Filonova L (2002) Review Developmental pathways of somatic embryogenesis. *Plant Cell, Tissue and Organ Culture* **69,** 233–249

Asokan MP, Kumari Jayasree P, Sushamakumari S (1992) Plant regeneration by somatic embryogenesis of rubber tree (*Hevea brasiliensis*). *International Natural Rubber Conference*, Bangalore, India, p 49

Asokan MP, Kumari Jayasree P, Thomas V, Sushamakumari S, Kala RG, Jayashree R, Sethuraj, MR, Thulaseedharan A (2002) Influence of 2, 4-D and sucrose on repetitive embryogenesis in rubber (*Hevea brasiliensis* Muell. Arg. Cv. GT1). *Journal of Tree Sciences* **21,** 18-26

Asokan MP, Narasimhan K, Kumari Jayasree P, Thulaseedharan A, Sushamakumari S, Kala RG, Jayashree R (2001) Isozyme markers for distinguishing embryogenic and nonembryogenic callus during somatic embryogenesis *Hevea brasiliensis* (Muell.) Arg. *Journal of Tree Sciences* **20,** 78-85

Asokan MP, Sobhana P, Sushamakumari S, Sethuraj MR (1988) Tissue culture propagation of rubber (*Hevea brasiliensis* Muell. Arg.) Clone GT1. *Indian Journal of Natural Rubber Research* **1,** 10-12

Auboiron E, Carron MP, Michaux-Ferriere N (1990) Influence of atmospheric gases particularly ethylene on somatic embryogenesis of *Hevea brasiliensis. Plant Cell, Tissue and Organ Culture* **21,** 31-37

Baudino S, Hansen S, Brettschneider R, Hecht VF, Dresselhaus T, Lörz H, *et al.* (2001) Molecular characterisation of two novel maize LRR receptor-like kinases, which belong to the SERK gene family. *Planta* **213**, 1-10

Blanc G, Lardet L, Martin A, Jacob JL, Carron MP (2002) Differential carbohydrate metabolism conducts morphogenesis in embryogenic callus of *Hevea brasiliensis* (Muell. Arg.). *Journal of Experimental Botany* **53**, 1453-1462

Blanc G, Michaux-Ferriere N, Teisson C, Lardet L, Carron MP (1999) Effects of carbohydrate addition on the induction of somatic embryogenesis in *Hevea brasiliensis. Plant Cell, Tissue and Organ Culture* **59**, 103-112

Blochl A, March GG, Sourdioux M, Peterbauer T, Ritcher A (2005) Inductio of raffinose oligosaccharide biosynthesis by abscisic acid in somatic embryos of alfalfa (*Medicago sativa* L.). *Plant Science* **168**, 1075-1082

Cailloux F, Guerrier JJ, Linossier L, Coudret A (1996) Long term embryogenesis and maturation of somatic embryos in *Hevea brasiliensis. Plant Science* **120**, 185-196

Cailloux M, Lleras E (1979) Fusão de protoplastos de *Hevea brasiliensise* e *Hevea pauciflore. Estabelecimento de Técnica Acta Amazonica* **9**, 9-13

Carron MP, d'Auzac J, Etienne H, EI Hadrami I, Housti F, Michaux-Ferriere N, Montoro P (1992) Biochemical and histological features of somatic embryogenesis in *Hevea brasiliensis. Indian Journal of Natural Rubber Research* **5**, 7-17

Carron MP, Enjalric E (1982) Studies on vegetative micropropagation of *Hevea brasiliensis* by somatic embryogenesis and *in vitro* on microcutting. In: Fujiwara A (Ed) *Plant Tissue Culture*, Maruzen, Tokyo, pp 751-752

Carron MP, Enjalric F (1983) Perspectives du microbouturage de l'*Hevea brasiliensis. Caoutchoucs et Plastiques*, pp 627-628

Carron MP, Enjalric E (1985) Somatic embryogenesis from inner integument of the seed of *Hevea brasiliensis* (Muell. Arg.). *Comptes Rendus de L'Academic des Sciences, Paris Series III* **300**, 653-658

Carron MP, Enjalric F, Lardet L, Deschamps A (1989) Rubber (*Hevea brasiliensis* Müll. Arg.). In: Bajaj YPS (Ed) *Biotechnology in Agriculture and Forestry* (Vol 5), Springer-Verlag, Berlin, pp 222-245

Carron MP, Etienne H, Michaux-Ferrière N, Montoro P (1995a) Somatic embryogenesis in Rubber tree (*Hevea brasiliensis* Müll. Arg.). In: Bajaj YPS (Ed)

Biotechnology in Agriculture and Forestry (Vol 30) Somatic Embryogenesis and Synthetic Seed I, Springer, Berlin, pp 353-369

Carron MP, Etienne H, Lardet L, Campagna S, Perrin Y, Leconte A, Chaine C (1995b) Somatic embryogenesis in rubber tree (*Hevea brasiliensis* Müll. Arg.). In: Jain S, Gupta P, Newton R (Eds) *Somatic Embryogenesis in Woody Plants* (Vol 2), Kluwer, Dordrecht, pp 117-136

Carron MP, Lardet L, Montoro P (2005) Different ways of integrating *in vitro* culture in *Hevea* planting material propagation. IRRDB Conference, Cochin, Nov. 7-11 2005, pp 79-82

Carron MP, Lardet L, Julien J, Boko C (2001) Somatic embryogenesis in *Hevea brasiliensis* (Muell.Arg.) Current advances and limits. In: Sainte-Beuve J (Ed) *Annual IRRDB Meeting*, CIRAD, Montpellier, France, pp 110-118

Carron MP, le Roux Y, Tison J, Dea BG, Caussanel V, Clair J, Keli J (2000) Compared root system architectures in seedlings and *in vitro* plantlets of *Hevea brasiliensis*, in the initial years of growth in the field. *Plant and Soil* **223**, 73-85

Charbit E, Legavre T, Lardet L, Bourgeois E, Ferrière N, Carron MP (2004) Identification of differentially expressed cDNA sequences and histological characteristics of *Hevea brasiliensis* calli in relation to their embryogenic and regenerative capacities. *Plant Cell Reports* **22**, 539-548

Chen Z, Qian C, Qin M, Wang C, Suo C, Xiao Y, Xu X (1981) Relationship between somatic cells and microspores in the process of of anther culture of *Hevea brasiliensis* Muell. Arg. *Acta Botanica* **1**, 1-7

Chen Z, Qian C, Qin M, Xu X, Xiao Y (1982) Recent advances in anther culture of *Hevea brasiliensis* (Muell. Arg). *Theoretical and Applied Genetics* **62**, 103-138

Chevallier MH (1988) Genetic variability of *Hevea brasiliensis* germplasm using isozyme markers. *Journal of Natural Rubber Research* **3**, 42-53

Clement-Demange A, Priyadarshan PM, Hoa TTT, Venkatachalam P (2007) *Hevea* rubber breeding and genetics. In: Janick J (Ed) *Plant Breeding Reviews*, John Wiley and Sons Inc., USA, pp 177-283

Colcombet J, Boisson-Dernier A, Ros-Palau R, Vera CE, Schroeder JI (2005) Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR KINASES1 and 2 are essential for tapetum development and microspore maturation. *Plant Cell* **17**, 3350-61.

Combe JC (1975) Demonstration of intraclonal variability in young grafted trees. *Revue Generale des Caouth. Plast.* **52**, 91-94

Cornish K (2000) Biochemistry of natural rubber, a vital raw material, emphasizing biosynthetic rate, molecular weight and compartmentalization in evolutionarily divergent plant species. Western regional research centre, Albany,USA

Das K, Sinha RR, Potty SN, Sethuraj MR (1994) Embryogenesis from anther derived callus of *Hevea brasiliensis* Muell. Arg. *Indian Journal of Hill Farming* **7**, 90-95

de Klerk G J, Schmitt A B, Lieberal R, Neuman K H (1997) *Biol. Plant.* **39**, 53–66

de Jong AJ, Schmidt EDL & de Vries SC (1993) Early events in higher plant embryogenesis. *Plant Molecular Biology* **22**, 367–377

Dodeman VL, Ducreux G & Kreis M (1997) Zygotic embryogenesis *versus* somatic embryogenesis. *Jounal of Experimental Botany* **48**, 1493–1509

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

Dudits D, Gyorgyey J, Bogre L & Bako L (1995) Molecular biology of somatic embryogenesis. In: Thorpe TA (ed) *In Vitro Embryogenesis in Plants* Kluwer Academic Publishers, pp 267–308

EI Hadrami I, Michaux-Ferriere N, Carron MP, d'Auzac J (1989) Polyamines a possible limiting factor in somatic embryogenesis of Hevea brasiliensis. *Comptes Rendus de l'Academic des Sciences*, Paris Series 308, 205-211

EI Hadrami I, Carron MP, d'Auzac J (1991) Influence of exogenous hormone on somatic embryogenesis in Hevea brasiliensis. *Annals of Botany* 67, 511-15

EI Hadrami I, d'Auzac J (1992) Effects of polyamine biosynthetic inhibitors on somatic embryogenesis and cellular polyamines in Hevea brasiliensis. *Journal of Plant Physiology* 140, 33-36

Emons AMC (1994) Somatic embryogenesis: cell biological aspects. *Acta Botany Neerl* **43**, 1–14

Engelmann F, Lartaud M, Chabrillange N, Carron MP, Etienne H (1997) Cryopreservation of embryogenic calluses of two commercial clones of Hevea brasiliensis. *Cryo Letters* 18, 107-116

Enjalric F, Carron MP (1982) Microbouturage in vitro de jeunes plants d'Hevea brasiliensis (Kunth) Muell. Arg. *Comptes Rendus de l'Academic des Sciences*, Paris Series 295, 259-264

Etienne H, Berger A, Carron MP (1991) Water status of callus from Hevea brasiliensis during induction of somatic embryogenesis. *Physiologia Plantarum* 82, 213-218

Etienne H, Montoro P, Michaux-Ferriere N, Carron MP (1993a) Effects of desiccation, medium osmolarity and abscisic acid on the maturation of Hevea brasiliensis somatic embryos. *Journal of Experimental Botany* 44, 1613-1619

Etienne H, Sotta B, Montoro P, Miginiac E, Carron MP (1993b) Relationship between exogenous growth regulators and endogeous indole-3-aectic acid and abscisic acid in the expression of somatic embryogenesis in Hevea brasiliensis (Muell. Arg.). *Plant Science* 88, 91-96

Etienne H, Lartaud M, Michaux-Ferriere N, Carron MP, Berthouly M, Teisson C (1997a) Improvement of somatic embryogenesis in Hevea brasiliensis (Muell. Arg.) using temporary immersion technique. *In Vitro Cellular and Developmental Biology – Plant* 33, 81-87

Etienne H, Lartaud M, Carron MP, Michaux-Ferriere N (1997b) Use of calcium to optimize long term proliferation of friable calluses and plant regeneration in Hevea brasiliensis (Muell. Arg.). *Journal of Experimental Botany* 48, 129-137

Feher A, Pasternac TP, Dudits D (2003) Transition of somatic plant cells to an embrygenic state , *Plant Cell Tissue Organ Culture* **74,** 201-228

Feher A (2006) Why somatic plant cells start to form embryos? In: *The Plant Plasma Membrane* vol 2, Springer Publishers

Fry SC (1995) Polysaccharide-modifying enzymes in the plant-cell wall. *Annual Review of Plant Physiology and Molecular Biology* **46**, 497–520

Gandimathi H, Paranjothy K (1975) Anther culture attempts at induction of haploidy in Hevea and other plants. *Proceedings of National Plant Tissue Culture Symposium, Kuala Lumpur*, Malaysia, pp 32-35

Gunatilleke ID, Samaranayake G (1988) Shoot tip culture as a method of micropropagation of Hevea. *Journal of Rubber Research Institure of Sri Lanka* 68, 33-44

Guzzo F, Baldan B, Mariani P, LoSchiavo F, Terzi M (1994) Studies on the origin of totipotent cells in explants of *Daucus carota* L. *Journal of Experimental Botany* **45,** 1427-1432

Hebant C (1981) Ontogeny of the primary laticiferous system of *Hevea brasiliensis*: an ultrastrutural and cytochemical study. *Journal of Canadian Botany* **59,** 974-985

Hecht V, Vielle-Calzada JP, Hartog MV, Schmidt ED, Boutilier K, Grossniklaus U, ***et al.*** (2001) The Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. *Plant Physiology* **127**, 803-816

He K, Gou X, Yuan T, Lin H, Asami T, Yoshida S, Russell SD, Li J (2007) BAK1 and BKK1 regulate brassinosteroid-dependent growth and brassinosteroid-independent cell-death pathways. *Current Biology* **17**, 1109–1115

Hu H, Xiong L, Yang Y (2005) Rice SERK1 gene positively regulates somatic embryogenesis of cultured cell and host defense response against fungal infection *Planta* **222**, 107–117

Huang S ***et. al.*** (2009) The genome of cucumber *Cucumus sativus* L. *Nature Genetics* **41,** 1275-1281

Jayashankar S, Marilyn van Aman, Zhijian Li, Dennis JG (2001) Direct seeding of grapevine somatic embryos and regeneration of plants. *In vitro Cellular and Developmental Biology – Plant* vol **37,** no: **4,** 476-479

Jayashree R, Rekha K, Sushamakumari S, Thulaseedharan A (2005) Establishment of callus cultures from isolated microspores of Hevea brasiliensis. *National Symposium on Biotechnological Interventions for Improvement of Horticultural Crops*, Trissur, pp 176-178

Jayashree R, Rekha K, Venkatachalam P, Uratsu SL, Dendekar AM, Kumari Jayasree P, Kala RG, Priya P, Sushamakumari S, Sobha S, Asokan MP, Sethuraj MR, Thulaseedharan A (2003) Genetic transformation and regeneration of rubber tree (Hevea brasiliensis Muell. Arg) transgenic plants with a constitutive version of an anti-oxidative stress superoxide dismutase gene. *Plant Cell Reports* 22, 201-209

Jheng FY, Do YY, Liauh YW, Chung JP, Huang PL (2006) Enhancement of growth and regeneration efficiency from embryogenic callus cultures of *Oncidium* 'Gower Ramsey' by adjusting carbohydrate sources. *Plant Science* **170**, 1133-1140

Kala RG, Leda Pavithran, Thulaseedharan A (2004) Effect of fungicides and antibiotics to control microbial contamination in Hevea cultures. *Plant Cell Biotechnology and Molecular Biology* 5, 51-58

Kala RG, Kumari Jayasree P, Sushamakumari S, Jayashree R, Rekha K, Sobha S, Thulaseedharan A (2005) In vitro regeneration of Hevea brasiliensis from leaf explants. *ICAR National Symposium on Biotechnological Interventions for Improvement of Horticultural Crops: Issues and Strategies,* Trichur, India, pp 105-106

Kala RG, Kumari Jayasree P, Sobha S, Rajani J, Thulaseedharan A (2006) Plant regeneration from leaf derived somatic embryos of Hevea brasiliensis. *International Symposium on Frontiers in Genetics and Biotechnology – Retrospect and Prospect*, Hyderabad, India, p 160

Karlova R, Boeren S, Russinova E, Aker J, Vervoort J, de Vries S (2006) The Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1 Protein Complex Includes BRASSINOSTEROIDINSENSITIVE1. *The Plant Cell* **18,** 626-638

Karlova R, Boeren S, van Dongen W, Kwaaitaal M, Aker J, Vervoort J, et al. (2009) Identification of in vitro phosphorylation sites in the Arabidopsis thaliana somatic embryogenesis receptor-like kinases. *Proteomics* **9,** 368-379

Kumari Jayasree P, Asokan MP, Sobha S, Sankariammal L, Rekha K, Kala RG, Jayashree R, Thulaseedharan A (1999) Somatic embryogenesis and plant regeneration form immature anthers of Hevea brasiliensis (Muell. Arg.). *Current Science* 76, 1242-1245

Kumari Jayasree P, Vinoth Thomas, Thulaseedharan A (2001) Optimization of parameters affecting somatic embryogenesis in Hevea brasiliensis. *Indian Journal of Natural Rubber Research* 14, 20-29

Kumari Jayasree P, Thulaseedharan A (2001) Gibberllic acid regulated embryo induction and germination in Hevea brasiliensis (Muell. Arg.). *Indian Journal of Natural Rubber Research* 14, 106-111

Kumari Jayasree P, Thulaseedharan A (2004) Initiation and maintenance of long term somatic embryogenesis in Hevea brasiliensis. *IRRDB, Biotechnology Workshop*, Kuala Lumpur, Malaysia, 56 pp

Kumari Jayasree P, Thulaseedharan A (2005) In vitro germination of Hevea somatic embryos: Effect of cytokinins. *Plant Cell Biotechnology and Molecular Biology* 6 61-64

Lardet L, Piombo G, Orioi F, Dechamp E, Carron MP (1999) Relations between biochemical characteristics and conversion ability in *Hevea brasiliensis* zygotic and somatic embryos. *Canadian Journal of Botany* **77**, 1168-1177

Lardet L, Martin F, Dessailly F, Carron MP, Montoro P (2007) Effect of exogenous calcium on post-thaw growth recovery and subsequent plant regeneration of cryopreserved embryogenic calli of *Hevea brasiliensis* (Mull. Arg.). *Plant Cell Reports* **26**, 559-569

Linossier L, Veisseire P, Cailloux F, Coudret A (1997) Effect of abscisic acid and high concentration of PEG on *Hevea brasiliensis* somatic embryo development. *Plant Science* **124**, 183-191

Lo Schiavo F, Pitto L, Giuliano G, Torti G, Nuti-Ronchi V,Marazziti D, Veraga R, Orselli S & Terzi M (1989) DNA methylation of embryogenic carrot cell cultures and its variations as caused by maturation, differentiation, hormones and hypomethylation drugs. *Theoretical and Applied Genetics* **77**, 325

Mahalakshmi A (1994) Ph D thesis. University of Delhi, Delhi **28**

Maillot P, Lebel S, Schellenbaum P, Jacques A, Walter B (2009) Differential regulation of SERK, LEC1-like and pathogenesis-related genes during indirect secondary somatic embryogenesis in grapevine. *Plant Physiology and Biochemistry* **47**, 743-52.

Martre P, Lacan D, Just D, Teisson C (2001) Physiological effects of temporary immersion on *Hevea brasiliensis* callus. *Plant Cell, Tissue and Organ Culture* **67**, 25-35

Mendanha ABL, de Almeida Torres RA, de Barros Freire A (1998) Micropropagation of rubber tree (*Hevea brasiliensis* Muell. Arg.). *Genetics and Molecular Biology* **21**, 1415

Merkele SA, Parrott WA & Flinn BS (1995) Morphogenic aspects of somatic embryogenesis. In: Thorpe TA (ed) *In vitro Embryogenesis in Plants* Kluwer Academic Publishers, Dordrecht, Boston, London. pp 155–203

Merkle FA, Parrott WA, Flinn BS (1995) Morphogenic aspects of somatic embryogenesis. In. Thorp (ed.) *In vitro embryogenesis in plants* Kluwer Academic Publishers, Netherlands 1 – 16

Michaux-Ferriere N, Carron MP (1989) Histology of early somtic embryogenesis in Hevea brasiliensis: The importance of the timing of the subculturing. *Plant Cell, Tissue and Organ Culture* **19**, 243-256

Michaux-Ferriere N, Grout H, Carron MP (1991) Origin and ontogenesis of somtic embryos in *Hevea brasiliensis* (*Euphorbiaceae*). *American Journal of Botany* **79**, 174-180

Montoro P, Etienne H, Michaux-Ferriere N, Carron MP (1993) Callus friability and somatic embryogenesis in *Hevea* brasiliensis. *Plant Cell, Tissue and Organ Culture* **33**, 331-338

Montoro P, Etienne H, Carron MP (1995) Effect of calcium on callus friability and somatic embryogenesis in *Hevea* brasiliensis Mull. Arg. Relations with callus mineral nutrition, nitrogen metabolism and water parameters. *Journal of Experimental Botany* **46**, 255-261

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

Muzik TJ, Cruzado HJ (1958). Transmission of juvenile rooting ability from seedlings to adults of Hevea brasiliensis. *Nature* (London) **181**, 1288

Namashivayam P (2007) Acquisition of embryogenic competence during somatic embryogenesis. *Plant Cell, Tissue, Organ Culture* **90**, 1 – 8

Nam KH, Li J (2002) BRI1/BAK1, a receptor kinase pair mediating brassinosteroid signaling. *Cell* **110**, 203–212

Nayanakantha and Seneviratne (2007) Tissue culture of rubber: past, present and future prospects. *Ceylon Journal of Science (Bio. Sci.)* **36 (2)**, 116-125

Nolan KE, Kurdyukov S, Rose RJ (2009) Expression of the SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1 (SERK1) gene is associated with developmental change in the life cycle of the model legume Medicago truncatula. *Journal of Experimental Botany* **60**, 1759-71

Nolan KE, Irwanto RR, Rose RJ (2003) Auxin upregulates MtSERK1 expression in both Medicago truncatula root-forming and embryogenic cultures. *Plant Physiology*; **133**, 218-230

Paranjothy K, Ghandimathi H (1975) Morphogenesis in callus cultures *Hevea* brasiliensis Muell. Arg. *Proc. Natl. Plant Tissue Culture Symposium*, Kuala Lumpur, pp 19-25

Paranjothy K, Gandhimathi H (1976) Tissue and organ culture of *Hevea*. *Proc. Intl. Rubber Conference 1975* (Vol 2), Kuala Lumpur, Malaysia, pp 59-84

Paranjothy K, Rohani O (1978) Embryoid and plantlet development from cell culture of Hevea. *4th International Congress Plant Tissue Cell Culture*, University Calgary, Abstract No 134

Paranjothy K (1974) Induced root and embryoid differentiation in *Hevea* tissue culture. *3rd International Congress Plant Tissue Cell Culture*, University, Leicester, Abstract No 67

Perez-Nunez MT, Souza R, Saenz L, Chan JL, Zuniga-Aguilar JJ, Oropeza C (2009) Detection of a SERK-like gene in coconut and analysis of its expression during the formation of embryogenic callus and somatic embryos. *Plant Cell Report* **28**, 9–11

Perrin Y, Lardet L, Enjalric F, Carron MP (1994) Rajeunissement de clones matures d'Hevea brasiliensis (Müll. Arg.) par microgreffage in vitro. *Canadian Journal of Plant Science* 74, 623-630

Perrin Y, Doumas P, Lardet L, Carron M P (1997) Endogenous cytokinins as a biochemical marker of rubber tree (Hevea brasiliensis Mull.Arg.) clone rejuvenation. *Plant Cell, Tissue and Organ Culture* 47, 239-245

Rains DW (1989) Plant tissue and protoplast culture: applications to stress physiology and biochemistry. In: *Jones HG, Flowers TJ & Jones MB (eds) Plants and Stress* Cambridge University Press, 181–196

Ramer H (1935) Cytology of *Hevea. Genetics* **17**, 193

Reinert J (1958) Untersuchungen über die Morphogenese an Gewebenkulturen. *Ber. Dtsch Bot. Ges.* **71,** 15

Reinert J (1959) Ober die controlle der morphogenese und die induction von adventive-embryonem am gevebeculturen aus carotten. *Planta* **53,** 318-333

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning; a Laboratory Manual, second edition.* Coldspring Harbour Laboratory press, Plain view, New York.

Santa-Catarina C, Hanai LR, Dornelas MC, Viana AM, Floh EIS (2004) SERK gene homolog expression, polyamines and amino acids associated with somatic embryogenic competence of Ocotea catharinensis Mez. (Lauraceae). *Plant Cell Tissue Organ Culture* **79**, 53-61

Santos MO, Romano E, Yotoko KSC, Tinoco MLP, Dias BBA, Aragão FJL (2005) Characterisation of the cacao somatic embryogenesis receptor-like kinase (SERK) gene expressed during somatic embryogenesis. *Plant Science* **168,** 723-9

Saraswathyamma CK, Markose VC, Licy J, Annamma Y, Panikkar AO (1984) Cytomorphological studies in an induced polyploidy of *Hevea brasiliensis* Muell. Arg. *Cytologia* **49**, 725-729

Schmidt ED, Guzzo F, Toonen MA, de Vries SC (1997) A leucine-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos. *Development* **124**, 2049-2062

Schultes RE (1977) The Odyssey of the Cultivated Rubber Tree. *Endeavour New Series* **1**, 133-138

Schultz RE (1990) A Brief Taxonomic View of the Genus Hevea. Kuala Lumpur, MRRDB *Monograph* **14**

Seguin M, Flori H, Legnaté, Clément-Demange A (2003) Rubber tree. In: Hamon P, Seguin MA, Perrier X, Glaszmann JC (Eds) *Genetic Diversity of Tropical Crops*, CIRAD, France and Science Publ., Enfield, USA, pp 277-306

Seneviratne P, Wijesekara G A S, De Zoysa GM (1995) Root system of hevea with special reference to micropropagated plants. *Journal of the Rubber Research Institute of Sri Lanka* **76**, 11-20

Seneviratne P, Flagmann A (1996) The effect of thidiazuron on axillary shoot proliferation of *Hevea brasiliensis in vitro*. *Journal of the Rubber Research Institue of Sri Lanka* **77**, 1-14

Seneviratne P, Wijesekara G A S (1996) The problem of phenolic exudates in *in vitro* cultures of mature *Hevea brasiliensis*. *Journal of Plantion Crops* **24**, 54-62

Seneviratne P, Wijesekara G A S (1997) Effect of episodic growth pattern on *in vitro* growth pattern of *Hevea brasiliensis*. *Journal of Plantation crops* **25**(1), 52-56

Seneviratne P (1991) Micropropagation of juvenile and mature *Hevea brasiliensis*. PhD Thesis, University of Bath, UK, 278 pp

Shah K, Schmidt ED, Vlak JM, de Vries SC (2001) Expression of the Daucus carota somatic embryogenesis receptor kinase (DcSERK) protein in insect cells. *Biochimie* **83**, 415-421

Sharp WR, Son Dahl MR, Caldas LF, Maraffa SB (1980) The Physiology of in vitro asexual embryogenesis. *Horticul. rev.* 268 – 310

Shijie Z, Zhenghua C, Xueng X (1990) A summary report on anther culture for haploid plants of *Hevea brasiliensis*. In: *Proc. IRRDB Symposium on the Breeding of Hevea brasiliensis*, Kumming, China, pp 69-78

Shimada T, Hirabayashi T, Endo T, Fujii H, Kita M, Omura M (2005) Isolation and characterization of the somatic embryogenesis receptor-like kinase gene homologue (CitSERK1) from Citrus unshiu Marc. *Science Horticulture* **103**, 233-238

Shiu SH, Bleecker AB (2001) Receptor-like kinases from Arabidopsis form a monophyletic gene family related to animal receptor kinases. *Procta National Academy of Sciences, USA* **98**, 10763–10768

Singla B, Khurana JP, Khurana P (2008) Characterization of three somatic embryogenesis receptor kinase genes from wheat, Triticum aestivum. *Plant Cell Report* **27**, 833-843

Smith DL and Kirkorian AD (1990) Somatic proembryo production from excised, wounded zygotic carrot embryos on hormone-free medium: evaluation of the effects of pH, ethylene and activated charcoal. *Plant Cell Report* **9**, 468–470

Sobhana P, Sinha PR, Sethuraj MR (1986) Micropropagation of *Hevea brasiliensis:* retrospect and prospects. In: *International Congress of Plant tissue and Cell Culture,* Minnesota, USA

Somleva MN, Schmidt EDL, de Vries SC (2000) Embryogenic cells in Dactylis glomerata L. (Poaceae) explants identified by cell tracking and by SERK expression. *Plant Cell Report* **19**, 718-726

Song D, Li G, Song F, Zheng Z (2009) Molecular characterization and expression analysis of OsBISERK1, a gene encoding a leucine-rich repeat receptor-like kinase, during disease resistance responses in rice. *Molecular Biology Report* **35**, 275-283

Steward FC, Mapes MO & Hears K (1958) Growth and organized development of cultured cells. II. Growth and division of freely suspended cells. *American Journal of Botany* **45**, 705–708

Sushamakumari S, Sobha S, Jayashree R, Asokan MP (1999a) Evaluation of parameters affecting the isolation and culture of *Hevea brasiliensis* (Muell. Arg.). *Currernt Science* **77**, 1580-1582

Sushamakumari S, Rekha K, Thomas V, Sobha S, Jayashree R (1999b) Multiple shoot formation from somatic embryos of *Hevea brasiliensis. Indian Journal of Natural Rubber Research* **12**, 23-28

Sushamakumari S, Asokan MP, Anthony P, Lowe KC, Power JP, Davey MR (2000a) Plant regeneration from embryogenic cell suspension-derived protoplasts of rubber. *Plant Cell, Tissue Organ Culture* **61**, 81-85

Sushamakumari S, Sobha S, Rekha K, Jayashree R, Asokan MP (2000b) Influence of growth regulators and sucrose on somatic embryogenesis and plant regeneration from immature inflorescence of *Hevea brasiliensis. Indian Journal of Natural Rubber Research* **13**, 19-29

Sushamakumari S, Sobha S, Rekha K, Jayashree R, Thulaseedharan A (2006) Plant regeneration via somatic embryogenesis from root explants in *Hevea brasiliensis. International Symposium on Frontiers in Genetics and Biotechnology - Retrospect and Prospect*, Hyderabad, India, 160 pp

Thomas TD (2006) Effect of sugars, gibberellic acid and abscisic acid on somatic embryogenesis in *Tylophora indica* (Burm.f.) Merrill. *Chinese Journal of Biotechnology* **22**, 465-471

Thorpe, T. A. (1978 ed.) Frontiers of plant tissue culture. Calgary: University of Calgary Printing Services 556 pp [Dedicated to Prof. H. E. Street, 1912-1977]

Thorpe, T. A. (1980) Organogonesis in vitro: structural, physiological and biochemical aspects. In: Vasil, I. K., ed. *Perspectives in plant cell and tissue culture* Int. Rev. Cytol. New York, Academic Press **Suppl.11 A** 71-111

Thorpe, T. A. (1981 ed.) Plant tissue culture: methods and applications in agriculture. New York, Academic Press, 379 pp

Veisseire P, Cailloux F Coudret A (1994a) Effect of conditioned media on the somatic embrogenesis of *Hevea brasiliensis. Plant Physiology and Biochemistry* **32**, 571-576

Veisseire P, Linossier L, Coudret A (1994b) Effect of absicsic acid and cytokinins on the development of somatic embryos in *Hevea brasiliensis. Plant Cell, Tissue Organ Culture* **39**, 219-223

Venkatachalam P, Thanseem I, Thulaseedharan A (1999) A rapid and efficient method for isolation of RNA from bark tissues of *Hevea brasiliensis. Current Science* **77**, 635 – 637

Wan AR, Ghandimathi H, Rohani O, Paranjothy K (1982) Recent developments in tissue culture of *Hevea.* In: Rao AN (Ed) *Tissue Culture of Economically Important Plants*, Costed, Singapore, pp 152-158

Wang Z, Zeng X, Chen C, Wu H, Li Q, Fan G, Lu W (1980) Induction of rubber plantles from anther of *Hevea. brasiliensis* Mulle. Arg. *in vitro*. *Chinese Journal of Tropical Crops* **1**, 25-26

Wang Z, Wu H, Zeng X, Chen C, Li Q (1984) Embryogeny and origin of anther plantlet of *Hevea brasiliensis*. *Chinese Journal of Tropical Crops* **5**, 9-13

Wang ZY, Chen XT (1995) Effect of temperatue on stamen culture and somatic plant regeneration in rubber. *Acta Agronimics Sincia* **21**, 723-726

Wang ZY, Wu HD, Chen XT (1998) Effects of altered temperatues on plant regeneration frequencies in stamen culture rubber trees. *Journal of Tropical and Subtropical Botany* **6**, 166-168

Wilson HM, Street HE (1974) The growth, anatomy and morphogenetic potential of callus and cell suspension cultures of *Hevea brasiliensis*. *Physiologia Plantarum* **36**, 399-402

Wilson ZA, Power JB (1989) Elimination of systemic contamination in explants and protoplast cultures of rubber (*Hevea brasiliensis)* Muell. Arg.. *Plant Cell Reports* **7**, 622-625

Wycherly PR, (1992) The genus Hevea :Botanical aspects *In:Natural Rubber Biology, Culitivation and Technology* (Eds. M.R.Sethuraj and N.M. Mathew). Elsevier, Amsterdam 50- 66

Zimmerman JL (1993) Somatic embryogenesis: a model for early development in higher plants. *Plant Cell* **5**, 1411–1423

Zhang S, Liu X, Lin Y, Xie G, Fu F, Liu H, Wang J, Gao S, Lan H, Rong T (2011) Characterization of a ZmSERK gene and its relationship to somatic embryogenesis in a maize culture. *Plant Cell, Tissue, Organ Culture* **105**, 29 – 37