EXPRESSION OF *LEA 5* AND *PEROXIDASE* GENES UNDER COLD STRESS IN *Hevea brasiliensis*

Dissertation submitted to Mahatma Gandhi University in
Partial fulfillment of the requirement for the award of the degree of

Master of Science in Biotechnology

BY

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2011

Dedicated to.....

My parents

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CERTIFICATE

This is to certify that the dissertation entitled, "Expression of *LEA 5* and *peroxidase* genes under cold stress in *Hevea brasiliensis*" is an authentic record of the project work done by Ms.Athira Krishnan.K.A at Rubber Research Institute of India, Kottayam, under the guidance of Dr.Mohamed Sathik.M.B, Senior Scientist (Botany), Crop Physiology Division, 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.

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DECLARATION

I, Athira Krishnan K. A hereby declare that ·eby decla the dissertation entitled "**Expression of *LEA 5* and *peroxidase* genes under cold stress in *Hevea brasiliensis*".** submitted to the Mahatma Gandhi University in partial fulfillment of the requirement for the award of the Master of Science in Biotechnology is a record of the original work done by me under the supervision and guidance of Dr. Mohammed Sathik M. B, Senior Scientist, RRII, Kottayam and it has not been previously included in a thesis, dissertation or report submitted to this university or any other institution for a degree, diploma or any other qualification.

Athira Krishnan K. A

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Athira Krishnan K. A

LIST OF ABBREVIATIONS

ABA : Abscissic acid

ABF : ABA binding factor

AFLP : Amplified fragment length polymorphism

APX : Ascorbate peroxidase

AREB : ABA responsive element binding protein

BAPTA : 1,2-bis (2-aminophenoxy) ethane N,N,N',N' tetraacetic acid

CAT : Catalase

CBF : C-repeat binding factor

CDPK : Calcium dependent protein kinase

COR : Cold regulated

CTE- : C-Terminal extension

DREB : Drought responsive element binding protein

GA : Gibberellin

GAPDH : Glyceraldehyde-3-phosphate dehydrogenase

GPX : Glutathione peroxidase

GSH : Glutathione

GST : Glutathione S transferase

HOS1 : High expression of osmotically responsive gene 1

ICE1 : Inducer of CBF expression 1

KIN1 : Cold induced gene

LEA : Late embryogenesis abundant

MAPK : Mitogen activated protein kinase

MAPKKK : MAPK kinase kinase

MPSS : Massively parallel signature sequencing

OsCDPK13: *Oryza sativa* calcium dependent protein kinase 13

P_N : Net photosynthetic rate

PRX : Peroxidase

qRT-PCR : Quantitative Real Time Polymerase Chain Reaction

RRII : Rubber Research Institute of India

RRIM : Rubber Research Institute of Malaysia

ROS : Reactive oxygen species

SA : Salicylic acid

SAGE : Serial analysis of gene expression

SOD : Superoxide dismutase

CONTENTS

INTRODUCTION

INTRODUCTION

Rubber tree (*Hevea brasiliensis* Muell. Arg.) is a perennial tropical tree which is indigenous to the tropical rainforests of the Great Amazon Basin of South America. It is the only major commercial source of natural rubber (NR), *cis*- 1, 4-polyisoprene. Natural rubber is a constituent of latex, a milky substance produced in laticiferous tissues i.e. in the cytoplasm of laticiferous cells. It is obtained from the mature trees through controlled wounding of trunk regions, a process called tapping. Rubber tree is one of the most recently domesticated crop species in the world. Rubber has been an undeniably beneficial commodity for the past 100 years. Rubber is produced in over 2000 plant species belonging to 300 genera of seven families *viz*., Euphorbiaceae, Apocynaceae, Asclepiadaceae, Asteraceae, Moraceae, Papaveraceae and Sapotaceae (Cornish et al. 1993). The para rubber tree, *(Hevea brasiliensis* Muell. Arg), belonging to the Euphorbiaceae family (spurge family) is the source of virtually all the world's rubber production.

The genus *Hevea* has 10 species, which are inter- crossable (Clement-Demange *et al.,* 2000). *Hevea* species are widely distributed among the countries of South America *Hevea* species are indigenous to Bolivia, Brazil, Colombia, French Guiana, Guyana, Peru, Surinam and Venezuela. All species, except *H. microphylla* occur in Brazil, the center of origin. Rubber tree was introduced to tropical Asia in 1876 by Sir Henry Wickham (through Kew garden) who brought the rubber seeds from Brazil. It is now cultivated in tropical regions of Asia, Africa and America. There are various other rubber producing species such as *Hevea benthamiana*, *Hevea guianensus* (Para rubber), *Manihot glaziovii* (Ceara rubber), *Manihot dichotoma* (Jeque rubber), *Castilla elastica* (Panama rubber), *Ficus elastica* (India rubber), *Cryptostegia grandiflora* (Madagascar rubber), *Parthenium argentatum* (Guayule), *Taraxacum kok-saghyz* (Russian dandelion), *and Palaquin gutta* (Gutta percha). Of these Guayule provides 10 percent of the world's natural rubber. Other species are not commonly exploited.

Hevea brasiliensis can grow to a height of 150 feet. The trees have a smooth greyish bark and trifoliate, palmately compound leaves. They are monoecious and have small inconspicuous flowers. The seeds are recalcitrant. Upon ripening, fruit capsule explodes and propels the seeds away from the tree. Rubber trees are deciduous and annual leaf fall is called wintering. The new leaves appear immediately after leaf fall in lush like growth pattern. It needs a high atmospheric humidity of about 80% with moderate wind, bright sunshine to about 2000 hours per year and a well-drained, fairly deep loamy soil with a pH value of 4.5-6.0. *Hevea* is propagated by bud grafting for which buds taken from high yielding mother plant are grafted to seedling stocks. The buds are allowed to grow into plants which will have all the characteristics of the mother plant. The trees get ready for harvesting in 5-7 years when they attain a girth of 50 cm at a height of 1.25 m from the bud union. All the trees that derive from a single mother plant are called clone. Each clone has its own characteristics of growth, yield, susceptibility to diseases and climatic stresses. The economic life period of rubber in plantations is around 32 years (upto 7 years of immature phase and about 25 years of productive phase).

Rubber tree yields both milk (latex) and honey (nectar). The latex is produced by the laticiferous cells in the bark and nectar exuded from the extra-floral nectaries at the junction of the trifoliate leaf petiole. The young leaves at half maturity (light green colour) start secreting nectar. There are three extra-floral nectaries at the junction of the petiole. An important side product of *Hevea* rubber production is rubber wood, which was originally perceived merely as a useful by-product for drying and smoking rubber and to provide a source of charcoal for local cooking. Rubber wood can be easily steam-bent, or stained to resemble any other timber, depending on consumer demand. Its favourable qualities and light colour make it a good timber for furniture making and other applications. The natural colour of rubber wood is one of the principal reasons for its popularity in Japan, where it is increasingly used to replace more traditional timbers. In 1998, Malaysia exported rubber wood furniture with a value of 683.3 million US $,

and in general rubber wood is one of the most successful export timbers of Southeast Asia.

Malaysia, Indonesia and Thailand are the major producers of natural rubber. Today India is the fourth highest producer of natural rubber in the world having a total of 6.87 lakh ha under rubber cultivation with a production of 831400t (Rubber Statistical News, 2009). The trees prefer warm, humid weather and perform best in climates closely resembling that of its original habitat. Climatic conditions necessary for optimum growth of rubber trees are: a) rainfall of 2000 mm or more, evenly distributed without any marked dry season and with 125-150 rainy days per annum; b) maximum temperature of about 29-34°C and minimum of about 20°C or more with a monthly mean of 25-28°C; c) increased atmospheric humidity of the order of 80 percent with moderate wind; d) bright sunshine amounting to about 2000h/annum at the rate of 6h per day throughout all the months (Webster and Baulkwill,1989).

In India, commercial cultivation of NR was introduced by the British planters, although the experimental efforts to grow rubber on a commercial scale in India were initiated as early as 1873 at the Botanical Gardens, Calcutta. The first commercial Hevea plantations in India were established at Thattekadu in Kerala in 1902. In India rubber trees are traditionally grown in two major zones namely, the traditional and nontraditional. On the basis of agro climatic conditions, the traditional zones include Kanyakumari district of Tamil Nadu, whole of Kerala and Dakshin Kannada and Coorg districts of Karnataka state. North eastern states, West Bengal, Konkan region of Goa and Maharashtra, parts of Andhra Pradesh, Madhya Pradesh and Odissa state comprise the non traditional region. Majority of rubber plantations concentrated in South Kerala comprise of districts of Trivandrum, Kollam, Pathanamthitta and Aleppey.

The yield of rubber is influenced by various factors such as climate, nutrient availability, rainfall system of tapping and genetic nature of the clone etc. Rubber trees are susceptible to biotic (fungal, bacterial, etc.) as well as abiotic (drought, cold,

wounding etc.) stress factors. Temperature is one of the key environmental factors which influence plant growth. *Hevea brasiliensis* being a species adapted to moderate temperature get affected by extremes in temperature. High temperature conditions result in higher rates of evapotranspiration, leading to severe soil moisture stress in absence of rainfall. High temperature above 37°C, coupled with soil moisture stress result in injury to leaf and drying of leaf margins. Thermal injury coupled with water deficit results in increased yield loss. In areas experiencing low temperature, growth rate increase with increase in temperature. The threshold temperature for growth is around 20°C.

A major limiting factor on tree growth in the North east region is stress due to low temperature. Because of shrinking availability of cultivable land in traditional tracts, rubber cultivation in India has been extended to areas of diverse agro climatic zones where near similar weather conditions prevail. Thus along with drought stress, salinity stress and water deficit, cold stress or low temperature stress has also gained much importance in India, since it is the major stress in the non traditional regions. Cold response mechanism may be related to various changes in for eg. the expression of kinases related to signal transduction, accumulation of osmolytes, or membrane lipid composition (Thomashow 1999). A number of cold responsive genes have been reported in various plant species: *cor* (cold regulated) genes, *lea* (late embryogenesis abundant) genes, regulatory genes, antifreeze protein genes and the genes encoding signal transduction proteins (Thomashow 1999; Shinozaki & Yamaguchi-Shinozaki 2000). With the advent of genomic methodology that permits the monitoring of expression of large number of genes simultaneously, it is now possible to identify sets of genes that are active during a specific developmental process. To isolate the genes that are differentially expressed because of abiotic stress, several methods have been used, including differential screening, subtractive hybridization, differential display, serial analysis of gene expression (SAGE) and microarrays. Gene expression analysis substantially improves our understanding of signaling and metabolic pathways underlying specific developmental and cellular processes. Identification of genes associated with cold stress is an important step towards understanding the cold tolerance

mechanism in *Hevea*. qPCR is currently one of the most powerful and sensitive techniques for analyzing gene expression. It is often used for validating output data produced by large scale gene expression analyses like micro and macroarrays of whole genomes and as primary source for detecting specific gene expression patterns.

Based on the results of previous investigations made in the laboratory of RRII, a few genes that wee responsive to various stresses have been identified. Among them peroxidase and LEA 5 protein were found to be stress responsive. But validation of its expression as a response to low temperature had not been studied. Hence this study was made with an aim to validate the expression of these two genes by qPCR method and to find if there expression has any relationship with the cold tolerance or susceptibility of the various clones of *Hevea* at RRII. For this study plants grown in polybags (cold tolerant and susceptible clones) were exposed to low temperature stress in an environmental chamber and the leaf samples were collected after determining the cold stress in the plants. The mRNA isolated from these samples were used for cDNA synthesis and eventually used in the qPCR analyses. The result of qPCR analyses is discussed in detail in the following chapters.

AIM AND OBJECTIVES

AIM AND OBJECTIVES

- To quantify the expression of peroxidase and LEA 5 protein under cold stress conditions.
- To find if there is any association between expression of these two stress related genes and cold stress tolerance.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Plants are frequently exposed to a plethora of stress conditions – both biotic and abiotic. Biological stress can be defined as an adverse force or a condition, which inhibits the normal functioning and well being of a biological system (Jones & Jones, 1989). Plants have a remarkable ability to cope with highly variable environmental stresses, including cold, drought, and soils with changing salt and nutrient concentrations (i.e. abiotic stress). Nevertheless, these stresses together represent the primary cause of crop loss worldwide (Boyer, 1982), reducing average yields for most major crop plants by more than 50%. In contrast, the estimated yield loss caused by pathogens is typically around 10% to 20% (Bray, 2000).

Cold, drought and salinity are the abiotic stresses which affect water relations of a plant on cellular as well as whole plant level causing specific as well as unspecific reactions, damage and adaptation reactions. Environmental stress tolerance is intricate and involves several changes at whole plant, cellular and molecular levels. These stresses have a negative impact on photosynthetic system in higher plants. Leaf photosynthesis is directly influenced by heat stress thereby decreasing final biomass yield. Temperature extremes and salinity are the major stress types which are great modulators of growth and productivity in crop plants (Zhang *et al.*, 2000). However the responses of plants to these stresses may be entirely different. In response to these stress factors various genes are upregulated, which can mitigate the effect of stress and lead to adjustment of the cellular milieu and plant tolerance. In nature stress does not generally come in isolation and many stresses act hand in hand with each other. In response to these stress signals that cross talk with each other, nature has developed diverse pathways for combating and tolerating them. These pathways act in cooperation to alleviate stress.

During temperature stress cellular homeostasis is disrupted because different pathways within cells have different temperature optimum. For example, due to the physical properties of membranes, membrane-associated processes such as

photosynthesis and respiration are more sensitive to temperature stress compared with pathways that are mainly carried out by soluble enzymes. When different pathways are uncoupled, electrons that have a high-energy state are transferred to molecular oxygen (O_2) to form reactive O_2 species (ROS) (Mittler, 2002). ROS, such as $^1O_2$, H_2O_2, O_2^-, and HO, are toxic molecules capable of causing oxidative damage to proteins, DNA, and lipids (Apel and Hirt, 2004). They are mainly produced at a low level in organelles such as chloroplasts, mitochondria, and peroxisomes, but during stress, their rate of production is dramatically elevated. ROS generated by temperature stresses such as heat, cold or freezing have been shown to injure cell membranes and proteins. High-light stress has the potential to enhance the production of ROS in cells and cause oxidative damage to chloroplasts. It was shown to enhance the production of ROS and ROS-associated injury during temperature stresses (Larkindale and Knight, 2002). Previous studies demonstrated that ROS-scavenging mechanisms have an important role in protecting plants against temperature stresses and a combination of high light and temperature stress (Iba, 2002; Yoshimura *et al.*, 2004). ROS play a key role in plants as signal transduction molecules involved in mediating responses to pathogen infection, environmental stresses, programmed cell death and developmental stimuli (Torres and Dangl, 2005).

Low temperature is one of the most important environmental factors that regulate plant growth and development and limit plant production (Cao *et al.,* 2002). Plants can respond and adapt to low temperature stress by several physiological, biochemical and molecular responses (Foyer *et al.*, 1998). There is increasing evidence that low temperature stress induced the increase generation of reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxide radicals (OH^-) and singlet oxygen ($^1O_2$) which have greater toxicity potentials on biomolecules and membranes in plants (Chaitanya *et al.*, 2001). Plants have antioxidant enzymes to keep the increase generation of ROS which includes superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX) (Zhou *et al.*, 2005).

Lowering temperatures will thermodynamically reduce the kinetics of metabolic reactions. Exposure to low temperatures will shift the thermodynamic equilibrium such

that there will be an increased likelihood that nonpolar side chain of proteins will become exposed to the aqueous medium of the cell. This will directly affect the stability and the solubility of many globular proteins (Siddiqui & Caviocchioli, 2006). This leads to a disturbance in the stability of proteins, or protein complexes and also to a disturbance of the metabolic regulations. Lower temperatures induce rigidification of membranes, leading to a disturbance of all membrane processes (e.g. opening of ion channels, membrane associated electron transfer reactions, etc.). Chilling is also associated with the accumulation of reactive oxygen species (ROS). The activities of the scavenging enzymes will be lowered by low temperatures, and the scavenging systems will then not be able to counterbalance the ROS formation that is always associated with mitochondrial and chloroplast electron transfer reactions. Moreover, the chloroplast electron transfer chain will be over reduced during chilling, which will lead to increased ROS formation. The accumulation of ROS will have deleterious effects, especially on membranes. This will result in ion leakage. In addition, the low temperatures will favour the formation of secondary structures in RNA, thus affecting gene and protein expression. Various phenotypic symptoms in response to chilling stress include reduced leaf expansion, wilting, chlorosis (yellowing of leaves) and may lead to necrosis (death of tissue). Chilling also severely hampers the reproductive development of plants for example exposure of rice plants to chilling temperature at the time of anthesis (floral opening) leads to sterility in flowers

Visible chilling symptoms on plants include surface lesions; water soaked appearance, desiccation, discoloration, tissue breakdown, accelerated senescence, shortened shelf life and faster decay due to leakage of plant metabolites (Aroca *et al.*,2003). Seedlings have been regarded as more chilling sensitive than young and mature plants. Under mild cold stress (e.g. 15°C), plants reduce root branching and growth. Furthermore, root length is more influenced by cold stress as well as its dry weight. These changes limit the roots capacity for water and mineral uptake including phosphorous, potassium and nitrogen and ultimately the overall plant growth. The success or failure of a seedling in the field is strongly related to the development of its root system under cold stress. Chilling-sensitive plants exposed to low temperatures

usually show water stress symptoms due to decreased root hydraulic conductance and leaf water and turgor potentials (Aroca *et al.*, 2003). In studies where shoots and roots are chilled, stomata remain open for up to 24–48 h after chilling despite low leaf water and turgor potentials. This loss of stomatal control of leaf transpiration not only decreases root hydraulic conductance but also further aggravates chilling-induced water deficit (Lee *et al.*, 2001). Increased viscosity of water partly accounted for an initial decrease in root hydraulic conductance during the first stages of chilling. However, further decreases in root hydraulic conductance due to prolonged exposure to chilling were unrelated to changes in water viscosity.

Low temperatures affect different aspects of photosynthesis. For example, low temperatures inhibit sucrose synthesis in the cytosol, leading to the accumulation of phosphorylated intermediates. This results in the depletion of the available inorganic phosphate and in the decreased cycling of inorganic phosphate between the cytosol and the chloroplast (Hurry *et al.*, 2000). This, in turn, impedes synthesis of the ATP necessary for the regeneration of ribulose- 1, 5 -bisphosphate to support CO_2 fixation. In addition, low temperatures can inhibit thylakoid electron transport by increasing membrane viscosity and restricting the diffusion of plastoquinone (Griffith *et al.*, 1984). In contrast, light energy trapping by the antenna of PSI and PSII and the use of this energy to drive charge separation within the reaction centre cores are largely temperature independent. Therefore an imbalance can be created by an exposure to low temperatures because the chlorophyll antenna complexes trap more energy that can be processed biochemically (Ensminger *et al.*, 2006). Under these conditions, thylakoid membranes become over energized. One of the consequences of this over-energized state is photodamage, caused primarily by the increased formation of ROS.

Freezing temperatures have even more damaging effects. Under natural conditions, plants freeze slowly and extracellular freezing occurs. Due to the difference in chemical potential created by a growing ice crystal, cellular water migrates to this extracellular ice, causing cell dehydration and shrinkage (Dowgert & Steponkus, 1984). The degree of dehydration produced by such extracellular freezing depends upon the temperature, and its severity increases with lower temperatures. Ultimately, ice can

penetrate the symplast (Gusta *et al.*, 2004), causing a deterioration of the intracellular structures and death of tissues. The major malicious effect of freezing is that it induces severe membrane damage (Steponkus *et al.*, 1993). This damage is largely due to the acute dehydration associated with freezing. The real cause of freeze-induced injury to plants is the ice formation rather than low temperatures. Dehydrated tissues such as seeds and fungal spores can survive at very low temperatures without any symptoms of injury. Freeze induced cellular dehydration results in multiple forms of membrane damage including expansion-induced-cell lyses and fracture lesions (Steponkus *et al.*, 1993; Uemura *et al.*, 1997) and lamellar-to-hexagonal-II phase transition. Although freeze exerts its effect largely by membrane damage due to severe cellular dehydration, certain additional factors may also contribute to damage induced by freeze. ROS produced in response to freeze stress contributes to membrane damage.

Plants differ in their tolerance to chilling (0-15°C) and freezing (< 0°C) temperatures. Plants from temperate regions are chilling tolerant, although most are not very tolerant to freezing but can increase their freezing tolerance by being exposed to chilling, non freezing temperatures, a process known as cold acclimation (Levitt, 1980), which is associated with biochemical and physiological changes (Gilmour *et al.*, 2000). Many of our existing staple crop species (e.g., corn (*Zea mays*), rice (*Oryza sativa*), and potatoes (*Solanum tuberosum*)) originate from tropical and subtropical regions and are susceptible to damage when temperatures fall below 15°C (McKersie & Leshem, 1994). The stage in the growth cycle most vulnerable to stress is the reproductive phase, which includes the formation of reproductive organs, flowering, fruiting, and seed development. Besides, these species also do not tolerate chilling temperatures and can suffer irreversible damage when the temperature goes down below 10°. Other species, such as *Pelargonium*, are able to tolerate chilling but not freezing temperatures and still others from temperate regions, such as spinach (*Spinacia oleracea*), winter wheat (*Triticum aestivum*), canola (*Brassica napus*) are able to survive freezing temperatures. During the course of evolution many plant species have developed an array of mechanisms that enable them to minimize the negative effects of cold stress. The ability to cold acclimate, i.e. to increase tolerance to severe cold (freezing) stress as a result of

prior exposure to moderately suboptimal (chilling) temperatures, is a multigenic trait and research over the last 20 years, primarily using *Arabidopsis thaliana* as a model species, has identified a large number of genetic and biochemical changes that take place during cold acclimation (Nakashima and Yamaguchi Shinozaki, 2006). In temperate latitudes, cold hardiness (or cold acclimation) is established in the autumn, when the temperatures are low but positive and the photoperiod decreases, and it reaches a maximum in winter

While current research has identified primary and secondary regulators responsible for modulating cold acclimation, the proteins and processes involved in sensing temperature change and initiating acclimation remain to be identified. Furthermore, the modulation of cellular metabolism and the regulation of transcriptome and proteome composition that is the result of acclimation is made even more complex by the presence of three different genomes within the cell (nucleus, chloroplast, and mitochondria), whose expressional activity must be coordinated depending on the status of the cell as a whole and of the individual organelles. Thus, proper cellular function can only be maintained in a fluctuating environment through the activity of a complex network of signalling pathways that respond to the metabolic status of the different compartments and regulate the expression of the different genomes in a coordinated fashion. Adding to this complexity, these regulators of cold acclimation have been identified in plants exposed to abrupt short term changes in temperature, not from plants exposed to prolonged cold such as experienced by herbaceous winter crops overwintering under snow or woody perennials overwintering above the snow cover.

The two distinct strategies taken by plants to combat low temperature stress are avoidance and tolerance. Stress avoidance entails preventing the freezing of sensitive tissues. Some succulent species (with thick tissue mass and abundant water content) are able to accumulate residual heat during the day and dissipate it slowly during the cold night (Nilsen & Orcutt, 1996); many annual herbs survive in the form of dormant organs or seed; others protect the shoot meristem with leaves (Kacperska, 1999). A more elaborate avoidance strategy involves super cooling, in which endogenous ice nucleation is prevented by inhibiting the formation of ice nucleators, even where the

temperature falls as low as 40°C. Extremely winter hardy species can generate within their cells so-called 'liquid glass', a highly viscous solution that prevents ice nucleation even at -196°C. Such cells become osmotically, thermally and mechanically desensitized to the presence of external ice (Wisniewski & Fuller, 1999). Where the severity of the stress is more progressive, tolerant plants have evolved the ability to acclimatize and are defined as as the non-heritable modification of structure and function in response to stress, in a way that reduces harm and thereby improves fitness (Kacperska 1999). The plant response to low-temperature stress can be divided into three distinct phases. The first is cold acclimation (pre-hardening), which occurs at low, but above zero temperatures. The second stage (hardening), during which the full degree of tolerance is achieved, requires exposure to a period of sub-zero temperatures. The final phase is plant recovery after winter (Li *et al.*, 2008). Some plants (especially trees) need a combination of short photoperiod and low temperature to fully develop their cold tolerance. In these cases, tolerance can be lost if the temperature is raised above zero and the photoperiod is lengthened (Kacperska, 1999). Plant organs differ in their level of tolerance – typically the roots are much more sensitive than the crown (McKersie & Leshem, 1994), which is understandable given that the crown is the site of the major meristem responsible for production of new roots and shoots at the end of the cold period. Other environmental stresses, including drought and pathogens, can also affect cold acclimation. The transcriptional profiles of stress-responsive genes indicate that cross-talk among signal transduction pathways is responsible for various stress responses (Xiong and Zhu, 2001).

Chilling tolerance refers to the plants strategies to cope with low temperatures stress. Plants have developed a number of mechanisms in order to avoid low temperature injuries. Most temperate plants while growing in the low temperature condition can acquire cold tolerance upon prior exposure to low but sublethal temperature. However, tropical or chilling sensitive plant species are incapable of cold-acclimation and they can not tolerate ice. The temperature threshold for chilling damage can be lowered even in some chilling-sensitive crop species by prior pretreatment at suboptimal low temperatures (Anderson *et al.*, 1994), a process called chilling-

acclimation. The chilling tolerance is a complex phenomenon, and involves a number of physiological and biochemical processes at whole plant, organ, cell and subcellular levels. These processes are reduced water loss by stomatal resistance, enhanced water uptake with the development of prolific root systems, and synthesis and accumulation of osmolytes. Amongst endogenous plant hormones, roles of SA and ABA have been implicated in chilling stress tolerance. Of these, ABA has a more direct role in cellular desiccation caused by freezing stress and control of gene expression during cold acclimation (Anderson *et al.*, 1994, Aroca *et al.*, 2003). ROS reduction by enzymatic and non-enzymatic systems, cell membrane stability, synthesis of functional aquaporins and stress proteins are vital mechanisms of cold tolerance. Lipids containing saturated fatty acids solidify at temperatures higher than those containing unsaturated fatty acids. Therefore, the relative proportion of unsaturated fatty acids in the membrane strongly influences the fluidity of the membrane (Steponkus *et al.*, 1993). The temperature at which a membrane changes from semi fluid state to a semi crystalline state is known as the transition temperature. Chilling sensitive plants usually have a higher proportion of saturated fatty acids and, therefore, a higher transition temperature. Chilling resistant species on the other hand are marked by higher proportion of unsaturated fatty acids and correspondingly a lower transition temperature.

The primary environmental factor responsible for triggering increased tolerance against freezing is the phenomenon known as 'cold acclimation.' It is the process where certain plants increase their freezing tolerance upon prior exposure to low non-freezing temperatures. The primary function of cold acclimation is to stabilize the membranes against freeze injury. Acclimation results in increase in proportion of unsaturated fatty acids and thereby a drop in transition temperature. It functions to prevent the expansion-induced lyses and formation of hexagonal II phase lipids in rye and other plants (Uemura *et al.*, 1997). Cold acclimation results in physical and biochemical restructuring of cell membranes through changes in the lipid composition and induction of other non-enzymatic proteins that alter the freezing point of water. Addition of solutes decreases the freezing point of water to a more negative value, thus preventing ice formation. Low temperatures induce a number of alterations in cellular components,

including the extent of unsaturated fatty acids, the composition of glycerolipids, changes in protein and carbohydrate composition and the activation of ion channels (Knight *et al.*, 1996). Accumulation of sucrose and other simple sugars that occurs with cold acclimation also contributes to the stabilization of membrane as these molecules can protect membranes against freeze-damage. Freezing tolerance is a multigenic trait. Low temperatures activate a number of cold-inducible genes (Jones & Inouye, 1994), such as those that encode dehydrins, lipid transfer proteins, translation elongation factors and the late-embryogenesis-abundant proteins. Moreover, intercellular ice formation can cause a mechanical strain on cell wall and membrane leading to cell rupture. There is also substantiation that protein denaturation occurs in plants at low temperature which could also result in cellular damage (Guy *et al.*, 1998). Overall, cold acclimation results in protection and stabilization of the integrity of cellular membranes, enhancement of the antioxidative mechanisms, increased intercellular sugar levels as well as accumulation of other cryoprotectants including polyamines that protect the intracellular proteins by inducing the genes encoding molecular chaperones (Guy & Li, 1998). All these modifications help the plant to withstand and surpass the severe dehydration associated with freezing stress.

Cold exposure induces an oxidative stress. ROS are either formed in the chloroplast, in the mitochondria, in peroxisome or in the cytosol. At low temperature the enzymatic systems that normally "destroy" the ROS will be less efficient (thermodynamically a lower temperature decreases enzymatic activity) and ROS accumulation in the cold by mitochondria has been reported. To counterbalance the ROS production, plants subjected to low temperatures induce and activate scavenging systems. Catalases and superoxide dismutases are induced by cold (Guo *et al.*, 2006; Goulas *et al.*, 2006). In *A. thaliana*, an increase in ascorbate is observed and is still observed after 14 days of exposure at 4°C (Kaplan *et al.*, 2004). Ascorbate is an abundant antioxidant in plants (Muller & Moule *et al.*, 2002). The detoxification of hydrogen peroxide produced in the chloroplasts relies exclusively on the activity of ascorbate peroxidase bound to thylakoid membranes in the vicinity of PSI (Miyake and Asada, 1992). Monodehydroascorbate reductase catalyzes the regeneration of ascorbate

in the chloroplast at the expense of NAD (P) H. Glutathione (GSH) is an intermediary in the cycle. In addition to its role in this cycle, GSH can react chemically with singlet oxygen, superoxide and hydroxyl radicals, and therefore function directly as a free radical scavenger. GSH may stabilise membrane structure by removing acyl peroxides formed by lipid peroxidation reactions (Price *et al.,* 1990). In a proteomics study performed in rice, ascorbate peroxidases and 2 Cys peroxyredoxin differentially accumulate during chilling (Yan *et al.,* 2006). Again in rice, ferritin and glutathione S transferase (GST) are induced in response to cold. GST with activity of GSH peroxidase is an enzyme catalyzing the conjugation of the glutathione to a variety of toxic substrates arising from oxidative stress, thereby reducing their toxicity (Cui *et al.,* 2005). A similar study of the dynamics of the soluble proteome of *Arabidopsis* during cold acclimation also identified a number of proteins involved in redox regulation and reactive oxygen scavenging that were differentially regulated by cold. These included monodehydroascorbate reductase and two 2 Cys peroxiredoxins (2 Cys Prx A and B) that were reduced in abundance (Goulas *et al.,* 2006). In plants, the 2 Cys Prx proteins are localized in the chloroplast stroma where, under low H_2O_2 conditions, they are present as dimers and function as peroxidases (Jang *et al.,* 2006). However, under oxidative stress eukaryotic 2 Cys Prx proteins undergo structural and functional changes leading to the formation of high molecular mass complexes with chaperone activity. It is likely that the reduction in abundance of the two 2 Cys Prx proteins in the stroma of these cold exposed *Arabidopsis* plants was the result of the formation of such super complexes. This, in turn, suggests a role for increased 2 Cys Prx chaperone activity in response to elevated oxidative stress. Responses of antioxidative defence systems to chilling studied in four cultivars of rice (*Oryza sativa* L.) showed that tolerance to chilling in rice is well associated with the enhanced capacity of antioxidative system under chilling condition (Guo *et al.,* 2006).

. Genes induced during stress conditions function not only in protecting cells from stress by producing important metabolic proteins, but also in regulating genes for signal transduction in the stress response. Thus, these gene products are classified into two groups (Fowler and Thomashow, 2002). The first group includes proteins that

probably function in stress tolerance, such as chaperones, LEA proteins, osmotin, antifreeze proteins, mRNA-binding proteins, key enzymes for osmolyte biosynthesis such as proline, water channel proteins, sugar and proline transporters, detoxification enzymes, enzymes for fatty acid metabolism, proteinase inhibitors, ferritin, and lipid-transfer proteins. Some of these stress-inducible genes that encode proteins, such as key enzymes for osmolyte biosynthesis, LEA proteins, and detoxification enzymes have been overexpressed in transgenic plants and produce stress tolerant phenotypes in the transgenic plants (Cushman and Bohnert, 2000). These results indicate that the gene products of the stress-inducible genes really function in stress tolerance. The second group contains protein factors involved in further regulation of signal transduction and gene expression that probably function in stress response. They include various transcription factors that regulate various stress-inducible genes cooperatively or separately, and may constitute gene networks. Some of these regulatory pathways are also involved in other stress responses including those of drought-, cold-, or high-salinity. Functional analysis of these stress-inducible transcription factors should provide more information on the complex regulatory gene networks that are involved in responses to drought, cold, and high-salinity stresses. The others are proteins kinases, protein phosphatases, enzymes involved in phospholipids metabolism, and other signaling molecules such as calmodulin-binding protein. At present, the functions of most of these genes are not fully understood. Some of these stress-inducible regulatory genes that encode proteins such as transcription factors have been overexpressed in transgenic plants and generate stress tolerant phenotypes in them (Tester and Bacic, 2005).

The discovery of gene expression change during cold acclimation was the starting of exploration of antifreezing molecular mechanisms. Global transcript profiling analyses indicate that > 10% of genes in the *Arabidopsis* genome are regulated during cold acclimation (Vogel *et al.*, 2005). Transcriptome analysis using microarray technology is a powerful technique, which has proven very useful for discovering many stress-inducible genes involved in stress response and tolerance Numerous genes that are induced by various abiotic stresses have been identified using various microarray

systems (Fowler and Thomashow, 2002; Vogel *et al.,* 2005).The technology available for the analysis of gene expression can be divided into two categories: closed and open systems .Closed systems are characterized by a finite number of genes that can be assessed by virtue of their inclusion by selection. Therefore, the coverage of genes will be related to the completeness of the knowledge of the genome being studied, limiting this kind of analysis to the most well characterized species or systems. Typically, closed systems such as microarrays and real-time polymerase chain reaction (PCR) have been extensively used in gene expression analysis in plants (Fernandez *et al.*, 2008). On the other hand, with open systems there is no need for previous knowledge of the genome or transcriptome of the organism. cDNA- AFLP (cDNA-Amplified fragment length polymorphism), MPSS (massively parallel signature sequencing), and specially SAGE (serial analysis of gene expression) have been successfully used to quantify transcript abundance and generate expression data across different tissue types or developmental stages in higher plants (Ritter *et al.*, 2008).

Quantification of gene expression levels is a fundamental step in virtually all aspects of molecular biological research. It is particularly important when genes that are expressed specifically under certain growth conditions are to be compared. Common experimental techniques used to evaluate gene expression levels include Northern blot hybridization and reverse transcription (RT)-mediated PCR (RT-PCR); both techniques are practical for analyzing expression of a small set of genes and are complemented by microarray analysis, which is routinely employed for large-scale, global expression profilings. In recent years, the qRT-PCR has been the method of choice for measuring gene expression levels in multiple samples involving a limited number of genes. It provides accurate and sensitive quantification of gene transcript levels, even for those genes with fairly low transcript levels (Nolan *et al.,* 2006). The qRT-PCR is broadly accepted as the method of choice for accurate and sensitive quantification of gene transcript levels, even for those genes whose transcript levels are low. For valid qRT-PCR analysis, accurate normalization of gene expression against an appropriate internal control is required. Most gene expression studies in the literature generally use a single internal control for normalization (Suzuki *et al.,* 2000) and the validity of the

conclusion depends highly on the control applied. Therefore, it is necessary to validate the expression stability of the control gene under specific experimental conditions prior to its use for normalization.

Low temperatures restrain the expression of the full genetic potential of plants owing to its direct inhibition of metabolic reactions and, indirectly, through cold induced osmotic (chilling-induced inhibition of water uptake and freezing induced cellular dehydration), oxidative and other stresses (Chinnusamy *et al.*, 2007). Using a pharmacological approach, plasma membrane rigidification has been shown to induce cold responsive (*COR*) genes and result in cold acclimation in alfalfa and oilseed (Orvar *et al.*, 2000). The *Arabidopsis fad2* mutant defective in oleate desaturase exhibited membrane rigidification and activation of diacylglycerol kinase at 18°C as compared with the wild type at 14°C and transgenic *Arabidopsis* overexpressing linoleate desaturase at 12°C (Vaultier *et al.*, 2006). These findings support the idea that plant cells can sense cold stress through its membrane rigidification effect (Williams *et al.*, 2005). Cold-induced Ca^{2+} increase in the cytosol can also be mediated through membrane rigidification. Subsequently Ca^{2+} signals amplification and phospholipid signaling might trigger cold-stress signaling (Komatsu *et al.*, 2007). Research studies over the past few years show that cold acclimation temperatures induce positive changes in the plant transcriptome. Significant progress has been made in the past decade in exploring the transcriptional networks regulating cold acclimation. Cold stress induces the expression of *APETALA2*/ethylene response factor family transcription factors, C repeat binding factors (CBFs), also known as dehydration-responsive element-binding protein 1s or DREB1s, which bind to *cis*-elements in the promoters of *COR* genes and upregulate them. Transgenic expression of Arabidopsis CBFs in different plant species was able to enhance chilling/freezing tolerance in an ectopic manner (Yamaguchi-Shinozaki and Shinozaki, 2006). CBFs regulate the expression of genes involved in a wide variety of physiological phenomena e.g., phosphoinositide metabolism, transcription, ROS detoxification, hormone metabolism, signaling and many others with known or anticipated protective functions (Lee *et al.*, 2005). Microarray analysis of transgenic Arabidopsis plants ectopically expressing CBFs revealed constitutive

induction of downstream cold-responsive transcription factor genes (*RAP2.1* and *RAP2.7*), which possibly control subregulons of the CBF regulon (Fowler and Thomashow, 2002). This suggested that CBFs play a crucial role in gene regulation during cold acclimation in genetically diverse plants. Among various CBFs, constitutive over-expression of *ICE1* enhanced the expression of *CBF3*, *CBF2* and *COR* genes during cold acclimation, and increased freezing tolerance of transgenic Arabidopsis. *ICE1* is constitutively expressed and nucleus localized, but causes CBFs expression only under cold stress, suggesting that cold stress-induced post-translational modification is necessary for *ICE1* to activate downstream genes in plants (Chinnusamy *et al.*, 2007).DREB/CBF type transcription factors, Egu CBF1a/b, were isolated from *Eucalyptus gunnii* and constitutively overexpressing in a cold sensitive *Eucalyptus* hybrid. The overexpression of EguCBF genes upregulated the putative target genes and enhanced freezing tolerance in the transgenic lines (Navarro *et al.*, 2011).

Studies on COR genes expression in Arabidopsis have shown that members of the CBF/DREB1 transcriptional factor family, which are induced early and transiently by cold stress, play a key role in cold acclimation (Fowler and Thomashow, 2002). In addition, evidence has accumulated indicates that plant hormones, such as ABA, play important roles in stress signal transduction. In higher plants, cold stress can cause an increasing accumulation of ABA. Recent genetic evidence suggests that the ABA-inducible bZIP transcription factors, ABF/AREB, regulate the stress-signaling pathways that activate LEA-like genes in higher plants (Yamaguchi-Shinozaki and Shinozaki, 2006). Brassinosteroids (BRs), which are steroidal compounds that appear to play a role in protecting plants from a variety of environmental stresses, could promote growth recovery of maize seedlings and tomato fruit setting after cold acclimation. The growth promoting effects of BRs have also been observed in rice during cold acclimation (Krishna, 2003).

Considerable efforts have been directed towards determining the nature of cold-inducible genes and establishing their role in freezing tolerance. The *Arabidopsis fad8* gene (Gibson *et al.*, 1994) encodes a fatty acid desaturase that contributes to freezing tolerance by altering the lipid composition. Cold-responsive genes encoding molecular

chaperones including a spinach *hsp70* gene (Anderson *et al.*, 1994), and a *Brassica napus hsp90* gene, contribute to freezing tolerance by stabilizing proteins against freeze-induced denaturation. Many cold-responsive genes encoding various signal transduction and regulatory proteins have been identified and this list includes the mitogen activated protein (MAP) kinase, MAP kinase kinase kinase (MAPKKK) (Mizoguchi *et al.*, 1996) and the calmodulin- related proteins (Polisensky, 1996). These proteins might contribute to freezing tolerance as well as tolerance to other stresses by controlling or regulating the expression and activity of the major stress genes as well their proteins. The largest class of cold induced genes encodes polypeptides that are homologs of LEA proteins and the polypeptides that are synthesized during the late embryogenesis phase, just prior to seed desiccation and also in the seedlings in response to dehydration stress (Close, 1997). These LEA like proteins are mainly hydrophilic, many have relatively simple amino-acid composition, and are composed largely of a few amino acids with repeated amino acid sequence motifs. Many of these proteins are predicted to contain regions capable of forming amphipathic a helices. The examples of cold responsive genes include: *COR15a*, (Artus *et al.*, 1996), alfalfa *Cas15* (Monroy *et al.*, 1993), and wheat *WCS120*. The expression of *COR* genes has been shown to be critical for both chilling tolerance and cold acclimation in plants (Thomashow, 1999). *Arabidopsis COR* genes include: *COR78/RD29*, *COR47*, *COR15a*, *COR6.6* and encode LEA like proteins (Thomashow, 1999). These genes are induced by cold, dehydration or ABA. COR15A polypeptide is targeted to the chloroplast. Formation of hexagonal II phase lipids is a major cause of membrane damage in non-acclimated plants. COR15a expression decreases the propensity of the membranes to form hexagonal II phase lipids in response to freezing (Uemura & Steponkus, 1997). The analysis of the promoter elements of *COR* genes revealed that they contain DRE (dehydration responsive elements) or CRT (C-repeats) and some of them contain ABRE (ABA-responsive element) as well (Stockinger *et al.*, 1997).

Induction of the *COR* genes was accomplished by over-expression of transcription factor CBF (CRT/DRE binding factor) (Stockinger *et al.*, 1997). CBF binds to the CRT/DRE elements present in the promoter of the *COR* genes and other

cold-regulated genes. The over-expression of these regulatory elements not only resulted in increased freezing tolerance but also an increase to drought tolerance (Liu *et al.*, 1998). This finding provides strong support that a fundamental role of cold-inducible genes is to protect the plant cells against cellular dehydration, Genetic analysis of *HOS1* (high expression of osmotically responsive genes) locus of *Arabidopsis*. showed that the *hos1* mutation resulted in sustained and super induction of *CBF2*, *CBF3* and their target regulatory genes during cold stress (Lee *et. al.*, 2001) Therefore, *HOS1* was identified as a negative regulator of *COR* genes by modulating the expression level of CBFs. (Chinnuswamy *et al.*, 2003). *HOS1* gene encodes a ring finger protein and is constitutively expressed but gets drastically down-regulated within 10min of cold stress. Genetic analysis led to the identification of ICE1 (inducer of CBF expression 1) as an activator of CBF3 (Chinnuswamy *et al.*, 2003). *ICE1* encoded a transcription factor that specifically recognized MYC sequence on the *CBF3* promoter. Transgenic lines overexpressing ICE1 did not express *CBF3* at warm temperature but showed a higher level of expression for *CBF3* as well as *RD29* and *COR15a* at low temperatures. This study suggests that cold induced modification of *ICE1* is necessary for it to act as an activator of *CBF3* in plants. Recently two CBF1-like cDNAs *CaCBFIA* and *CaCBFIB* have been cloned and characterized (Kim *et al.*, 2004) from hot pepper. These were induced in response to low temperature stress (4 °C) and not in response to wounding or ABA. Two-hybrid screening led to the isolation of a homeodomain leucine zipper (4D-Zip) protein that interacts with CaCBFIB. The expression of 4D-Zip was elevated by low temperature and drought (Kim *et al.*, 2004). Calcium-dependent protein kinases (CDPKs) play an important role in the signal transduction and recently the function of *OsCDPK13* (*Oryza sativa CDPK 13*) has been characterized. The gene expression as well as protein accumulation of OsCDPK13 were up-regulated in response to cold and gibberellin (GA) but suppressed under salt and drought stress and also in response to ABA. The overexpressing transgenic lines of OsCDPK13 had higher recovery rates following cold stress in comparison with the vector control rice. Cold-tolerant rice varieties exhibited higher expression of *OsCDPK13* than the cold sensitive ones. Antisense OsCDPK13 transgenic lines were

shorter in comparison with the vector control lines. Moreover, dwarf mutants of rice also had lower level of OsCDPK13 than in wild type. However, there has been no mention of the sensitivity of OsCDPK13 antisense lines in response to cold stress (Abbasi *et al.,* 2004). We however expect that these antisense lines should be hypersensitive to cold stress as the gene has been shown to play an important role in mediating tolerance in response to cold stress which is evident due to higher recovery rates following cold stress than the vector control lines.

Mutagenesis study resulted in the identification of a gene, *eskimo 1* (*esk1*), which has a major effect on freezing tolerance. These plants were more freeze tolerant than the wild type plants without cold acclimation. The concentration of free proline (Xin & Browse, 1998) in the *esk1* mutant was found to be 30-fold higher than in the wild-type plants. Proline has been shown to be an effective cryoprotectant and this is also one of the major factors imparting freezing tolerance. In addition to the total sugars, which were elevated, the expression of *RAB18* cold-responsive *LEA II* gene was also found to be elevated three fold. This suggests that *esk1* may act as a negative regulator. Significantly, the *esk 1* mutation did not appear to affect the expression of *COR* genes. This suggests that multiple signaling pathways are involved in response to cold stress and they may cross talk with each other as well as with genes involved in other stresses.

Calcium is an important messenger in a low temperature signal transduction pathway. The change in cytosolic calcium levels is a necessary first step in a temperature sensing mechanism, which enables the plant to withstand future cold stress in a better way. In both *Arabidopsis* (Knight *et al.,* 1996) and alfalfa (Monroy & Dhindsa, 1995) cytoplasmic calcium levels increase rapidly in response to low temperature, largely due to an influx of calcium from extracellular stores. Through the use of pharmacological and chemical reagents, it has been demonstrated that calcium is required for the full expression of some of the cold induced genes including the *CRT/DRE* controlled *COR6* and *KIN1* genes of *Arabidopsis* (Knight *et al.,* 1996). For example, Ca^{2+} chelators such as BAPTA and Ca^{2+} channel blockers such as La^{3+} inhibited the cold-induced influx of calcium and resulted in the decreased expression of the cold inducible *Cas15* gene and blocked the ability of alfalfa to acclimate in cold. In

addition *Cas15* expression can be induced at a much higher temperature, i.e., 25°C by treating the cells with A23187, a Ca^{2+} ionophore that causes a rapid influx of calcium (Monroy & Dhindsa, 1995).

LEA stands for late embryogenesis abundant, as coined by Galau *et al.*, 1986, the discoverers of the LEA proteins in the cotton plant *Gossypium hirsutum* (Galau and Dure, 1981). As the first reports, many similar proteins and their genes or complementary DNAs (cDNAs) have been described in other plant species (Wise, 2003). Their name reflects the fact that the proteins originally described are expressed at high levels during the later stages of embryo development (post-abscission) in plant seeds. As, at this stage in the development process, orthodox seeds acquire the ability to withstand extreme dehydration, LEA proteins have been associated with desiccation tolerance (Cuming, 1999). LEA protein groups 1, 2 and 3 were identified, and members of each group were categorized by the presence of particular sequence motifs (Dure *et al.,* 1989): Group 1 proteins are characterized by a hydrophilic 20-amino-acid motif; group 2 proteins have at least two of three distinct sequence motifs, named Y, S and K by (Close, 1997); and members of group 3 contain multiple copies of an 11-amino-acid motif. A more sophisticated version of this idea has been the recent appearance of Pfam motifs for the respective LEA protein groups; each defined by a hidden Markov model based in the first instance on a curated multiple sequence alignment (Bateman *et al.,* 2004).

LEA proteins were first identified in seeds, when mRNA and proteins that occur in large quantities were investigated. LEA proteins are located in the cytoplasm, nucleus, mitochondria, vacuoles (Egerton-Warburton *et al.*, 1997), near the cellular membrane, as well as in amyloplasts (Rinne *et al.,* 1999), but primarily they accumulate in the cytoplasm and nucleus. After synthesis they are transported to cell organelles and membranes, where they stabilize cell structures and molecules. The partition of LEA proteins into various cell compartments determines their possible function in the protection or regulation of essential biochemical processes, like replication or respiration. LEA5 gene was rarely reported though it was postulated related with both

stress response and hormone response. The over-expression of this gene resulted in enhanced growth ability under salt- and drought-stress conditions.

LEA proteins have been grouped into various families on the basis of sequence similarity (Cuming, 1999). Although significant similarity has not been detected between the members of the different families, a unifying and outstanding feature of most of them is their high hydrophilicity and high content of Gly and small amino acids like Ala and Ser (Dure, 1993). Most LEA proteins are part of a more widespread group of proteins called "hydrophilins." The physicochemical characteristics that define this set of proteins are Gly content greater than 6% and a hydrophilicity index greater than 1. By database searching, it was shown that this criterion selects most LEA proteins, as well as additional proteins from different taxa (Garay-Arroyo *et al.*, 2000). The genomes of *Escherichia coli* and *Saccharomyces cerevisiae* contain five and 12 genes, respectively, encoding proteins with the characteristics of hydrophilins. The fact that the transcripts of all these genes accumulate in response to osmotic stress suggests that hydrophilins represent a widespread adaptation to water deficit (Garay-Arroyo *et al.*, 2000). Remarkably, now it is known that these proteins are distributed across archeal, eubacterial, and eukaryotic domains. Although little is known about the Group 5 (hydrophobic or atypical LEA proteins) proteins, the available data indicate that their transcripts accumulate during the late stage of seed development and in response to stress conditions, such as drought, UV light, salinity, cold, and wounding (Kim *et al.*, 2005). Cotton LEA D-34 and D-95 proteins are representatives of the group V LEA proteins (Galau *et al.*, 1993).

Ectopic expression of some plant hydrophilins (LEA proteins) in plants and yeast confers tolerance to water-deficit conditions (Zhang et al., 2000), and their presence has been associated with chilling tolerance (Nakayama *et al.*, 2007). An osmosensitive phenotype is caused by the deletion of the RMF hydrophilin gene in *E. coli* (Garay-Arroyo *et al.*, 2000) and by the absence of a LEA protein in the moss *Physcomitrella patens* (Saavedra *et al.*, 2006). The original definition of the LEA proteins involved two characteristics: gene expression at a specific stage of plant seed embryogenesis and sequence similarity to canonical LEA proteins. Hughes and Galau

(1989) proposed reserving the Lea designation for only those genes expressing post-abscission and using the term LEA A to describe genes that also have a smaller expression spike during the earlier maturation stage (associated with abscisic acid) in addition to the much larger post-abscission peak. While the proposal never caught on, induction by abscissic acid came to be one of the other expression hallmarks for LEA protein genes. Responses to desiccation stress, salt stress and cold stress have also been noted (Bray 2000; Wise 2003), and improved stress tolerance often correlates with LEA protein expression. When abscisic acid was used to activate LEA protein gene expression prematurely in immature soybean seeds, a corresponding improvement in cell integrity was noted after desiccation stress (Blackman *et al.,* 1995).

Over time, many new examples have been defined as LEA proteins on the basis of only one of the above characteristics, i.e. according to either expression pattern or sequence similarity alone. In the former case, where no sequence relatedness to previously recognised LEA proteins was evident, such proteins have led to the designation of new groups such as Lea5 or Lea14 (Galau *et al.*, 1993), or at least to a recognition that they fall outside the existing categories. Where the expression profile has proved different to that of the original prototypes, these proteins are sometimes referred to as "LEA-like" proteins. For example, dehydrin Xero1 (XERO1, ARATH; P25863) has often been included among the group 2 LEA proteins. However, neither is it found in seeds, nor is it inducible by desiccation or cold stress or by application of abscissic acid; rather, it appears to be constitutively expressed (Welin *et al.,* 1994). Similarly, Q06431 BETVE, the BP8 protein from birch, a homologue of the group 3 LEA protein LEAD8, DAUCA, is itself only constitutively expressed and at low levels (Puupponen-Pimia *et al.,* 1993).

More recently, global analyses of transcriptomes and proteomes have become technically possible. For example, in *Arabidopsis*, whose genome contains more than 50 LEA protein or LEA-like genes, expression of the whole set can be queried in different tissues and at developmental stages under a variety of conditions. The LEA protein gene set divides roughly into those with seed-specific expression and those expressed in vegetative tissues, with surprisingly little overlap. This confirms that LEA proteins also

have a role to play in vegetative tissues of plants like *Arabidopsis thaliana*, which are not desiccation tolerant. Intriguingly, at least one *Lea* gene expressed in seeds of *A. thaliana* has an orthologue that is up-regulated in desiccating leaves of the resurrection plant, *Xerophyta humilis* (Illing *et al.*, 2005). This is consistent with the hypothesis that resurrection plants acquired systemic desiccation tolerance by reprogramming seed-specific gene sets, although this presumes that LEA proteins normally expressed in seeds are more potent desiccation protectants than those expressed in non-seed tissues. LEA protein gene expression also figured prominently in the transcriptome of desiccation-tolerant bryophytes undergoing rehydration, suggesting a role for these proteins in recovery from desiccation, as well as the drying process per se (Oliver *et al.*, 2004).It was already noted that proteins from the three groups (group 6, Lea5, Lea14) have fewer polar residues and higher average hydrophobicity than sequences from the three principal LEA protein groups (Wise 2003). Furthermore, in contrast to groups 1, 2 and 3 proteins, which are largely natively unfolded, a Lea14 protein from *Arabidopsis thaliana* is structured, as determined by NMR (PDB entry 1xo8; Singh *et al.*, 2005). It seems likely that the Lea5 and group 6 proteins will also be structured. When O03983ARATH, the Lea14 sequence corresponding to 1xo8 (above), the canonical group 6 protein LE34GOSHI and the Group 3 LEA sequence LEA1APHAV are submitted to FoldIndex (Prilusky *et al.*, 2005), the first is judged to be completely folded, the second is predicted to have an unstructured N-terminal region of 39 aminoacid, but to be otherwise completely structured, while the group 3 LEA protein is found to be totally unstructured, the latter prediction fitting experimental observations (Goyal *et al.*, 2003). Recalling that LEA proteins have been recognised by comparison with a canonical set of sequences and recognising both that many other (i.e. non-LEA) proteins are expressed in plants during embryogenesis and that Lea5, Lea14 and group 6 (D34) proteins are very different from groups 1, 2, and 3 LEA proteins.

Peroxidase plays a key role in plant disease resistance, cold stress and some developmental processes, and cold-regulated protein functions necessarily in reaction of plants on cold or heat stress. Recent studies showed that these processes in plant cells were involved in programmed cell death (PCD). Peroxidases (PRXs) are

members of a large group of heme containing glycoproteins that catalyze oxidoreduction between H_2O_2 and various reductants and exist in almost all living organisms (Hiraga *et al.,* 2001). Unlike most other enzymes, PRXs may have roles in both the production and scavenging of reactive oxygen species (ROS). Plant PRXs (EC 1.11.1.7), designated class III PRXs (Duroux and Welinder 2003), are members of a large gene family. For example, the recently sequenced genomes in the dicot *Arabidopsis thaliana* (ecotype Columbia) and the monocot rice (*Oryza sativa* subsp. *japonica*) contain 73 and 138 peroxidase like genes, respectively (Passardi *et al.,* 2004), and show diverse expression profiles (Valerio *et al.*, 2004). The existence of a large group of isoforms suggests that the proteins encoded by these genes are involved in a broad spectrum of physiological processes, requiring abundant or redundant members to act efficiently during normal and stress conditions. The proposed functions of class III PRXs include lignification, suberization cross-linking of cell wall structural proteins (Fry 1986), auxin catabolism (Lagrimini *et al.*, 1997), defense against pathogen attack, salt tolerance, oxidative stress protection (Amaya *et al.*, 1999), cell elongation (Hiraga *et al.*, 2001), and ROS generation (Mittler *et al.*, 2004).

Plant PRX protein sequences are characterized by the presence of highly conserved amino acids, including two histidine residues interacting with the heme group and eight cysteine residues forming disulphide bridges (Welinder *et al.*, 2002). A variable region might reflect diversified substrate specificities and biological function of individual PRXs (Longu *et al.*, 2004). Other structures in the protein also might be important for the compartmentalization and function of the enzymes, although there is not much direct evidence to support this. For example, a short span of propeptide at N-terminus has been proposed to be necessary for sorting the protein through endoplasmic reticulum (ER) to the outside of plant cell, and a C-terminal extension (CTE) probably to be responsible for vacuolar targeting. To date plant cDNAs encoding GPX like proteins have been isolated from *Citrus sinensis* (Holland *et al.*, 1993), *Nicotiana sylvestris* (Crique *et al.,* 1992), *Spinacea oleracea* (Sugimoto *et al.,* 1997), *Lycopersicon esculentum* (Depege *et al.,* 1998), *Helianthus annus*, *Arabidopsis thaliana* (Sugimoto & Sakamoto, 1997), *Brassica campestris* (Eshdat *et al.,* 1997), *Avena fatua*

(Johnson *et al.*, 1995), *Hordeum vulgare* (Churin *et al.*,1999), *Oryza sativa* (Li *et al.*, 2000) and *Brassica napus* (Jung *et al.*, 2002)

The knowledge generated through these studies could be employed to develop plants that can tolerate various stress conditions without compromising on growth and yield characteristics. A thorough understanding of molecular mechanisms involved in stress tolerance in the particular crop is very much essential to develop strategies to evolve plants that can tolerate extreme stress conditions. As a pre-requisite to this, genes or factors contributing for stress tolerance have to be identified. Although some mechanism of stress tolerance is common to all *Hevea* clones, the degree of tolerance varies between clones. Hence in this study, attempts were made to assess the relevance of two stress specific genes (LEA 5 and peroxidase) in cold treated plants belonging to two *Hevea* clones.

MATERIALS AND METHODS

MATERIALS AND METHODS

Plant material

Leaf samples were collected from control and cold treated plants of RRII 105 and RRIM 600. Six months old poly bag plants of RRII 105 (cold susceptible) and RRIM 600 (cold tolerant) were acclimatized in a growth chamber for three days with a minimum temperature of 15° C during night (for 3 hours) and a gradual rise to maximum temperature up to 25°C in the day time. Fourth day onwards, cold treatment was imposed: 8° C for 3 hours between 2 and 5 am followed by a gradual increase of maximum temperature up to 16° C in the day time for five consecutive days mimicking natural conditions in winter. The control plants were allowed to grow at stress free and ambient weather conditions of RRII. Cold induced inhibition of photosynthesis in the low temperature treated plants when compared to the control plants was confirmed by analyzing the CO_2 exchange rate using a portable photosynthesis system

Determination of CO_2 assimilation and stomatal conductance

Net CO_2 exchange rate (A) and stomatal conductance (g_s) were measured using a portable IRGA (Infrared Gas Analyzer, Li 6400, Li- COR Inc.)

mRNA isolation

RNA extraction buffer (1X)

Component	Stock concentration	Volume/amount	Final concentration
Tris HCL	1M	5ml	50mM
$LiCl_2$	8M	1.875ml	150mM
EDTA	0.5M	1ml	5mM
SDS	20%	25ml	5%
DEPC	-	40.125	-
PVP(soluble)	-	1g	1%
ß mercaptoethanol	2%	500µl	-

Protocol for mRNA isolation (Dynabeads mRNA DIRECT™ Kit)

Sample preparation

1. An appropriate amount of ground sample was weighed in an Oakridge tube.
2. 1x RNA extraction buffer (1:10) was added, vortexed and homogenized thoroughly.
3. The sample was spun at 12,000 rpm for 30 minutes at room temperature.
4. The supernatant was transferred to a fresh tube(if there was considerable amount of debris in the transferred supernatant spun it once more in 12000 rpm for 15 minutes).
5. About 10 ml of the clear supernatant was transferred to a round bottom falcon tube (15 ml).

BEAD PREPARATION

1. The required amount of beads (200µl beads for 1g sample) was transferred into 2 ml eppendorf tube.
2. The storage buffer from the beads was removed after capturing the beads by keeping the tube in magnetic stand.
3. The beads were activated by adding 500µl buffer (RNA extraction buffer used in sample preparation).
4. The beads were transferred to the supernatant in the round bottom falcon tube.
5. Kept it for mixing in a rocking platform or a rotator for about 20 minutes to allow the mRNA (poly A tail) to anneal with the oligo-dT on the beads.
6. The beads were captured by keeping in the magnetic stand (roughly 10 minutes).
7. The supernatant was removed after the whole beads have been captured.
8. The beads were transferred into a new 2ml tube along with the last 1ml of the sample.
9. The beads were captured and removed the sample.
10. The beads were washed with wash solution A [1ml] twice and wash solution B [1ml] once.

ELUTION OF mRNA

1. Elute mRNA thrice after incubating with 100 µl of hot elution buffer (95°C) for 2 to 3 minutes and transferred the supernatant containing the m RNA after capturing the beads on a magnetic column each time.
2. The concentration was checked.

PRECIPITATION OF m RNA

1. 0.1 volume 3M sodium acetate (pH 5.2) and 2.5 volume ethanol was added into the eluted supernatant, inverted for mixing and kept in -80°C for one hour or -20°C overnight.
2. The sample was spun at 14000 rpm for 30 minutes at 4°C.
3. The supernatant was discarded and washed with 80% ethanol.
4. The sample was spun at 14000 rpm for 10 minutes at 4°C.
5. The supernatant was discarded and the pellet was dried.
6. The pellet containing the mRNA was resuspended in the required amount of nuclease free water
7. The concentration was checked spectrophotometrically.

QUANTIFICATION OF m RNA

Isolated m RNA was dissolved in DEPC treated water ,quantity and quality were determined using NanoDrop ND-1000 spectrophotometer (USA) according to the manufactures instructions .it is measured 1µl samples with high accuracy and reproducibility. A ratio of ~2.0 was generally accepted as " pure" for RNA.

cDNA SYNTHESIS

cDNA synthesis was done using superscript III First strand c DNA synthesis kit (Invitrogen, USA).

PROTOCOL FOR c DNA SYNTHESIS

1. Combine the following in a 0.5 ml tube.
    a. m RNA (250ng) - 5µl
    b. Primer (oligo dT) - 1 µl
    c. 10 m M d NTP mix - 1 µl
    d. DEPC treated water - 3µl
2. Incubate at 65°C for 5 min., then place on ice for at least 1 min.
3. A cDNA synthesis mix is prepared using the following components

    10X RT buffer - 2µl
    25mM $MgCl_2$ - 4µl
    0.1 M DTT - 2µl
    RnaseOUT (40U/µl) - 1µl
    Superscript IIIRT(200U/µl)- 1µl
4. Aliquot 10µl of cDNA Synthesis Mix to each RNA mix and spin.
5. Incubate at 50°C for 50 minutes (oligo dT).
6. Terminate the reactions at 85°C for 5 min. chill on ice.
7. Collect the reactions by brief centrifugation. Add 1µl of RNase H to each tube and incubate for 20 min. at 37°C.
8. cDNA was stored at -20°C or used for PCR immediately.

REAL TIME PCR

Quantitative PCR (qPCR) was performed by using real-time PCR (Applied Biosystems, USA, and Model No. 7500). GADPH was used as endogenous control in the relative quantification studies. The primers were designed (determined on the 3' regions of the genes with an amplicon size of 75-150 bp) using the Primer Express software (Version 3.0) and synthesized by Ocimum Biosolutions, Hyderabad.

Gene	Forward primer	Reverse primer
LEA5	CTCGCTTTCCCTCCAATG	TTCCTCACCATACCACTCC
PEROXIDASE	AATTGGCACGAATTTCCC	CACGGATAAGAGAACAAGG
GADPH	GCCTGTGATAGTCTTCGGTGTTAG	GCAGCCTTATCCTTGTCAGTGAAC

NORMAL PCR

Normal PCR was carried out to check the amplifiability of the specific gene primers. The amplified products were run in 2% agarose gel and the bands were visualized in UV transilluminator.

PCR thermal profile

Step1	Initial denaturation	94°C	3minutes
Step2	Denaturation Annealing Extension	94°C 55°C 72°C	30sec 30sec 1min30sec
Step3	Repeat the step 2	36 cycles	
Step4	Final elongation	72°C	10 minutes

Reaction mixture

PCR REAGENTS	QUANTITY
PCR buffer(10X)	1.5µl
10mM dNTP	0.375µl
Taq DNA polymerase	0.15µl
Template(100ng)	1µl
Forward primer	1µl
Reverse primer	1µl
Water	9.975µl

The real time PCR reaction was then standardized for

- Template concentration
- Primer concentration
- Primer specificity and primer efficiency

Standardization of template concentration

- Serially diluted template concentrations were used for standardization
- Template concentration for relative quantification (RQ) analysis was decided based on C_T value in the optimum range.

Standardization of primer concentration

- Various combinations of forward and reverse primer in the range of 50/50, 50/25, 25/50, 25/25, 12.5/12.5, 6.25/6.25nM were attempted to find out the optimum primer concentration.
- Primer combinations that did not produce a fluorescence peak in non template control (NTC) were selected for further use in qPCR.

Primer efficiency

Efficiency for each primer without peak in non template control was checked by calculating slope using 1:10 serially diluted template concentrations. Primers having slope values between -3.2 and -3.5 are suitable for selection. Efficiency was calculated using the equation

Efficiency= $10^{(-1/slope)}$-1

qRT PCR Mix (20µl)

Template (50ng/µl) -1µl
Forward primer (6.25nM) -1µl
Reverse primer (6.25nM) -1µl
Sterile water -7µl
SYBR Green master mix (2X)-10µl

The Power SYBR Green PCR Master Mix is supplied in a 2X concentration and it contains:

- SYBR Green I Dye
- AmpliTaq Gold DNA polymerase
- dNTPs
- Passive reference
- Optimized buffer components

RT-PCR was performed in a 20µl reaction mixture containing 1µl of 1/20 dilution of first –strand cDNA reaction, 6.5nM each of forward and reverse primer and 10µl Power SYBR Green PCR Master Mix (Applied Biosystems, USA) on an Applied Biosystems 7500 Real Time PCR System. The cycling conditions comprised 10 min polymerase activation at 95°C and 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. This was followed by a melt curve analysis (95°C for 15 seconds, 60 °C for one minute, 95°C for 30 seconds and 60 °C for 15 seconds). Each experiment was repeated two to three times, and each PCR reaction was performed in triplicate with null-template controls. Primers with slope values between -3.2 and -3.5 only were employed for these reactions. Relative quantification of the genes of interest was calculated by comparative C_T method ($2^{-\Delta\Delta C}{}_T$, C_T- Threshold cycle, ΔC_T (C_T Target - C_T Endo).The relative quantification (RQ) values were analyzed using the software of Applied Biosystems (SDS 7500 ,v.2.0.3) and were exported in excel sheet to study the fold change in the expression levels between the treatments.

INSTRUMENTS:

Following instruments were used for the present study.

- Autoclave : Equitron, Medical Instrument MGF. Co. Mumbai
- Bio Imaging System : Syngene,UK
- Biomedical Freezer(-20°C) : Sanyo, Electric Biomedical Co., Ltd, Japan

- Centrifuge : Biofuge Stratos, Heraeus
- Centrifuge : Eppendorf, Hamburg, Germany.
- Centrivap DNA Concentrator : Labconco corporation,USA.
- Electronic Balance (max-220g) : Sartorius
- Freezer and Refrigerator : CoolTechBio,Samsung.
- Hot Air Oven : Kemi
- Ice machine : Icematic F125 Compact
- Laminar Air Flow : Labline Instruments, Kochi.
- Magnetic Stirrer : Remi Equipments
- Microcentrifuge : Eppendorf,Germany Spinwin
- Microwave Oven : Kenstar
- Spectrophotometer : NanoDrop, U.S.A.
- PCR Insrument : Eppendorf (Model : Mastercycler gradient)
- Pharmaceutical Refrigerator (4 °C) : Sanyo, Electric Co., Ltd, Japan.
- Powerpack 100(Electrophoresis Unit) : Biorad
- Real time PCR : Applied biosystems, 7500, SDS Software,v.2.0.3
- Thermomixer : Eppendorf AG ,Germany
- Ultra Low temperature Freezer(-80°C) : Sanyo Electric Biomedical Co., Ltd, Japan.
- UV Illuminator : Uvitec
- Vortex mixer : Gene 2, scientific Industries, Inc.USA

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

There are many reports to prove that repeated and prolonged exposure to abiotic stresses may make the repair mechanisms inadequate resulting in irreversible damage to the photosynthetic machinery leading to senescence and thus affecting the dry matter production (Long *et al.*, 1994). The crop growth rate of *Hevea* plants is lesser during winter than the stress-free post monsoon season in Agartala. It is known that the inadequacy of biochemical mechanisms to scavenge the reactive oxygen species such as superoxide, hydroxyl radical etc. which are produced during photochemical reactions in the thylakoid causes serious damage in plants experiencing abiotic stresses.

In this study, both the cold susceptible (RRII 105) and tolerant (RRIM 600) plants were exposed to a minimum temperature of 8° C for 3 hours between 2 and 5 am followed by a gradual increase of maximum temperature up to 16° C with a light range of 400-800 μ mol m^{-2} s^{-1} in the day time for five consecutive days. The plants started showing photobleaching in the leaves from fifth day onwards. Among the control plants, RRIM 600 exhibited slightly better (12.1 μ mol m^{-2} s^{-1}) photosynthetic rate (P_N) than RRII 105 (10.7 μ mol m^{-2} s^{-1}) (Fig. 1). Stomatal conductance (g_s) was almost same (0.1 mol m^{-2} s^{-1}) in both the clones (Fig. 2). After the imposition of cold condition in the growth chamber, the P_N reduced to almost 98 and 90 % in RRIM 600 and RRII 105 respectively. There was a complete reduction of stomatal conductance in both the clones after cold treatment.

The leaf samples collected from these plants were frozen in liquid nitrogen and were stored in minus eighty freezer. mRNA was isolated from these samples as mentioned in the materials and methods, quantified and its purity was checked using a minispectrophotometer (Nanodrop, USA). After confirming its quality and quantity, these samples were used for cDNA synthesis. The cDNA synthesized were used as template after proper dilution for PCR and qPCR analysis. PCR amplification was performed using specific primers designed for both LEA5 and peroxidase using standard protocols to confirm the PCR amplifiability of designed primers (Fig. 3). The primers were checked for their specificity by performing a melting curve analysis in a

Real Time PCR machine. For this purpose, both the forward and reverse primers alone were run with SYBR green dye (without template DNA) to see if they produce any signal that might interfere with the signals produced by regular amplification in the presence of template DNA. The melting curve analysis indicated that both the primers did not produce any signal under non template control run.

Further the PCR efficiency of these primers (both target and endogenous control genes) was evaluated. For this purpose, serial dilutions with different concentrations of cDNA (0.01 ng to 100 ng) were made and used for qPCR to workout the slope value (Fig. 4 and Fig. 5). The PCR efficiency was calculated using the formula, Efficiency=$10^{(-1/slope)}$-1 and primers with a slope value between -3.2 and -3.5 were employed for the downstream reactions. In this attempt, both the primers employed yielded a slope value that is closer to 3.5 and hence were included for the analysis. The GAPDH gene selected as endogenous control gene showed a slope of -3.2.

The qPCR data of both the genes attempted is given in table 1 and 2 and figure 6 and 7. In the case of LEA 5 protein, the data given in Table 1 indicates that it is not much altered in RRII 105 plants when subjected to low temperature stress. Whereas in clone RRIM 600, the levels of LEA 5 protein was about six and sixty times higher in the control and treated plants respectively. Such higher level of LEA 5 protein in a tolerant clone like RRIM 600 when compared to its reduction in a susceptible clone like RRII 105 indicates that LEA 5 protein is in fact cold stress responsive.

In the case of peroxidase, when the data was analyzed by keeping RRII 105 control plants as calibrator (Table 1), about 20 and 160 fold increase in peroxidase expression was found in control and cold treated plants of RRIM 600 when compared to RRII 105 control plants which showed an increase of about 10 fold under low temperature conditions. Higher levels of peroxidase in RRIM 600 plants also indicate its association with stress tolerance. The comparatively higher levels of peroxidase in RRIM 600 also suggest the existence of increased levels of reactive oxygen species under low temperature conditions in *Hevea* and the higher levels of free radical scavenging mechanisms being triggered in a tolerant clone like RRIM 600.

Figure 1: CO_2 assimilation rate (A) of clone RRIM 600 and RRII 105 under control and low temperature conditions.

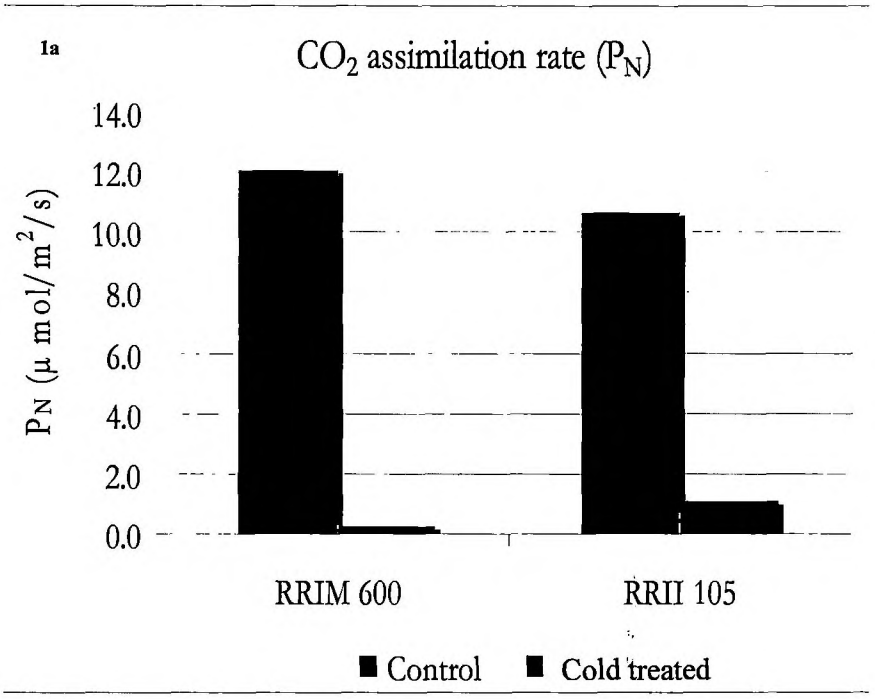


Figure 2: Stomatal Conductance (g_s) of clone RRIM 600 and RRII 105 under control and low temperature conditions.

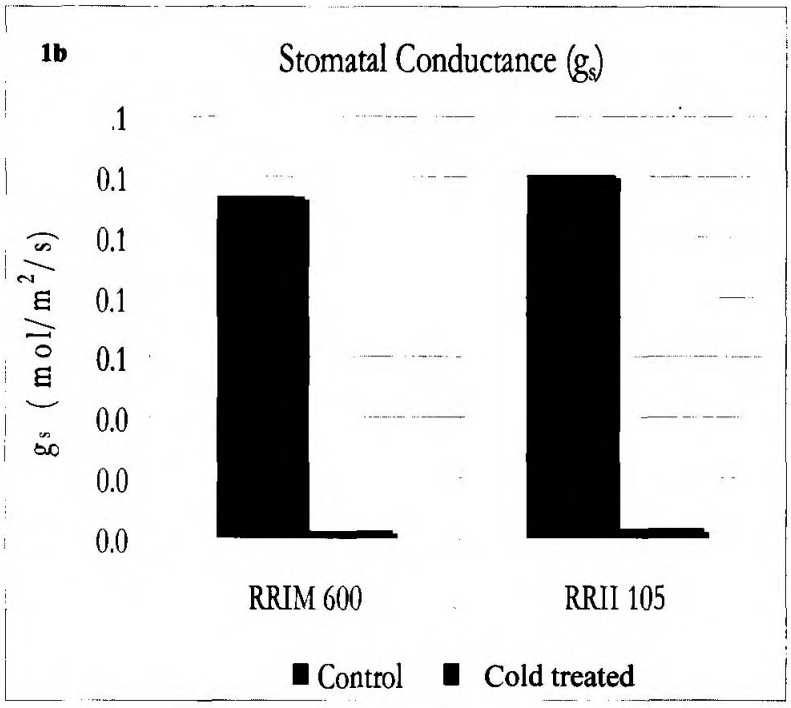
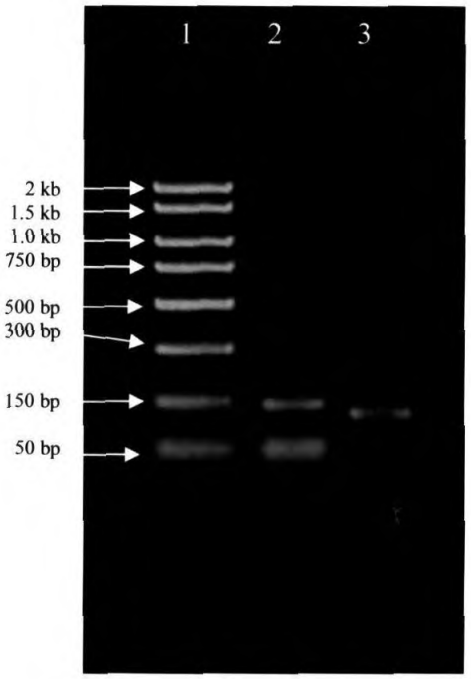


Figure 3: Agarose gel photograph of PCR amplified products of peroxidase and LEA 5 protein



1 – DNA Marker
2 - Peroxidase
3 – LEA 5 Protein

Figure 4: Primer efficiency analysis for the primers of LEA 5 gene

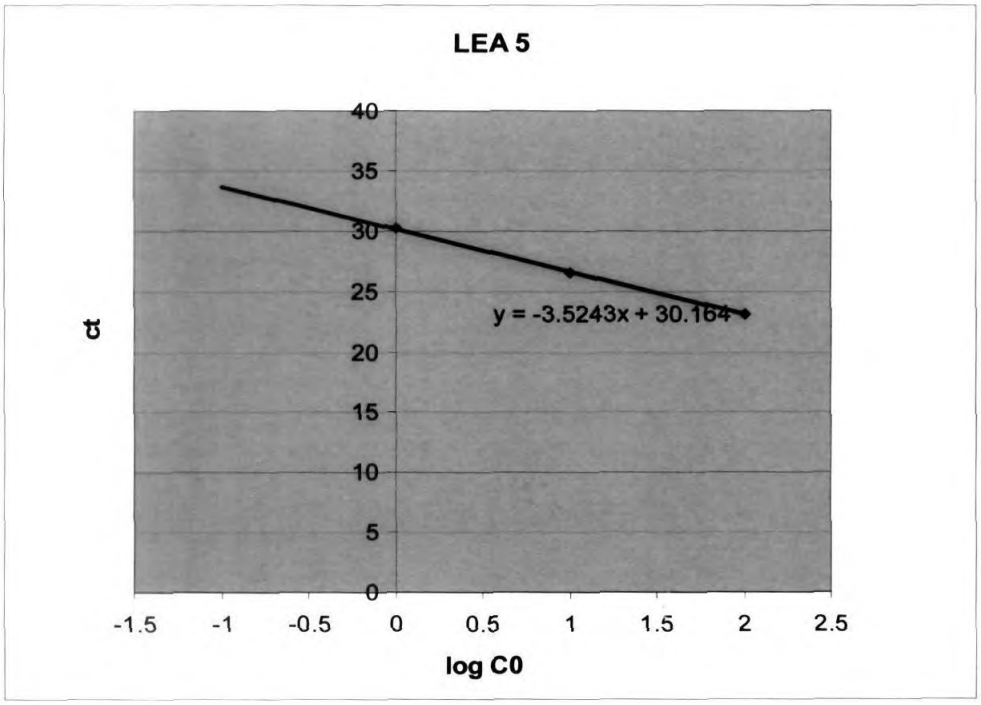


Figure 5: Primer efficiency analysis for the primers of Peroxidase gene

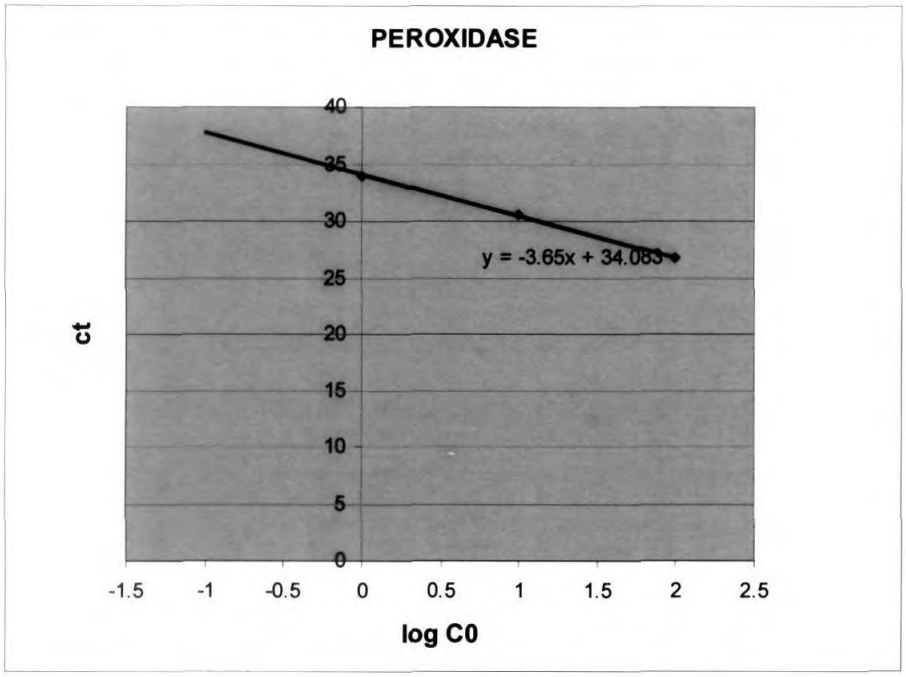


Figure 6: Primer efficiency analysis for primers of endogenous control gene, GAPDH

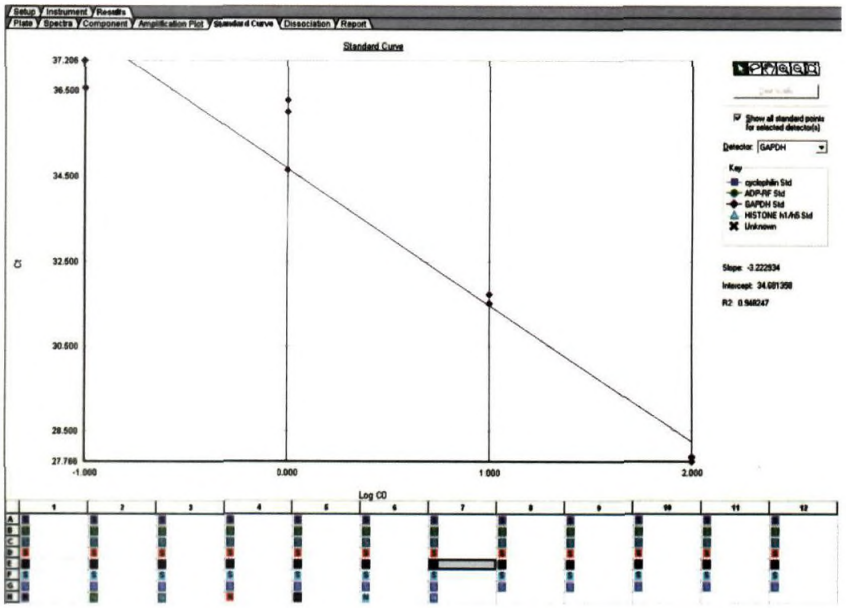


Table1. Relative gene expression (fold change) of LEA 5 and Peroxidase in low temperature treated plants of RRII 105 and RRIM 600 plants (LT) using RRII 105 control (C) as calibrator.

Gene	105 Control	105 LT	600 Control	600 LT
LEA5	1	1.48	5.98	58.9
PEROXIDASE	1	9.48	19.87	158.3

Table2. Relative gene expression (fold change) of LEA 5 and Peroxidase in low temperature treated plants (LT) of RRII 105 and RRIM 600 with their respective control as calibrator

Gene	105 Control	105 LT	600 Control	600 LT
LEA5	1	1.48	1	9.85
PEROXIDASE	1	9.48	1	7.96

Figure 7: Quantitative Expression analysis of LEA 5 protein in clones RRII 105 and RRIM 600 under cold stress. Data of RRII 105 control plants was used as calibrator

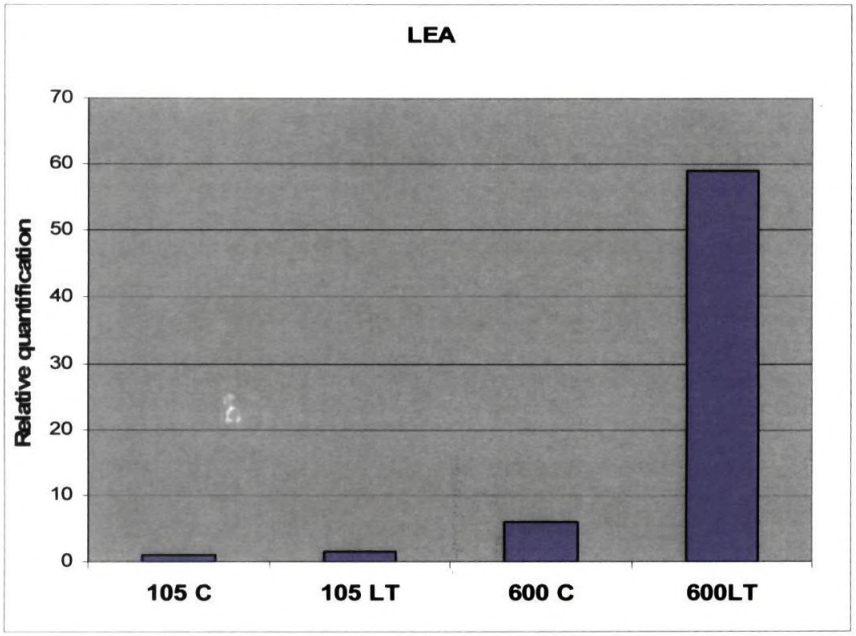
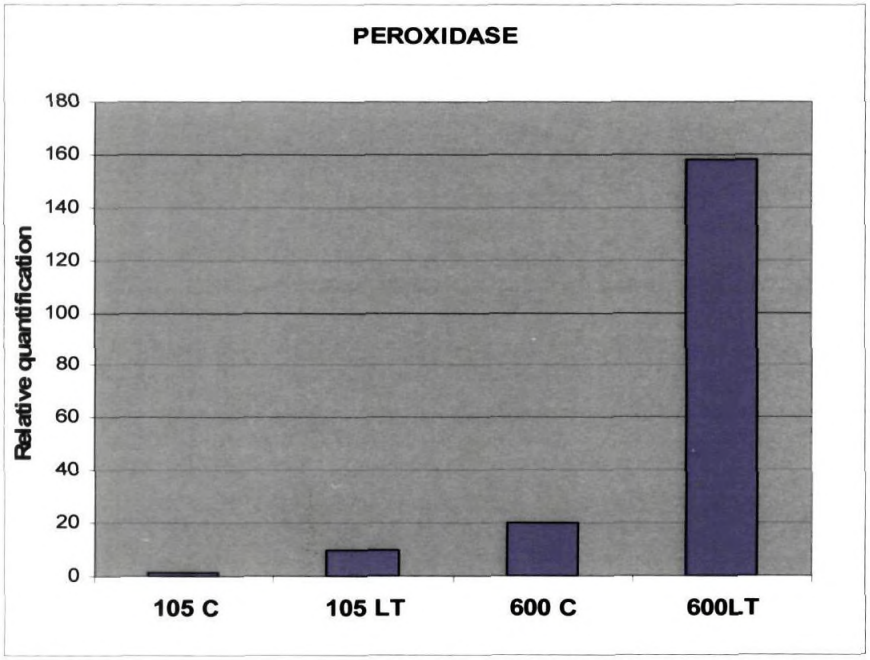


Figure 8: Quantitative Expression analysis of Peroxidase in clones RRII 105 and RRIM 600 under cold stress. Data of RRII 105 control plants was used as calibrator.



The reports from other plants also indicate similar results. Under low temperature conditions, levels of SOD and ascorbate peroxidase activity increases in tomato cultivars after chilling, but particularly in those with a lower level of chilling tolerance The increase in the levels of ROS scavenging enzymes in response to freezing or chilling has been studied in several laboratories (Amaya *et al.,* 1999). A higher level of peroxidase activity was observed in chilling stressed cucumber plants, in parallel to an increase in the levels of H_2O_2 (Li *et al.,* 2000). Ascorbate peroxidase activity has been shown to increase in response to a number of stress conditions, such as drought, air pollution, high light intensity combined with chilling or deficiency in microelements iron stress, excessive light, UV-B light and salt stress. In some cases post-translational components are involved in the regulation of the APX activity (Passardi *et al.,* 2004). However, increases in APX activity are usually accompanied by transcriptional activation of the gene. The expression of APX gene in rice is mediated by high temperature, furthermore, rice seedlings previously subjected to increased temperature displayed increased level of tolerance to chilling stress (Longu *et al.,* 2004).

LEA-type proteins have been reported to be expressed in response to water deficit resulting from desiccation, cold and osmotic stress in a wide range of plant species. Such proteins may preserve protein structure and membrane integrity by binding water, preventing protein denaturation or renaturing unfolded proteins, and sequestering ions in stressed tissues (Mayra Rodriguez, 2005). Late embryogenesis abundant (LEA) protein of HVA1 from barley has been shown to be effective in increasing cold tolerance when introduced to rice plants (Xu *et al.,* 1996). *In vitro* and *in vivo* experiments showed that LEA proteins play important role in normal seed development and plant's response to environmental stress conditions, such as dehydration, salinity, osmosis and low temperature (Battaglia *et al.* 2008; Bies-Ethève *et al.* 2008; Hundertmark and Hincha 2008; Shimizu *et al.* 2010).

Most of the LEA V proteins are acidic and are distinctively different from the other four groups of LEA proteins. They contain a high proportion of hydrophobic residues and lack the feature of boiling solubility. LEA I, II, III, and IV proteins are hydrophilic,

whereas LEA V are hydrophobic (Tunacliffe & Wise, 2007). ABA and oxidant induced *Arabidopsis* LEA V gene, AtLEA6-1 (previously called Sag21 or AtLEA5), when introduced into *Arabidopsis* increased the root growth and shoot biomass, both in optimal condition and under H_2O_2 stress (Shih *et al.*, 2008).

It can be concluded from this report that under low temperature conditions, production of free radicals increases and the system is struggling hard enough to get rid of these radicals by up-regulation of production of free radical scavenging enzymes. Proteins like LEA5 protein and peroxidase are also expressed in higher levels indicating its role in stress tolerance. Increased levels of these two genes (proteins) in tolerant clone like RRIM 600 indicate its association with cold stress tolerance in *Hevea*. Probably this kind of study has to be extended to more number of genes. The recent innovative techniques like microarray, transcriptome sequencing and real time PCR techniques can be employed to identify the exact candidate genes that are specifically up-regulated in a tolerant clone and not being over expressed in a susceptible clone. Such genes can be identified and selected genes can be further validated for selection of the suitable candidate gene for stress tolerance. It could be a triggering factor or regulatory element that is up-regulated in a tolerant clone. So, with the advent of new generation technologies, we should be able to identify the genes that are contributing for stress tolerance.

Among the two genes studied, it has been identified that both are cold stress responsive. But, only more studies (validation of its expression) in other clones that display cold tolerance in field conditions. As a preliminary level study, this investigation indicates that both are cold responsive and LEA 5 protein is highly expressed in a tolerant clone like RRIM 600.

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

- The relative quantification PCR study (using GAPDH as endogenous control) was done for two genes (LEA 5 protein and peroxidase) in two *Hevea* clones with varying levels of cold tolerance (susceptible clone RRII 105 and tolerant clone RRIM 600).
- The results indicated the up-regulation of LEA 5 and peroxidase genes under cold stress in *Hevea* and especially in tolerant clone RRIM 600.
- Relatively higher levels of both LEA 5 and peroxidase genes in tolerant clone (RRIM 600) indicate its association with cold stress tolerance.
- Extension of these studies to other cold tolerant clones and inclusion of more stress related genes would provide better knowledge about role of these genes towards stress tolerance in *Hevea*.

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