ISOLATION AND CHARACTERIZATION OF WATER STRESS ACTIVATED PROTEIN KINASE GENE FROM BLACK PEPPER *Piper nigrum* L. VAR. KALLUVALLY

By

BANKAR ASHOK DNYANDEO
(2009-11-101)

THESIS

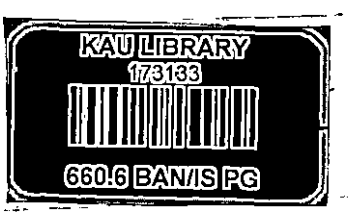
Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture

Kerala Agricultural University, Thrissur



CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY

COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR - 680 656

KERALA, INDIA

2011

DECLARATION

I, hereby declare that this thesis entitled **"Isolation and characterization of water stress activated protein kinase gene from black pepper (*Piper nigrum* L.) var. Kalluvally"** is a bonafide record of research work done by me during the course of research and that it has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title of any other University or Society.

Vellanikkara
Date: 15/ 8 /2011

Bankar Ashok Dnyandeo
(2009-11-101)

CERTIFICATE

Certified that this thesis entitled **"Isolation and characterization of water stress activated protein kinase gene from black pepper (*Piper nigrum* L.) var. Kalluvally"** is a bonafide record of research work done independently by **Mr. Bankar Ashok Dnyandeo** under my guidance and supervision and that it has not formed the basis for the award of any degree, diploma, fellowship or associateship to him.

Vellanikkara
15/8/11

Dr. P. A. Nazeem
(Major Advisor, Advisory Committee)
Professor and Head
Centre for Plant Biotechnology and Molecular Biology
College of Horticulture
Vellanikkara, Thrissur

CERTIFICATE

We, the undersigned members of the advisory committee of **Mr. Bankar Ashok Dnyandeo** a candidate for the degree of **Master of Science in Agriculture,** with major field in **Plant Biotechnology**, agree that the thesis entitled **"Isolation and characterization of water stress activated protein kinase gene from black pepper (*Piper nigrum* L.) var. Kalluvally"** may be submitted by **Mr. Bankar Ashok Dnyandeo** in partial fulfilment of the requirement for the degree.

Dr. P. A. Nazeem
(Major Advisor, Advisory Committee)
Professor and Head
Centre for Plant Biotechnology and Molecular Biology
College of Horticulture
Vellanikkara, Thrissur

Dr. D. Girija
(Member, Advisory committee)
Professor and Head
Department of Agricultural Microbiology
College of Horticulture
Vellanikkara, Thrissur.

Dr. R. Keshavachandran
(Member, Advisory committee)
Professor
Centre for Plant Biotechnology and Molecular Biology
College of Horticulture
Vellanikkara, Thrissur.

Dr. K. Nandini
(Member, Advisory committee)
Professor
Department of Plant Physiology
College of Horticulture
Vellanikkara, Thrissur.

External Examiner
Dr. George Thomas
Scientist C.
Dept. of Plant Molecular Biology
RGCB, Thycaud. P.O.
Tvm - 695014

ACKNOWLEDGEMENT

Words cannot express my deep sense of gratitude and indebtdness to ***Dr. P.A. Nazeem****, Professor and Head, CPBMB and chairperson of my Advisory Committee. I wish to place my heartfelt thanks to her for her inspiring guidance, untiring help, patience, encouragement, constructive criticism and valuable suggestions during the period of the investigation and preparation of the thesis. My sincere and heartfelt gratitude ever remains with her.*

I am ever grateful to ***Dr. D. Girija****, Professor and Head, Department of Agricultural Microbiology and member of my advisory committee for her invaluable help, guidance and critical assessment throughout the period of the work. I thank her for all the help and cooperation she has extended to me.*

I express my sincere gratitude to ***Dr. R. Keshavachandran,*** *Professor, CPBMB and member of my advisory committee for his valuable suggestions and guidance rendered me for the completion of the research programme and preparation of the thesis.*

I am deeply obliged to ***Dr. K. Nandini****, Professor and Head, Department of Plant Physiology and member of my advisory committee for her unfailing support and enthusiasm, relevant suggestions and whole hearted cooperation throughout the period of investigation.*

I also avail this opportunity to pay my sincere obligations and heartfelt thanks to ***Dr. Sujatha, Dr.Valsala, Dr. Shylaja, Dr. Abida and Dr. Deepu*** *of CPBMB for their encouragement and kind help offered at different stages of the study.*

I wish to express my sincere thanks to all the non-teaching staff members and labourers of CPBMB for their whole hearted cooperation and timely assistance.

I express my deep sense of gratitude to ***Sherin, Anju, Surya, Shidi, Deepa, Priya, Sukanya, Nithin, Harish, Saju, Varun, Shrinath, Nitha, Shailaja, Simi and others*** *of CPBMB, who helped me in several ways for the completion of this venture.*

I have no words to express my deep sense of gratitude to all my senior friends ***Kiran, Shivaji, Sunil, Gajanan, Jusna, Sudha, Prathmesh, Arya, Alex and Rajesh*** *for their valuable assistance and guidance extended throughout the investigation.*

Words fall short as I place on record my indebtedness to my class mates and friends, ***Rakesh, Achintya, Manjunath, Pradeep, Sarveshwar, Somya, Lubina, Sahna, Renisha, Vishnuda, Adarsha, Kannan, Danish and Elavarsan,*** *for their prompt help and cooperation for the entire period of study.*

I am in dearth of words to thank my friends, ***Raju, Shekhar, Sachin, Yogesh, Onkar, Abhijeet, Sopan, Nilesh*** *and my juniors* ***Amar, Ashok, Meera, Renju, Reshmi, Pramod, Pawan, Narendra, Rohit, Parimal, Ravindra, Sharada and others*** *for their encouragement and unliving support.*

With gratitude and affection, I recall the boundless affection, constant encouragement, warm blessings and motivatuion from my parents and advisor Dr. P. A. Nazeem without which this endeavour would never have become a reality.

Ashok D. Bankar

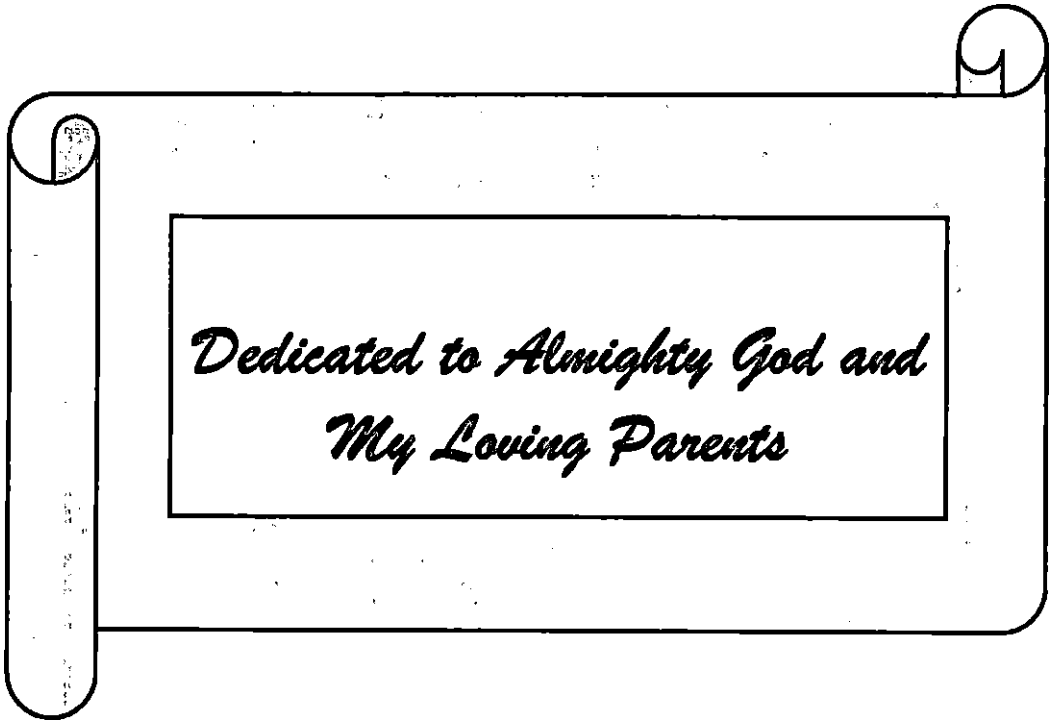

Dedicated to Almighty God and
My Loving Parents

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDIX

ABBREVIATIONS

A	Adenine
ABA	Abscisic Acid
A-PCR	Anchored-Polymerase Chain Reaction
APK	Arabidopsis Protein Kinase
ATPase	Adenine Triphosphatase
β	Beta
BLAST	Basic Local Alignment Search Tool
C	Cytosine
°C	degree Celsius
cm	Centimeter
CDD	Conserved Domain Database
CDK	Calmodulin Dependent Kinase
CDPK	Calcium Dependent Protein Kinase
cDNA	Complementary DNA
CDS	Protein coding sequence
cds	Complete cDNA sequence
CPBMB	Centre for Plant Biotechnology and Molecular Biology
DAG	Diacylglycerol
DEPC	Diethyl pyro-carbonate
DIC	Distributed Information Centre
DMSO	Dimethyl sulfoxide
DNA	Deoxy Nucleotide Triphosphate
DREB	Dehydration Responsive Element Binding
ds	Double stranded
E. coli	*Escherichia coli*
EDTA	Ethylene Diamine Tetra Acetic acid
ESTs	Expressed Sequence Tags
G	Guanine
g	Gram

HSP	Heat Shock Protein
H_2O_2	Hydrogen peroxide
I-PCR	Inverse-Polymerase Chain Reaction
IPTG	Isopropyl thio galactoside
KAU	Kerala Agricultural University
kb	kilo base
kDa	kilo Dalton
LB	Luria Bertani
LEA	Late Embryogenesis Abundant
M	Mole
MAPKKK	Mitogen Activated Protein Kinase Kinase Kinase
MDa	Mega dalton
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimole
M-MuLV RT	Murine-Molodeny Lukemia Virus Reverse Transcriptase
MOPS	(2-(N-morpholino) ethanesulfonic acid
mRNA	Messenger RNA
MSL	Mean Sea Level
µg	Microgram
µl	Microlitre
µM	Micromole
NAD	Nicotinamide Adenine Diphosphate
NCBI	National Centre for Biotechnology Information
ng	Nanogram
nm	Nanometer
OD	Optical Density
ORF	Open reading frame
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration

PNK	*Piper nigrum* Kalluvally
%	Percentage
RACE	Rapid Amplification of cDNA Ends
RNA	Ribo Nucleic Acid
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	Rotations per minute
RT- PCR	Reverse Transcriptase Polymerase Chain Reaction
SDS	Sodum Dodecyl Sulphate
sec	Second
SnRK	Sucrose non-fermenting 1 Related Kinase
SSH	Suppression Subtractive Hybridization
T	Thymine
TAE	Tris Acetate EDTA
TE	Tris EDTA
U	Unit
UTR	Untranslated Region
UV	Ultra violet
V	Volts
v/v	Volume by volume
w/v	Weight by volume
X-gal	5- bromo 4 chloro 3- indolyl β-D galactosidase

Introduction

1. INTRODUCTION

The beginning of the 21 century is marked by global shortage of water resources, environmental pollution and increased level of salts in soil and water. Abiotic stress is already a major limiting factor in plant growth and will soon become even more severe as desertification covers more and more of the world's terrestrial area (Vinocur and Altman, 2005).

Drought is a serious problem that affects many regions of world, decreasing the rate of photosynthesis and limiting the productivity of crops worldwide, reducing yield up to 50 per cent (Bray *et al.*, 2000). Drought means water loss and dehydration at normal or even elevated temperatures. Shrinking of cells leads to loss of turgor, osmotic stress and a potential change of membrane potentials. Severe water loss from the cells results in membrane disintegration and abolition of metabolic processes (Mahajan and Tuteja, 2006).

Black pepper is the most important spice of the world and is commonly known as the 'black gold'. It therefore is rightly called the 'King of Spices', as the volume of international trade of it is the highest among all the spices known.

Ninety percent of the area under black pepper is rainfed. These areas frequently experience severe water deficit due to uncertain and uneven rainfall distribution pattern and the occurrence of drought in such areas may devastate the plantations of black pepper completely. Seasonal water stress from December to May is regarded as one of the major constraints in increasing the productivity of black pepper (Vasantha *et al.*, 1989).

Genotypic variations for drought tolerance has been reported in black pepper and the variety Kalluvally is one among the six genotypes identified as drought tolerant (Thankamani *et al.*, 2003) based on physiological and biochemical parameters. Unraveling the mechanisms involved in drought tolerance in the resistant genotypes, could help the molecular breeding of economical crops in a long way.

The cellular and molecular responses of plants to environmental stress has been investigated mainly using *Arabidopsis thaliana* as a model plant (Ingram and Bartels, 1996; Shinozaki and Yamaguchi- Shinozaki, 1997; Zhu, 1997, 2002). These signalling pathways can be divided into ABA-dependent pathways and ABA-independent pathways. Gene products that are activated by these pathways can be classified as functional proteins and regulatory proteins (Shinozaki and Yamaguchi, 1997, 2007). Various genes are involved in water stress response in plants. These genes encode two groups of proteins.

The first group includes proteins that probably function in stress tolerance, such as the enzymes required for osmolyte biosynthesis, chaperones, LEA proteins, mRNA binding proteins, water channel proteins, sugar and proline transporters, detoxification enzymes and various proteases (Ingram and Bartels, 1996; Seki *et al.*, 2002).

The second group includes protein factors such as protein kinases, transcription factors and enzymes involved in the regulation of signal transduction and gene expression that probably function in stress response and phospholipid metabolism (Shinozaki and Yamaguchi-Shinozaki, 1997). The existence of a variety of water-inducible genes suggest that the responses of plants to water stress are rather complex. Regulatory proteins include transcription factors, protein kinases and phosphatases. These are involved in the further regulation of signal transduction and gene expression in stress tolerance (Shivmani *et al.*, 2000). Zhu (2002) and Xiong *et al.* (2002) have reviewed the role of signal transduction in the regulatory network of water stress.

It is important to identify drought resistance genes in black pepper and investigate their role in drought stressed and irrigated conditions. Through this strategy, we may be able to understand the molecular mechanism of differential gene expression under drought conditions. The differential genes expressed during water stress condition in black pepper var. Kalluvally have been characterised by subtractive PCR (Kushwah, 2008).

Several plant protein kinases were found to be activated by osmotic stress (Zhu, 2002). In present study, several putative protein kinases which were among the stress up-regulated transcripts identified in black pepper used for the enrichment.

Protein kinase can modify many proteins, including enzymes, through phosphorylation on serine, threonine, or tyrosine residues (Schenk and Snaar-Jagalska, 1999). Changes of proteins in their phosphorylation state can alter their properties and activities and then trigger a cascade of reactions.

The present study was undertaken to enrich protein kinase gene sequence information from drought tolerant genotype of black pepper (*Piper nigrum* L.) var. Kalluvally by using Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR) and Rapid Amplification of cDNA Ends (RACE) techniques.

We hope that after enrichment, the structure and function of these genes involved in water stress regulation could be studied thoroughly and which will contribute to bring us closer to the understanding of the molecular mechanism of water stress tolerance in black pepper.

Review of literature

2. REVIEW OF LITERATURE

Black pepper (*Piper nigrum* L.) the king of spices is one of the important commodities of commerce and trade in India since pre-historic period. It originated in the tropical evergreen forests of the Western Ghats of India. India is the largest producer of black pepper, growing in about 2.15 lakh hectares. In 2008 the export of spices from India has been 525,750 tonnes valued Rs.6840.71 crores (US $ 1502.85 Million) (Spice Board, 2008). Productivity of black pepper in the country is very low (less than 500 gm) as compared to other producing countries (Madan, 2007). One of the main reasons for the low productivity is losses caused due to biotic and abiotic stresses.

Black pepper grows successfully between 20° North and 20° South of equator and from sea level up to 1500 m above MSL. It is a plant of humid tropics, requiring 2000-3000 mm of rainfall, tropical temperature and high relative humidity with little variation in day length throughout the year. Black pepper does not tolerate excessive heat and dryness and in India, the growing areas receive 1500 mm to more than 4000 mm rainfall. Rainfall after stress induces profuse flowering in the crop (Pillay *et al.,* 1988). Growth of fruit bearing lateral shoots and photosynthetic rate are maximum during peak monsoon (June to July) in India (Mathai, 1983). A relative humidity of 60 to 95 per cent is optimum at various stages of growth. The crop tolerates temperature between 10 and 40°C, the ideal being 23 to 32°C with an average of 28°C. Optimum soil temperature for root growth is 26 to 28°C (Wahid and Sitepu, 1987).

The economic costs of drought can be enormous. It has been historically associated with food shortages of varying intensities, including those that have resulted in major famines in different parts of Asia and Africa. In India, major droughts in 1918, 1957 to 1958 and 1965 resulted in famines during the 20th century. The 1987 drought affected almost 60 per cent of the total cropped area and 285 million people across India. Similarly, the average annual drought-affected area in China during 1978 to 2003 was estimated to have been 14 million

ha and the direct economic cost of drought is estimated to have been 0.5 to 3.3 per cent of the agricultural sector gross domestic product. In Thailand, the drought of 2004 was estimated to have affected 2 million ha of cropped area and over 8 million people.

Plants do have to deal with various environmental stresses during their life span. Drought, salinity and cold are major environmental stresses that severely reduce agricultural productivity worldwide. A common element in response to many environmental stresses is cellular dehydration. A lack of soil moisture, low relative humidity, water loss from wounding, elevated temperatures, and reduced xylem conductivity caused by pathogens are among the conditions and treatments that can affect tissue water potential in plants and cause water deficiency symptoms (Shinozaki and Yamaguchi-Shinozaki, 1996; Bray, 1997). Plants have evolved many mechanisms to defend themselves against stresses.

To cope up with water deficit, plants have developed various mechanisms to protect cellular activities and maintain whole plant integrity. Many stress-induced genes have been identified, including those encoding fundamental enzymes of abscisic acid (ABA) biosynthesis (Bray, 1997), proteins involved in osmotic adaptation, tolerance of cellular dehydration (Shinozaki and Yamaguchi, 1997), cellular protective enzymes (Ingram and Bartels, 1996), a range of signalling proteins such as transcription factors (Soderman *et al.,* 1996) and protein kinases/protein phosphatases (Hong *et al.*, 1997).

The molecular aspects of drought tolerance and the relevance of the protein kinase gene has been dealt in this chapter under the following heads.

1.1 Physiology of plants under drought

1.2 Gene network involved in drought tolerance

1.3 Systematic analysis of kinase superfamily in plants

1.4 Strategies to amplify the cDNA sequence to obtain full length gene

1.5 Gene discovery leading to improved drought tolerance

1.1 Physiology of drought stressed plants

'Drought' is a meteorological term that denotes a period without rain during which plants suffer from lack of water owing to reduced soil water content. Frequently, soil dryness is coupled with strong evaporation caused by the dryness of the air and high levels of solar radiation (Larcher, 1995). In constrast to many other stressful events, stress caused by drought does not occur suddenly, but rather develops slowly and increases in intensity with time. Drought stress triggers responses ranging from altered gene expression to changes in plant metabolism and growth. These responses may occur within a few seconds (e.g. a change in the phosphorylation state of a protein), minutes, hours (e.g. a change in gene expression) or several days (e.g. leaf senescence). Many factors affect plant responses to drought, including the duration and the magnitude of the stress. Recent results suggest that the molecular and metabolic responses observed under a combination of stresses like drought and heat are unique and cannot be extrapolated from plant response to the individual stress (Mittler, 2006).

According to Lichtenthaler (1996), plants may be exposed to short-term and long term stress effects, as well as to low stress events, which can be partially compensated for by acclimation, adaptation and repair mechanisms and strong stress or chronic stress events, which cause considerable damage and may eventually lead to plant death.

Accelerated leaf senescence in response to drought is considered to be of adaptive survival value because it reduces the water demand at the whole plant level. In fact, one of the early responses during drought is stomatal closure, which is particularly notable in the oldest senescing leaves. In this way, young leaves can retain more of the limited amount of water absorbed by roots in drought (Kozlowski, 1976; Proebsting and Middleton, 1980).

1.1.1 Symptoms of drought-induced leaf senescence

Chlorophyll degradation and increase in the ratio of carotenoids to chlorophylls have been reported during drought-induced leaf senescence in several species, including field-grown plants (Smirnoff, 1993; Rood *et al*; 2000; Munne-Boch *et al.*, 2001; Yang *et al.*, 2002).

Drought-induced leaf senescence contributes to plant survival under drought stress in several species, since it allows an early diversion of resources from vegetative to reproductive development, thus contributing to the completion of plant life-cycle in monocarpic species even under stressful conditions, remobilisation of nutrients from senescing leaves to young leaves, thus contributing to plant survival in perennials and reductions in water loss at the whole-plant level, especially when it is accompanied by leaf abscission (Munne-Bosch and Alegre, 2004).

1.1.2 Shoot growth

A primary response to water deficit is the inhibition of shoot growth. This response can benefit drought survival by progressively limiting the leaf area available for evaporative loss of limited soil water reserves. In addition, the photosynthetic production of sugars is limited under water deficit and root uptake of mineral ions is likely to be similarly impaired. The inhibition of leaf growth may then allow diversion of essential solutes from growth requirements to stress-related housekeeping functions, such as osmotic adjustment that improves cell water retention and turgor maintenance.

Shoot growth inhibition in response to water deficits may therefore extend the period of soil water availability and plant survival and can be considered as an adaptive response (Chapin, 1991; Neumann, 1995; Aachard *et al.*, 2006). However, stress adaptations that increase the chances of plant survival under stressful conditions by inhibiting growth will also decrease plant size and hence limit yield potential. The inhibition of shoot growth could certainly be a counter-

productive response in the case of crop plants exposed to episodes of moderate water deficit. In such cases, plant survival might not be threatened but stress-induced reductions in shoot growth would still limit yield potential

1.1.3 Root growth

Root growth is essential for effective plant retrieval of untapped water and mineral nutrient reserves in the soil. Paradoxically, root growth is inhibited by water deficits. Root growth inhibition involves spatially variable reductions in growth rate, outward proton pumping, wall extensibility and gene expression (Wu *et al.*, 1996; Fan and Neumann, 2004; Fan *et al.*, 2006; Ma and Bohnert, 2007). The inhibition by water deficits of growth and wall extensibility in the more basal regions of the maize (*Zea mays*) root elongation zone is associated with down-regulation of wall acidification, up-regulation of CCR gene transcription, lignin deposition and phenolic cross-linking in the cell walls (Fan *et al.*, 2006). These authors suggested that the basally localized inhibition of growth could promote the survival of the more apical meristem region by increasing the relative availability of water, minerals and sugars no longer required for growth. Increased meristem survival would serve to maintain the important ability to rapidly renew root growth after renewal of irrigation. However, for plants in deep soils, the inhibition of root growth can have negative implications. As the upper soil layers dry down, the remaining water reserves may be increasingly situated in deeper soil layers. In this situation, the onset of more severe water stress could be delayed and survival enhanced by an improved ability to maintain root growth towards the water in the deeper soil layers. Such effects have been predicted by computer simulations and quantified in field experiments (Sinclair and Muchow, 2001; Padilla and Pugnaire, 2007). Thus, the selection, breeding or genetic engineering of varieties that better maintain root growth rates during drought situations could be a rewarding approach for crops on deep soil profiles.

1.1.4 Root to shoot signals regulating stomatal aperture and shoot growth

A particularly attractive hypothesis in this context is that roots can sense water deficits in the rhizosphere and can then induce appropriate adaptive responses by transmitting chemical, hydraulic or electrical signals to the shoot. Research into the identities of stress-induced signals from the roots and their effects on stomatal closure and/or shoot growth inhibition, can suggest new approaches to regulating whole-plant responses to drought. For example, the plant hormone abscisic acid, which can inhibit stomatal opening and possibly growth in water-stressed leaves, is present in the xylem sap that is transported from the roots to the shoots of transpiring plants. Moreover, sap levels of ABA can increase in response to root water deficits. Thus, the xylem transport of root-derived ABA signals (possibly modulated by xylem pH and nitrate levels) has been considered a primary effecter of shoot changes induced by root water deficits (Davies and Zhang, 1991; Wilkinson and Davies, 2002; Davies *et al.*, 2005).

1.1.5 Photosynthesis under water stress: regulation mechanisms from whole plant to cell

Photosynthesis, together with cell growth, is among the primary processes to be affected by drought (Chaves, 1991). The carbon balance of a plant during a period of water stress and recovery may depend as much on the velocity and degree of photosynthetic recovery, as it depends on the degree and velocity of photosynthesis decline during water depletion. In general, plants subjected to mild stress recover fast (within 1 or 2 d) after stress is alleviated, but plants subjected to severe water stress recover only 40 to 60 per cent of the maximum photosynthesis rate during the day after re-watering and recovery continues during the next days, but maximum photosynthesis rates are not always recovered (Kirschbaum, 1987, 1988; Delfine *et al.*, 1999; Sofo *et al.*, 2004; Grzesiak *et al.*, 2006; Bogeat-Triboulot *et al.*, 2007; Galle′ *et al.*, 2007).

Bogeat-Triboulot *et al.* (2007) have shown that recovery after water stress, determined 10 d after re-watering, was associated with the increase in some

photosynthetic proteins, particularly rubisco activase and proteins of the water splitting complex. In the cases where photosynthesis recovery is slow and/or incomplete, sustained photoprotection and/or oxidative stress have been suggested as possible causes (Sofo *et al.*, 2004; Galle´ *et al.*, 2007). The influence of previous water stress severity on the velocity and extent of photosynthesis recovery has been illustrated in kidney bean by Miyashita *et al.* (2005) and Grzesiak *et al.* (2006).

1.1.6 Biochemical responses of plants against drought

Compatible solutes, or osmolytes, accumulate in organisms in response to osmotic stress. The primary function of compatible solutes is to maintain cell turgor and thus the driving gradient for water uptake. Recent studies indicate that compatible solutes can also act as free-radical scavengers or chemical chaperones by directly stabilizing membranes and/or proteins (Lee *et al.*, 1997; Hare *et al.*, 1998; Bohnert and Shen 1999; McNeil *et al.*, 1999 and Diamant *et al.*, 2001).

Compatible solutes fall into three major groups: amino acids (e.g. proline), quaternary amines (e.g. glycine betaine, dimethylsulfoniopropionate) and polyol/sugars (e.g. mannitol, trehalose).

Proline is considered as a compatible solute (Samaras *et al.*, 1995) as well as an osmoprotectant (Serrano and Gaxiola, 1994; Okuma *et al.*, 2003). In plants proline helps in maintaining low water potentials when water potential becomes low, allowing additional water to enter from the environment. This helps in minimizing the immediate effect of shortage of water within the organism (Kumar *et al.*, 2003).

In two genotypes of *Helianthus annus* (cv. Nantio F1 and cv. Ozdemirbey) accumulation of amount of proline under less water availability condition has suggested that a strong correlation exist in proline and water deficiency (Unyayar *et al.*, 2004).

In transgenic tobacco, overproduction of proline has resulted in enhanced root biomass and development of flower, which enhanced the drought tolerance by helping the cell to maintain water potential (Kavi Kishor *et al.*, 1995).

The transgenic rice plant showed an increase in biomass under stress conditions (Zhu *et al.*, 1998). These results support the idea that performance of plants during salinity and water stress was enhanced by over expressing P-5-CS gene that resulted in accumulation of proline. Increased amount of proline accumulation has been reported in water-stressed *Gossypium hirsutum* (Ronde *et al.*, 1999), wheat (Hamada, 2000), sorghum (Yadav *et al.*, 2005), bell pepper (Nath *et al.*, 2005) and in salt stressed *Catharanthus roseus* (Jaleel *et al.*, 2007).

Betaines are quaternary ammonium compounds, i.e. amino acid derivatives in which the nitrogen atom is fully methylated. In plants, glycine betaine, a representative member of this group of osmolytes is synthesized in the chloroplast from choline by a two-step process. The first step (choline to betaine aldehyde) is mediated by choline monooxygenase (CMO), which can be induced by drought and salinity (Russell *et al.*, 1998). The second step (betaine aldehyde to glycine betaine) is catalyzed by betaine aldehyde dehydrogenase (BADH), an NAD-dependent dehydrogenase. Many important crops, such as rice, potato and tomato, do not accumulate glycine betaine and are therefore potential candidates for the engineering of betaine biosynthesis (McCue and Hanson, 1990).

A number of sugar alcohols (mannitol, trehalose, myo-inositol and sorbitol) have targeted for the engineering of compatible-solute overproduction. Transgenic tobacco plants carrying a cDNA encoding myo-inositol O-methyltransferase (IMT1) accumulated D-ononitol and as a result, acquired enhanced photosynthesis protection and increased recovery under drought and salt stress (Sheveleva *et al.*, 1997).

Salt, drought, heat and oxidative stress are accompanied by the formation of ROS such as O_2, H_2O_2, and OH) (Price *et al.*, 1989; Moran *et al.*, 1994, Mittler, 2002), which damage membranes and macromolecules. Plants have developed

several antioxidation strategies to scavenge these toxic compounds. Enhancement of antioxidant defense in plants can thus increase tolerance to different stress factors. Antioxidants (ROS scavengers) include enzymes such as catalase, superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase, as well as non-enzyme molecules such as ascorbate, glutathione, carotenoids, and anthocyanins. Additional compounds, such as osmolytes, proteins (e.g. peroxiredoxin) and amphiphilic molecules (e.g. tocopherol) can also function as ROS scavengers (Bowler *et al.*, 1992; Noctor and Foyer, 1998).

In plants, protons are used as coupling ions for ion transport systems and the proton gradient, generated by proton pumps found in the cell membrane is the driving force for nutrient uptake (Serrano *et al.* 1999). Three distinct proton pumps are responsible for the generation of the proton electrochemical gradients (Sze *et al.* 1999): (i) the plasma membrane H-ATPase pump (PM H-ATPase) which extrudes H^+ from the cell and thus generates a proton motive force; (ii) the vacuolar-type H-ATPase pump (V-ATPase) and (iii) the vacuolar H-pumping pyrophosphatase pump (H-PPase). The latter two acidify the vacuolar lumen and other endomembrane compartments. Arabidopsis plants were transformed with a vacuolar H^+-PPase pump that is encoded by a single gene, AVP1 which could generate an H^+ gradient across the vacuolar membrane, similar in magnitude to that of the multisubunit vacuolar H^+-ATPase pump. These transgenic plants expressed higher levels of AVP1 and were more resistant to salt and drought than wild-type plants. It was also found that the resistant phenotypes had an increased vacuolar proton gradient, resulting in increased solute accumulation and water retention (Gaxiola *et al.*, 2001).

Heat-shock proteins (Hsps) and late embryogenesis abundant (LEA)-type proteins are two major types of stress-induced proteins that accumulate upon water, salinity and extreme temperature stress. Dysfunction of enzymes and proteins usually accompanies abiotic stress. Therefore, maintaining proteins in their functional conformations and preventing aggregation of non-native proteins are particularly important for cell survival under stress. Many stress-responsive

proteins, especially Hsps, have been shown to act as molecular chaperones, which are responsible for protein synthesis, targeting, maturation and degradation in a broad array of normal cellular processes. Furthermore, molecular chaperones function in the stabilization of proteins and membranes and in assisting protein refolding under stress conditions (Vierling, 1991; Hendrick and Hartl, 1993; Boston *et al.*, 1996; Hartl, 1996; Waters *et al.*, 1996 and To¨ ro¨ k *et al.*, 2001). Among five conserved families of Hsps (Hsp100, Hsp90, Hsp70, Hsp60 and sHsp), the small heat-shock proteins (sHsps) were found to be most prevalent in plants. sHsps are Hsps that vary in size from 12 to 40 kDa (Vierling, 1991). Various studies have shown that plant sHsps are not only expressed in response to heat shock but also under water, salt and oxidative stress and at low temperature (Almoguera *et al.*, 1993; Alamillo *et al.*, 1995; Sabehat *et al*; 1998; Ha¨ rndahl *et al.*, 1999; Hamilton and Heckathorn, 2001).

LEA-type proteins have been found in a wide range of plant species in response to water deficit resulting from desiccation, cold and osmotic stress. LEA-type proteins fall into a number of families, with diverse structures and functions (Close, 1996; Ingram and Bartels, 1996 and Thomashow, 1998). Predictions of secondary structures suggest that most LEA proteins exist as random coiled α-helices (Bray *et al.*, 2000). It was therefore proposed that most LEA and dehydrin proteins exist as largely unfolded structures in their native state, although a few members exist as dimers or tetramers (Ceccardi *et al.*, 1994; Kazuoka and Oeda 1994). Hydrophilicity is a common characteristic of LEA type and other osmotic stress-responsive proteins. Heat stability is another notable feature of LEA proteins and in most cases their related gene expression is transcriptionally regulated and responsive to ABA (Mundy and Chua, 1988; Skriver and Mundy, 1990; Leung and Giraudat, 1998). It has been suggested that LEA-type proteins act as water-binding molecules, in ion sequestration and in macromolecule and membrane stabilization (Dure, 1993a, 1993b; Close, 1996; Ingram and Bartels, 1996; Thomashow, 1998, 1999).

Xu *et al.*, (1996) and Park *et al.*, (2005) reported that the expression of HVA1, a LEA III family protein in barley, confers tolerance to water deficiency and salt stress in transgenic rice plants. Constitutive expression of the HVA1 protein in transgenic wheat plants improved biomass productivity and water-use efficiency under water-deficit conditions (Sivamani *et al.*, 2000).

1.2 Gene network involved in drought tolerance

Plants also respond and adapt to water deficit at both the cellular and molecular levels, for instance by the accumulation of osmolytes and proteins specifically involved in stress tolerance. An assortment of genes with diverse functions are induced or repressed by these stresses (Shinozaki *et al.*, 2003; Bartels and Sunkar, 2005; Yamaguchi-Shinozaki and Shinozaki, 2005). Most of their gene products may function in stress response and tolerance at the cellular level. Significantly, the introduction of many stress-inducible genes via gene transfer resulted in improved plant stress tolerance (Zhang *et al.*, 2004; Umezawa *et al.*, 2006).

Recently, a number of stress-inducible genes have been identified using microarray analysis in various plant species, such as Arabidopsis and rice. Dehydrin genes, which are also known as late-embryogenesis-abundant protein group II (LEA), have been most extensively studied in relation to drought and cold stresses. Now analysing the functions of these genes is critical to further understanding of the molecular mechanisms governing plant stress response and tolerance, ultimately leading to enhancement of stress tolerance in crops through genetic manipulation.

Drought triggers the production of the phytohormone abscisic acid (ABA), which in turn causes stomatal closure and induces expression of stress-related genes. Several drought-inducible genes are induced by exogenous ABA treatment, whereas others are not affected. Indeed, evidence exists demonstrating the presence of both ABA-independent and ABA-dependent regulatory systems governing drought-inducible gene expression. Both cis-acting and trans-acting

regulatory elements functioning in ABA-independent and/or ABA-responsive gene expression induced by drought stress have been precisely analysed at the molecular level (Yamaguchi-Shinozaki and Shinozaki, 2005).

Expression of several plant protein kinase genes has been shown to be induced by dehydration. These protein kinases include Arabidopsis calcium-dependent protein kinase genes (Urao *et al.*, 1994; Holappa and Walker-Simmons, 1995; Hwang and Goodman, 1995; Mizoguchi *et al.*, 1996). Whereas the structures of the protein kinases encoded by these genes imply that they are intracellular proteins and there has been no report on a receptor-like protein kinase that is induced by osmotic stress.

Interpretation of ever-increasing raw sequence information generated by modern genome sequencing technologies faces multiple challenges, such as gene function analysis and genome annotation. Indeed, nearly 40 per cent of genes in plants encode proteins of unknown function. Functional characterization of these genes is one of the main challenges in modern biology. In this regard, the availability of full-length cDNA clones may fill in the gap created between sequence information and biological knowledge. Full-length cDNA clones facilitate functional analysis of the corresponding genes enabling manipulation of their expression in heterologous systems and the generation of a variety of tagged versions of the native protein. In addition, the development of full-length cDNA sequences has the power to improve the quality of genome annotation.

1.2.2 Functions of drought-inducible genes in plant system

The products of the drought-inducible genes identified through the recent microarray analyses in Arabidopsis can be classified into two groups (Shinozaki *et al.*, 2003). The first group includes proteins that most probably function in abiotic stress tolerance. These include molecules such as chaperones, late embryogenesis abundant (LEA) proteins, osmotin, antifreeze proteins, mRNA-binding proteins, key enzymes for osmolyte biosynthesis, water channel proteins, sugar and proline transporters, detoxification enzymes and various proteases. The second group is

comprised of regulatory proteins, i.e. protein factors involved in further regulation of signal transduction and stress-responsive gene expression. These include various transcription factors, protein kinases, protein phosphatases, enzymes involved in phospholipid metabolism and other signalling molecules such as calmodulin-binding protein. Many transcription factor genes were stress inducible, suggesting that various transcriptional regulatory mechanisms may function in regulating drought, cold or high salinity stress signal transduction pathways. These transcription factors could govern expression of stress-inducible genes either cooperatively or independently and may constitute gene networks in plant system.

Similar to the Arabidopsis findings, the products of stress inducible genes identified in rice can also be classified into functional proteins and regulatory proteins (Rabbani *et al.*, 2003). Comparative analysis of stress-inducible genes in Arabidopsis with those in rice revealed a considerable degree of similarity in stress responses between the two genomes at the molecular level. Among the 73 genes identified as stress inducible in rice, 51 have already been reported in Arabidopsis to perform a similar function. These results confirm that plants share common stress-inducible genes though they have evolved separately over millions of years.

1.3 Systematic analysis of kinase superfamily in model plants

Determination of gene function is particularly problematic when studying large-gene families because redundancy limits the ability to assess the contributions of individual genes experimentally. Phylogenomics is a phylogenetic approach used in comparative genomics to predict the biological functions of members of large gene-families by assessing the similarity among gene products. The rice MAPKKK genes play a key role in a broad range of stress responses, including responses to biotic/abiotic stresses. Rice might have evolved an enhanced response to these stresses by retaining multiple gene family members that perform critical functions (Jung *et al.*, 2010).

The eukaryotic protein kinases comprise one of the largest superfamilies of homologous proteins and genes. Within this family, there are now hundreds of different members whose sequences are known. Although there is a rich diversity of structures, regulation modes and substrate specificities among the protein kinases there are also common structural features. These conserved structural motifs provide clear indications as to how these enzymes manage to transfer the phosphate of a purine nucleotide triphosphate to the hydroxyl groups of their protein substrates.

Several reports include monumental task of analyzing and collating the amino acid sequences of all reported protein kinases and defining the conserved structural features that characterize the portion of these proteins that is responsible for their catalytic activity (Hanks *et al.*, 1988; Hanks, 1991; Hanks and Quinn, 1991).

The currently accepted classification of the eukaryotic protein kinase superfamily considers eight 'conventional' protein kinase groups (ePKs) and four 'atypical' groups (aPKs) (Manning *et al.*, 2002; Miranda-Saavendra and Barton, 2007). Among the ePKs are the AGC group (including cyclic nucleotide and calcium-phospholipid-dependent kinases, ribosomal S6-phosphorylating kinases, G protein-coupled kinases and all close relatives of these sets); the CAMKs (calmodulin-regulated kinases); the CK1 group (casein kinase 1, and close relatives); the CMGC group (including cyclin-dependent kinases, mitogen-activated protein kinases, glycogen synthase kinases and CDK-like kinases); the RGC group (receptor guanylate cyclase); the STEs (including many kinases functioning in MAP kinase cascades); the TKs (tyrosine kinases) and the TKLs (tyrosine kinase-like kinases).

However, there is a significant proportion of kinases which, whilst exhibiting some degree of sequence similarity to the eight groups above, could not be classified easily into particular groups. These form a ninth group called 'Other'. The aPKs are a small set of protein kinases that do not share clear

sequence similarity with ePKs, but have been shown experimentally to have protein kinase activity.

The bona fide aPKs (Miranda-Saavendra and Barton, 2007) are the alpha-kinase group (exemplified by myosin heavy chain kinase of Dictyostelium discoideum), PIKK (phosphatidyl inositol 30 kinase-related kinases), RIO and PHDK (pyruvate dehydrogenase kinases). The sequencing of complete genomes for many eukaryotic species has allowed the determination and comparison of their complete kinase complements (kinomes). These include the kinomes of *Saccharomyces cerevisiae* (Hunter and Plowman, 1997), *Caenorhabditis elegans* (Plowman *et al.*, 1999), *Drosophila melanogaster* (Morrison *et al.*, 2000), *Mus musculus* (Caenepeel *et al.*, 2004), *Homo sapiens* (Manning *et al.*, 2002), *Dictyostelium discoideum* (Golberg *et al.*, 2006), *Strongylocentrotus purpuratus* (Bradham *et al.*, 2006), *Tetrahymena thermophila* (Eisen *et al.*, 2006) and the plants *Arabidopsis thaliana* and *Oryza sativa* (Krupa *et al.*, 2006).

1.3.1 PKC isotypes

The first PKCs to be identified and cloned were a, b and c isotypes, which were initially isolated from brain cDNA libraries. This tissue has proven to be a rich source of PKC isotypes and low-stringency screening of brain cDNA libraries with probes derived from the a, b and c isotypes yielded three additional PKCs, the d, e and f isotypes. Further low stringency screens of other tissue cDNA libraries has delivered PKCg, PKCh, PKCi (of which PKCk is the mouse homologue) and most recently, the PKC-related kinases (PRKs). The mammalian PKC isotypes have been grouped into smaller subfamilies on the basis of their enzymic properties. The best understood and most studied of these groups is the conventional PKCs (cPKCs), which comprise a, bI, bII and c isotypes (the PKCb gene is alternatively spliced to produce two gene products which differ only in their extreme C terminal ends. These PKC isotypes are activated by PS in a Ca^{2+}-dependent manner; they also bind DAG, which both increases the specificity of the enzyme for PS and also shifts the affinity for Ca^{2+} into the physiological

range. The cPKCs are targets of the tumour-promoting phorbol ester PMA, which activates these enzymes by eliminating the requirement for DAG and decreasing the concentration of Ca^{2+} needed for activation (Mellor and Parker, 1998).

1.4 Strategies to amplify the cDNA sequence to obtain full length gene

Isolation of full-length gene transcripts is important to determine the protein coding region and study gene structure. However, isolation of novel gene sequences is often limited to expressed sequence tags (ESTs) (i.e., short cDNA fragments that predominantly represent the 3′ end of the transcript). PCR-based techniques are most popular methods for isolation of DNA sequences flanking a known region.

Conventional PCR allows the amplification of sequences within known boundaries. Several methods have been developed for the amplification of DNA sequences that flank regions of known sequences. These include TGW-PCR (targeted gene walking PCR) (Parker *et al.*, 1991), UP-PCR (unpredictably primed PCR) (Dominguez and Lopez-Larrea, 1994), Inverse PCR (Triglia *et al.*, 1988; Ochman *et al.*, 1988; Silver and Keerikatte, 1989), ligation-mediated PCR (LM-PCR), randomly primed PCR (RP-PCR), anchored PCR and rapid amplification of cDNA ends (RACE) etc.

Inverse PCR is a convenient and versatile method of cloning unknown sequences upstream or downstream of known sequences (Triglia *et al.*, 1988). It circumvents the laborious procedures of producing and screening genomic libraries (Forster *et al.*, 1994). It has successfully been used to isolate the seed lipoxygenase promoter from pea as well as a wound-inducible promoter from *Asparagus* (Forster *et al.*, 1994). One of the drawbacks of Inverse PCR is the requirement for two restriction enzyme sites that flank the priming region. The lack of data on restriction sites as well as the size of chromosomal DNA greatly reduces the successful cloning rates. An additional problem with Inverse PCR is the inefficient PCR amplification of closed circular double-stranded DNA. Forster *et al.* (1994) found that digestion of the self-ligated DNA improved the yields of

PCR amplification product. The presence of introns in the target gene must also be considered when carrying out Inverse PCR from genomic DNA. IPCR was the first method developed for this kind of purpose. However, it is now rarely used because of the difficulty in finding suitable restriction sites in the target region or poor circularization of the template molecule.

LM-PCR and RP-PCR are more frequently used nowadays, yet they also have some limitations. For example, LM-PCR depends on restriction sites within a reasonable distance in the flanking regions, while the amplified products of RP-PCR are generally small «1 kb). Moreover, both methods often result in excessive amplification of non-specific molecules, which greatly reduces their efficiencies in obtaining sequences of interest. To resolve these problems, some new strategies have emerged in the past few years, such as Vectorette-PCR, biotin-capture PCR, TAIL-PCR and T-linker PCR. These improved methods are more efficient than their old versions; however, most of them are still limited by restriction digestion or ligation. Although the intervening steps are avoided in TAIL-PCR, the amplified fragments are often small because of the use of random primers.

Anchored PCR (A-PCR) is a method for chromosome walking, which is free from restriction digestion or ligation steps. The method uses the same homo-oligomeric tailing principle as for the traditional 5'-RACE (rapid amplification of cDNA ends). However, the fundamental difference from 5'-RACE is that the described method uses genomic DNA as the starting material to amplify the flanking sequence of a known region. It could be a very useful tool for cloning the regulatory sequence of a gene and analysis of T-DNA or transposon insertion sites in known or unknown genomes, while 5'-RACE was designed only to obtain the ends of cDNA, using RNA as the starting material. In brief, the A-PCR could be an alternative method for chromosome walking from high complex genome, especially in the absence of any restriction information (Ohora *et al.,* 1889).

Another approach known as ''Genomic DNA Splicing'' protocol which avoids RNA preparation and reverse transcription steps, and the entire assembly

process can be finished within hours. Since genomic DNA is more stable than RNA, it may be a more practical cloning strategy for many genes, especially the ones that are very large and difficult to generate a full length cDNA using oligo-dT primed reverse transcription. With this technique, the full-length wild type coding sequence of human polymeric immunoglobulin receptor was successfully cloned, which was 2295 bp in length and composed of 10 exons (An *et al.*, 2007).

Rapid amplification of cDNA ends (RACE) is today by far the most popular approach for obtaining full-length cDNAs when only part of the transcript's sequence is known. Since its original description numerous modifications and improvements of the method have been developed and consist of a collection of PCR-based cloning procedures that extend a known cDNA fragment toward the 3′ (3′ RACE) or the 5′ (5′ RACE) cDNA end.

The original method is based on attachment of an anchor sequence to one end of the cDNA that can be used as a primer binding template in PCR with a second gene-specific primer from the known part of the gene. Although this procedure seems in theory fast and simple, it is technically difficult and usually requires substantial optimization and several repetitions before satisfactory results could be obtained. This is particularly due to the use of a universal primer corresponding to the anchor sequence present in all cDNAs, which may result in a high background of nonspecific products even after a nested PCR with a gene-specific primer internal to the first gene-specific primer is performed. Another drawback of the method is the difficulty of obtaining the full-length 5′ end of the transcript due to the presence of many truncated transcripts in the messenger RNA (mRNA) pool.

Several strategies aimed at eliminating these problems have been developed and have proven to be very useful in certain applications. One improvement is based on the utilization of a pair of gene-specific primers in inverse PCR on circularized cDNA templates, which would avoid the use of a

universal primer and the problems it may generate. This strategy also allows the simultaneous isolation of both cDNA ends in a single reaction.

Some of these procedures require the generation of double-stranded cDNA, including the use of template-switching reverse transcription or a post-reverse transcription adaptar ligation step. Methods that are performed directly of first-strand cDNA are complicated by the low efficiency of RNA ligase for the circularization reaction or the need for bridging oligonucleotides for this step.

Furthermore, existing inverse- RACE methods typically require nested PCR to amplify the transcript of interest, and only a limited number of transcripts can be isolated from a single reverse transcription reaction, making it difficult to analyze rare transcripts from scarce tissue. So an improved inverse-RACE method, known as Rolling circle amplification-RACE (RGA-RACE) is used for simultaneous isolation of 5′ and 3′ cDNA ends from amplified cDNA templates (Polidoros *et al.*, 2006).

RNA Ligase Mediated Rapid Amplification of cDNA Ends (RLM-RACE) represents a major improvement to the classic RACE technique (Maruyama and Sugano, 1994, Shaefer, 1995). RLM-RACE is designed to amplify cDNA only from full-length, capped mRNA, usually producing a single band after PCR.

A polygalacturonase-inhibiting protein (*pgip*) gene from *Malus domestica* cv Granny Smith apple plants was cloned by degenerate oligo-primed polymerase chain reaction (PCR) and Inverse PCR (Arendse *et al.*, 1999).

The cDNA of MAPK (mitogen activated protein kinase) gene have been isolated and characterized in halotolerent alga *Dunaliella salina* using degenerate oligonucleotide primers in a polymerase chain reaction (PCR) amplification approach and rapid amplification of cDNA ends (RACE) method (Lei *et al.*, 2008).

A cDNA clone encoding a dehydrin gene was isolated and characterised from a cDNA library prepared from white spruce (*Picea glauca*) needle mRNAs (Richard et al., 2000). It involved the use cDNA sequence as a probe to screen total RNA for desired clone. And the dehydrin cDNA was serendipitously isolated from the library while screening 1x 10^6 clones of the library with a 358 bp PCR fragment probe bearing homology to a protein kinase gene (Richard and Séguin, unpublished).

The FirstChoice RLM-RACE kit (Ambion, USA) was used for rapid amplification of cDNA ends to get full length gene involved in water-stress tolerance in wild barley (*Hordium spontaneoum*) (Suprunova *et al.*, 2007).

Full length cDNA of betaine aldehyde dehydrogenase gene (*OjBADH*) from *O. japonicas* have been isolated using Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Rapid Amplification of cDNA Ends (RACE) techniques (Liu et al., 2010).

1.5 Gene discovery leading to improved drought stress tolerance in plants via gene transfer

Introduction by gene transfer of several stress-inducible genes has demonstrably enhanced abiotic stress tolerance in transgenic plants (Zhang *et al.*, 2004; Bartels and Sunkar, 2005; Umezawa *et al.*, 2006b). These particular genes encode key enzymes regulating biosynthesis of compatible solutes such as amino acids (e.g. proline), quaternary and other amines (e.g. glycinebetaine and polyamines) and a variety of sugars and sugar alcohols (e.g. mannitol, trehalose, galactinol and raffinose).

Genes encoding LEA proteins and heat shock proteins have also been used to improve drought tolerance in transgenic plants. A gene encoding galactinol synthase (GolS), a key enzyme involved in raffinose family oligosaccharide biosynthesis, was introduced to improve drought-stress tolerance in transgenic Arabidopsis (Taji *et al.*, 2002).

Other studies demonstrated that overexpression of some LEA class genes results in enhanced tolerance to dehydration, although the precise mechanism is still unknown. LEA proteins may also function as chaperone-like protective molecules to combat cellular damage (Umezawa *et al.*, 2006).

Transcription factors have also proven quite useful in improving stress tolerance in transgenic plants, through influencing expression of a number of stress-related target genes (Shinozaki *et al.*, 2003; Yamaguchi-Shinozaki and Shinozaki, 2005).

Other regulatory factors, such as protein kinases and enzymes involved in ABA biosynthesis, are also useful for improving stress tolerance by regulating many stress-related genes in transgenic plants. ABA is synthesized *de novo* primarily in response to drought and high salinity stress. Genes involved in ABA biosynthesis and catabolism were identified based on genetic and genomics analyses (Nambara and Marion-Poll, 2005). It was demonstrated that overexpression of the gene encoding 9-cisepoxycarotenoid dioxygenase (NCED), a key enzyme in ABA biosynthesis, improves drought stress tolerance in transgenic Arabidopsis plants (Iuchi *et al.*, 2001).

Recently, a cytochrome P450 CYP707A family member was identified as ABA 8-hydroxylase, an enzyme that degrades ABA during seed imbibition and dehydration stress (Kushiro *et al.*, 2004; Saito *et al.*, 2004). A T-DNA insertion mutant of CYP707A3 was the most abundantly expressed gene amongst the four CYP707A members under stress conditions, exhibited elevated drought tolerance with a concomitant reduction in transpiration rate (Umezawa *et al.*, 2006b).

The ABA-activated SnRK2 protein kinase (OST1/ SRK2E) functions in the ABA signal transduction pathway controlling stomatal closure (Mustilli *et al.*, 2002; Yoshida *et al.*, 2002). SnRK2 is a member of the SNF1-related PKase family, which contains 10 members in Arabidopsis and rice. SnRK2s were activated by drought, salinity, and ABA (Yoshida *et al.*, 2002). SRK2E/OST1 was involved in stomatal closure, but not seed germination. Another SnRK2, SRK2C,

was activated by osmotic stress, salt stress, and ABA treatment (Umezawa *et al.*, 2004). SRK2C was strongly expressed in the root tip and was involved in the root response to drought stress. SRK2C also functioned in transgenic plants to improve stress tolerance, as many of the downstream genes it influences were stress inducible. In addition, SnRK2 protein kinases may activate transcription factors influencing osmotic stress-responsive gene expression.

In eukaryotes, reversible protein phosphorylation is central to perception of and response to environmental stresses and constitutes a major mechanism for the control of cellular functions, such as responses to environmental stimuli and pathogens and hormonal control of pathway (Sheen, 1998; Kwak *et al.*, 2002). As a counterpart, various stress-inducible protein kinase families such as mitogen-activated protein kinase (MAPK) (Wrzaczek and Hirt, 2001), calcium-dependent protein kinase (CDPK) (Ludwig *et al.*, 2004) and SNF1-related protein kinase (SnRK), which were first analysed in yeast from where the name originated, are activated by ABA and diverse stress signal metabolism (Cohen, 1988). Genetic evidence clearly shows that type 2C and 2A protein phosphatases function in the early ABA signalling.

Yeast SNF1 protein kinase, mammalian AMP-activated protein kinase (AMPK) and plant SnRK protein are highly conserved and play pivotal roles in growth and metabolic responses to cellular stress. In yeast, SNF1 is involved in a variety of functions, including regulation of glucose responsive genes, control of pseudohyphal growth under nutrient limitations (Cullen and Sprague, 2000) and regulation of meiosis (Honigberg and Lee, 1998).

In plants, SnRKs were grouped into three subfamilies: SnRK1, SnRK2 and SnRK3 (Hrabak *et al.*, 2003). SnRK1 kinase has well characterized at the molecular and biochemical levels and evidence indicates that SnRK1s have roles in regulating energy metabolism (Hardie *et al.*, 1998). SnRK2 and SnRK3 are unique to plants and are involved in responses to environmental stresses. Several

SnRK3 members were extensively characterized and the well-known SOS2 was required for Na+ and K+ homeostasis in Arabidopsis (Gong *et al.*, 2002).

Current studies indicate that the SnRK2 family is involved in hyperosmotic stress responses and ABA signalling (Boudsocq *et al.*, 2004, 2007; Kobayashi *et al.*, 2004). Ten SnRK2s were identified in Arabidopsis; nine of them were activated by hyperosmotic and salinity stresses and five of the nine were activated by ABA, whereas none was activated by cold stress (Boudsocq *et al.*, 2004). AtSRK2.6/ AtSnRK2E/OST1 and *Vicia faba* AAPK were activated by ABA and were involved in ABA regulation of stomatal closing and ABA-regulated gene expression (Li *et al.*, 2000; Mustilli *et al.*, 2002; Yoshida *et al.*, 2002).

Overexpression of AtSnRK2.8/AtSnRK2C enhances drought tolerance in Arabidopsis (Umezawa *et al.*, 2004). In rice, 10 SnRK2 members were identified; all were activated by hyperosmotic stress and three were also activated by ABA (Kobayashi *et al.*, 2004). With overexpression of SAPK4, one rice member significantly enhanced salt tolerance of transgenic plants (Diedhiou *et al.*, 2008).

In maize, 10 SnRK2 members were cloned, and most ZmSnRK2 genes were induced by one or more abiotic stresses (Huai *et al.*, 2008). In soybean (*Glycine max*), four SnRK2 members were isolated and all were activated by hyperosmotic stress (Yoon *et al.*, 1997; Monks *et al.*, 2001). NtOSAK, identified in tobacco, was involved in the response to hyperosmotic stress (Kelner *et al.*, 2004).

In wheat, only one SnRK2 member, PKABA1, was induced by ABA and hyperosmotic stress and it repressed the activities of gibberellic acid inducible promoters when transiently overexpressed in barley aleurone layers (Gomez-Cadenas *et al.*, 1999, 2001; Shen *et al.*, 2001; Johnson *et al.*, 2002).

Previous findings suggest that many SnRK2 members are involved in the response to environmental stimuli and different members exhibit diverse

expression patterns, suggesting that they may play different roles in response to abiotic stresses. However, knowledge of specific functions of SnRK2s is fragmentary and their role in stress signalling is still enigmatic.

Materials and methods

3. MATERIALS AND METHODS

The present study was aimed at enrichment of sequence information on water stress activated protein kinase gene in the drought tolerant black pepper (*Piper nigrum* L.) var. Kalluvally, by various methods. The study was undertaken based on the availability of expressed sequence tag (EST) characterized during water stress. The chemicals used in this study were of good quality (AR grade), and obtained from different reputed firms like, Ambion Inc., USA; Applied Biosystems, USA; Bangalore Genei, India; GE Healthcare, USA; Merck, Germany; Invitrogen, USA; Sigma Aldrich, USA and Sisco Research Laboratory (SRL), India. Plastic ware and glassware used in the study were obtained from Applied Biosystems, USA; Axygen Biosciences, USA; Borosil and Tarsons India Ltd. Instruments such as PCR thermal cycler (Eppendorf), gel documentation system (Biorad), electrophoresis system (Biorad) available at the Centre for Plant Biotechnology and Molecular Biology (CPBMB) were used for the work. The softwares necessary for primer designing and gene sequence analysis were accessed at Distributed Information Centre (DIC) attached to the Centre. The details of the work carried out are presented in this chapter.

3.1 Maintenance of source plants

Potted plants of *P. nigrum* var. Kalluvally (Plate 1.) were raised from the cuttings and maintained at CPBMB, College of Horticulture (COH), Vellanikkara which served as the source material. Leaves of fresh sprouts were used for further experiments.

3.2 Selection and sequencing of cDNA clones

Protein kinase clones (named PNK21 and PNK49) specific to water stress were selected from water stress specific cDNA library of Kalluvally (Kushwah, 2008) maintained at the Centre. The clones were already subjected to single pass sequencing and the length of the ESTs was 721 and 698 bp for PNK21 and PNK49 respectively. The available sequence served as the background sequence

Plate 1. Kalluvally potted plants

for further experiments. The details of the sequence information available on selected clones and composition of different media used in the study are given in Appendix I and II respectively.

The clones were revived on LB/ampicillin medium. Colony PCR was performed to confirm the presence of insert from single colony. The stabs were made from the same colony selected and it was sent for double pass sequencing. The composition of master mix and PCR set for colony PCR are given in Appendix III.

The sequence data obtained was processed to remove any vector and adaptor sequences by using VecScreen and Bioedit- Biological sequence alignment editor tool. The sequences were designated as PNK21A and PNK49 and were used to design degenerate primers for RT-PCR.

3.3 Primer designing

Primers were designed based on the high sequence conservation among protein kinase genes from different plant species showing significant homology with PNK21A and PNK49A sequences. Blastn and Blastx analysis was performed to select the homologous sequences for primer designing. The primer designing was done with ClustalW multiple sequence alignment program and Primer 3 software.

3.3.1 Criteria considered for designing primers

- Amplicon length >200 bp for optimum PCR efficiency
- Optimal primer length- 18 bases
- Tm (melting temperature)- 55 to 60^0C
- GC content 50 per cent

- The last five nucleotides at the 3' end had no more than two G + C residue
- Avoid runs of identical nucleotides. If repeats were present, they were fewer than four consecutive G residues
- The amplicon expected had one or more introns to avoid amplification of the target gene from the contaminated genomic DNA
- If the gene did not contain intron, then ran RT minus control to observe contamination of RNA with genomic DNA

3.3.1.2 Procedure

Primers were designed to amplify 5' and 3' ends of available EST sequences. Gene fragments were attempted to amplify under moderate stringency conditions. Available complete cDNA sequences (cds) for protein kinase genes from different crops like *Vitis vinifera, Ricinus communis,* Arabidopsis and rice were retrieved from NCBI GenBank and used for primer designing for both PNK21 and PNK49 partial clones.

- The sequences showing significant homology with available EST clones based on which degenerate primers are to be designed were copied in the Fasta format.
- Complete cds of homologous genes from related crops were used to find out open reading frame (ORF) and they were used for multiple sequence alignment using ClustalW online tool.
- Entirely conserved column was selected which served as forward or reverse primers to amplify 5' and 3' cDNA ends respectively. Parameters for primers were set according to the requirement (as detailed in section 3.3.1).

- The degenerate primers designed were ordered in lyophilized form from Sigma Aldrich Chemicals Pvt Ltd.

3.4 Drought treatment

Kalluvally is a drought tolerant variety of black pepper (Thankmani *et al.*, 2003). Based on the previous study (Kushwah, 2008) daily watered plants were starved from two to seven days under the open sunlight during the summer months (Jan to May) and light green, tender dehydrated leaves were collected for RNA extraction.

3.5 Isolation of Total RNA

Intact RNA was isolated from the stressed plants using TRIzol method originally given by Chomczynski and Sacchi (1986) with slight modifications. TRI® (Sigma Aldrich, USA) is a phenol-based reagent containing a unique combination of denaturants and RNase inhibitors and used a convenient, single-step disruption procedure.

3.5.1 Procedure:

I. Treatment with diethyl pyrocarbonate (DEPC)

DEPC, a highly reactive alkalyting agent, was used to inactivate RNases in buffers and on glasswares. Mortar, pestle, micro tips, microfuge tubes, cylinders and glasswares were treated with 0.025 per cent DEPC solution overnight and washed well using milli Q water to remove the traces of DEPC and then were double autoclaved. Electrophoresis unit and pipettes were cleaned with RNaseZap (Ambion, USA) followed by rinsing with double autoclaved DEPC treated water. Gloves were used throughout the experiment to avoid contamination with RNases.

II. Isolation:

1. Young leaves were collected early morning or at late evening hours.

2. Weighed 0.1 g of leaf tissue and ground with liquid nitrogen using sterile mortar and pestle.

3. Immediately added 1 ml of TRI® reagent to the homogenate and kept at room temperature to thaw.

4. Suspension was transfered into a 1.5 ml microfuge tube and centrifuged at 11,000 g for 15 min at 4°C.

5. Supernatant was transfered to a new tube and added 0.2 ml of chloroform.

6. Mixed by inversion for 15 seconds and kept for 2 min at room temperature and centrifuged at 11,000 g for 12 min at 4°C.

7. Steps 4 and 5 were repeated.

8. Carefully removed upper aqueous phase to a new tube and gently added 0.5 ml of ice-cold isopropanol.

9. Tube was allowed to incubate at room temperature for 10 to 15 min.

10. Centrifuged at 12,000 g for 15 min at 4°C.

11. Pellet was washed with 1ml 75 per cent ethanol by centrifugation at 7500 g for 7 min at 4°C.

12. Tube was decanted and pellet was air dried to remove traces of ethanol.

13. Pellet was dissolved in 30µl autoclaved DEPC treated water.

3.5.1.2 Agarose gel electrophoresis of RNA

The RNA sample was electrophoresed on 0.7 per cent agarose gel. Preparation of 10X MOPS buffer is given in Appendix IV. The details of procedure are as follows.

- Agarose (0.21 g) was dissolved in 30 ml 1X MOPS buffer by heating in a microwave oven.
- The solution was then allowed to cool up to 65^0C and then 5µl of ethidium bromide added.
- The dissolved agarose gel was poured into a presterilized casting tray containing positioned comb and allowed to solidify. The gel was then placed into a 1X MOPS buffer in a buffer tank.
- 5µl RNA was mixed with gel loading dye and loaded into well. RNA molecular weight marker was also loaded.
- The electrophoresis was performed at 30 V until dye ran up to 75 per cent of the total gel (~ one hour).
- Integrity of RNA was observed in gel documentation unit.

3.5.1.3 Quality analysis of total RNA

The quality of RNA was checked with agarose gel electrophoresis using MOPS buffer. The RNA samples run on 0.7 per cent agarose gel with MOPS buffer at 30V. The gel observed under UV light to check the integrity of bands.

3.5.1.4 Spectrophotometric analysis of total RNA

The quality and quantity of RNA was estimated using Nanodrop spectrophotometer (NanoDrop Technologies Inc., USA). The ratio of OD_{260}/OD_{280} and OD_{260}/OD_{230} was recorded to assess the quality of RNA. A ratio of absorbance 1.8-2.0 and 2.0 indicate good quality of RNA at OD_{260}/OD_{280} and OD_{260}/OD_{230} respectively.

3.6 cDNA synthesis from total RNA by reverse transcription (Genie AMV RT-PCR kit)

The isolation of intact mRNA is very essential for successful first strand cDNA synthesis and subsequent PCR amplification. Mature mRNA molecules carry poly (A) at their 3' termini, to which oligo dT primers anneal. Reverse transcriptase enzyme can synthesize single stranded cDNA from isolated RNA under favorable conditions. The single stranded cDNA synthesized was used immediately for PCR without further purification.

3.6.1 Materials

- RNA sample
- Random hexamer (1 μg/μl)
- Oligo $(dT)_{18}$ primer
- 5X M-MuLV RT buffer
- 30 mM dNTP mix (7.5 mM each)
- DTT (0.1 M)
- M-MuLV reverse transcriptase (50 units/μl)
- Autoclaved DEPC treated water

Procedure:

1. 9μl of total RNA (~5μg) was added to a 0.2ml microfuge tube.
2. Added 1μl of oligo dT primer.
3. The reaction mix was incubated at 65^0C for 10 min and at room temperature for 2 min to remove any secondary structure present in the RNA sample.

4. The reaction mix was spinned briefly to collect the content at the bottom of the tube and the reagents added one by one as follows.

   - 1 µl RNase inhibitor
   - 1 µl DTT (0.1 M)
   - 4 µl RT buffer (5X)
   - 2 µl dNTP mix (30 mM)
   - 0.5 µl M-MuLV reverse transcriptase
   - 1 µl nuclease free water

5. The content was mixed well, spinned and incubated at 37^0C for 1 hour (lid temperature of PCR was kept at 92^0C during incubation).

6. Final incubation was done at 95^0C for 2 min to denature RNA-cDNA hybrids.

7. The reaction mix was spun briefly and transferred immediately on to ice.

8. cDNA was then stored at -20^0C for long term storage and was later used for PCR assay.

9. In order to detect contamination with genomic DNA, NAC (No Amplification Control) was included in the reaction without adding reverse transcriptase.

3.6.2 PCR amplification of the internal gene sequences using degenerate primers

RT reaction product (single stranded cDNA) of section 3.6 was used directly for PCR amplification within two hours after cDNA synthesis.

Degenerate oligonucleotide primers designed in 3.3.1.2 were used for the purpose.

Procedure:

- Master mix was prepared and spun briefly for proper mixing. Later, aliquot of the master mix as described under 3.6.2.1 was added to each tube and cDNA template added separately.
- A negative control was maintained to ensure that no non-specific amplification has occurred due to contamination in any of the reagents.
- A positive control reaction was maintained to ensure proper cDNA synthesis.
- Special thin walled tubes of uniform thickness were used to ensure rapid and equal temperature changes throughout the reaction volume.
- PCR reaction was optimized by gradient PCR and by changing the concentration of template, primers, Taq DNA polymerase and number of cycles.

3.6.2.1 PCR reaction mix (20µl)

- 10x reaction buffer : 2 µl
- dNTP mix : 1.0 µl
- Forward primer : 0.5 µl (1000nM)
- Reverse primer : 0.5 µl (1000nM)
- cDNA : 3.0 µl

- Taq DNA polymerase : 0.3 µl
- Nuclease free water : 12.7 µl

PCR product was stored at -20^{0}C and electrophoresed using 1.0 per cent agarose; visualized by staining with ethidium bromide and observed under UV transilluminator. Preparation of buffers and dyes used in gel electrophoresis is given in Appendix IV.

3.6.3 Gel elution of PCR amplified fragments

cDNA fragment amplified with gene specific primers was eluted using AxyPrep DNA Gel Extraction Kit (Axygen, Biosciences). It employs optimized reagents in combination with a convenient AxyPrep spin column to purify DNA fragments on agarose gels. Procedure was followed as per the manufacturer's guidelines.

- Agarose gel slice containing the DNA fragment characterized by molecular weight marker was excised with a sterile, sharp scalpel by a brief exposure to UV light.
- Gel slice was weighed in a 1.5 ml microcentrifuge tube.
- Added 3X gel volume of gel solubilization buffer (DE-A buffer).
- The gel was resuspended in gel solubilization buffer by vortexing. The mix was heated at 75^{0}C until the gel was completely dissolved. Intermittent vortexing was given every 2 to 3 min to accelerate gel solubilization.
- Added 0.5 X Buffer DE-A volume of Buffer DE-B and mixed properly.
- A spin column was placed in a 2 ml microfuge tube. The solubilized gel slice was transferred into the spin column that was assembled in the 2 ml collection tube and centrifuged at 12,000 g for 1 min.

- The filtrate was discarded and added 500 µl of wash Buffer (W1) to the spin column and centrifuged at 12,000 g for 30 sec.
- The filtrate was discarded and added 700 µl of desalting Buffer (W2) and centrifuged at 12,000 g for 30 sec.
- A second wash was given by adding 700 µl of desalting buffer (W2), followed by centrifugation at 12,000 g for 30 sec to ensure the complete removal of salt. The filtrate was discarded and spin column was again placed in collection tube.
- Column was again centrifuged for 1 min at 10,000 g to remove any residual buffer.
- Spin column was transferred to a fresh 1.5 ml microcentrifuge tube. The eluent was prewarmed at 65^0C to improve the elution efficiency.
- DNA was eluted by adding, 15 to 25 µl of prewarmed eluent to the centre of the membrane and allowed to stand for 1 min at room temperature and centrifuged at 12,000 g for 1 min.
- Eluted fragments were checked spectrophotometrically and sent for double pass sequencing, primer details were also provided with DNA sample.

3.7. Sequence analysis

The forward and reverse sequence data obtained for RT PCR were not overlapping with each other, so it was separately compared with PNK21A fragment and then two overlapping sequences were merged using EMBOSS merger tool. The merged sequence was designated as PNK21B.

RT-PCR reaction performed could enrich only 66 bp sequence information towards 5' end of the mRNA transcript and a gene fragment towards 5' end was still unknown.

So, the second strategy was attempted to get full length coding sequence by using rapid amplification of cDNA ends (RACE).

3.8 Rapid amplification of cDNA ends (RACE)

FirstChoice RLM-RACE Kit (Ambion, Inc., USA) which is a major improvement of Classic RACE (Maruyama and Sugano 1994) was used to determine the remaining sequence towards and up to the 5' and 3' end subsequently. RLM-RACE has designed to amplify cDNA only from full-length, capped mRNA, usually producing a single band after PCR. The procedure was followed as per the instructions given by the manufacturer. The reagents and primers used for RLM-RACE reaction are given in Appendix V.

3.8.1 Primer Designing

Following PCR primer design recommendations were used

- 20-24 bases in length
- 50 per cent G:C content, with no secondary structure
- Avoid placing more than three G or C residues in the 3'-most five bases
- Avoid primers with a G as the 3' terminal base
- Avoid sequences with 3' ends that can self-hybridize to the 3' ends of the other primer in the PCR (forming primer dimers)
- Finally, using primer design software, evaluated the gene-specific primers in combination with the corresponding primers

3.8.1.1 Procedure

The sequence PNK21B analysed and confirmed as the part of protein kinase gene was used for designing gene specific primers for both 5' and 3' RACE reaction.

Alignment of the PNK21B sequence with homologous sequences from other plant sources indicated that the fragment was located in the centre of the gene, with around 500 bases flanking the 5' end and around 200 bases flanking the 3' end. This led to the designing of further experiments to deduce the sequence towards both the ends. Two sets of gene specific primers (GSP) each for 5' and 3' RACE were designed to amplify the flanking region.

3.8.2 5' RLM-RACE

3.8.2.1 RNA Processing

1. Following reagents were prepared:

- Ice cold 100 per cent isopropanol
- Ice cold 70 per cent ethanol

2. Treatment with CIP at 37°C for one hour.

a) Following components were assembled in an RNase-free microfuge tube.

Component	Volume (µl)	Amount
Total RNA (880.3ng/µl)	11	~10 µg
10X CIP buffer	2	
Calf intenstine alkaline phospatase (CIP)	2	
DEPC H_2O	5	

b) Content was mixed gently and spun briefly.

c) Allowed to incubate at 37^0C for one hour.

3. Termination of CIP reaction and extraction of RNA

a) Following components were assembled in an RNase free microfuge tube on ice:

Component	Volume (µl)
Ammonium acetate solution	15
DEPC H_2O	115
Acid phenol:chloroform (AM9720)	150

b) The homogenate was vortexed thoroughly and centrifuged for 5 min at top speed in microfuge (≥10,000 g) and aqueous phase (top layer) transfered to a new tube.

c) 150 µl chloroform was added, vortexed thoroughly.

d) Centrifuged 5 min at top speed in microfuge (≥10,000 g) and aqueous phase (top layer) was transfered to a new tube.

4. Precipitatation with isopropanol

a) Isopropanol 150 µl was added, vortexed thoroughly and chilled on ice for 10 min.

b) Centrifuged for 20 minutes at maximum speed and the pellet was rinsed with 0.5 ml cold 70 per cent ethanol. Again centrifuged for 5 min at maximum speed.

c) Ethanol was removed carefully and pellet allowed to air dry.

5. TAP treatment

Resuspended the RNA pellet in Nuclease-free water and 1µl of CIP-treated RNA was reserved at -20^{0}C for a minus-TAP control reaction.

a) Following components were assembled in an RNAase-free microfuge tube:

Component	Standard rxn (µl)	TAP (-) control
CIP treated RNA	5	4
10X TAP buffer	1	-
Tobacco Acid Phosphatase	2	1
DEPC H_2O	2	-

b) Components were mixed gently, spun briefly and incubated at 37^{0}C for one hour.

c) Reaction was stored at -20^{0}C or proceeded to ligation step.

6. RACE Adapter Ligation

a) Following components were assembled in an RNAase-free microfuge tube on ice:

Component	Actual volume (µl)
CIP/TAP-treated RNA	2.5
5'RACE Adapter	0.5
10X RNA Ligase Buffer	0.5
T_4 RNA Ligase (2.5U/µl)	1
DEPC H_2O	0.5

(Before use, 10X RNA Ligase buffer was warmed quickly by dipping the ligase vial in the small water bath to resuspend any precipitate. Since it contained ATP, heated $\leq 37^0$C)

b) Components were mixed gently and spun briefly.

c) Reaction was kept for incubation at 37^0C for 1 hour.

d) Stored at -20^0C or proceeded to reverse transcription.

3.8.2.2 Reverse Transcription

a) RT reaction (20µl reaction) was assembled by combining primer, total RNA, dNTP mix and DEPC water in tube placed on ice. The reaction mix consisted of

- Ambion Random Decamer (50µM) - 2µl
- Ligated RNA - 2µl
- dNTP mix (10mM) - 4µl
- 10X RT Buffer - 2 µl

- RNase inhibitor - 1 µl
- M-MLV Reverse Transcriptase - 1 µl
- Nuclease-free water - 8 µl

b) Reaction was mixed gently and spun briefly.

c) Incubated at 42^0C for one hour and stored at -20^0C.

7) The cDNA synthesized was then used as a template for amplification in PCR.

3.8.2.3 PCR amplification

PCR reaction was performed with the nested primers which were designed one within another to amplify the same transcript sequence. In nested PCR spurious products will be avoided and correct sequence having overlap will be amplified. So outer PCR was performed with first set of nested pimers and inner PCR was performed with second set of primers. A minus-template control was kept to check contamination of reaction with one or more PCR reagents.

3.8.2.3.1 Outer 5' RLM-RACE PCR

a) Following components were assembled in the PCR tubes on ice:

- RT reaction (from the previous step) - 1 µl
- 10X PCR Buffer - 5 µl
- dNTP mix - 4 µl
- 5' RACE gene specific outer primer (10µM) - 2 µl
- 5' RACE Outer primer - 2 µl
- Nuclease-free water - to 50 µl
- Thermostable DNA polymerase (5U/µl) - 0.25 µl

c) Components were mixed gently, spun briefly to return the contents to the bottom of the tube.

d) PCR was cycled as follows:

	Stage	Cycles	Temp	Time
Initial denaturation	1	1	94^0C	3 min
Amplification	2	35	94^0C 60^0C 72^0C	30 sec 30 sec 30 sec
Final extention	3	1	72^0C	7 min

3.8.2.3.2 Inner 5' RLM-RACE PCR

Outer PCR product from previous cycle was used as template (3 to 5 µl). Nested 5' RACE gene specific inner antisense primer along with sense 5' RACE inner primer was used for amplification. PCR cycling profile used was same as in outer 5' RLM-RACE PCR.

3.8.2.3.3 Gel analysis of products

After PCR was complete, 10 µl of each sample was run in a 2 per cent high resolution agarose gel containing 1µg/ml ethidium bromide and visualized on UV transilluminator. A sample of the outer PCR was also run for evaluation since the product may be sometimes visible after the primary PCR. There should be one to a few bands from nested PCR from the experimental samples and the minus control sample should have no visible PCR product.

3.8.3 3' RACE

First strand cDNA was synthesized from total RNA, using 3' RACE adapter supplied. The cDNA was then subjected to PCR using 3' RACE outer primer which was complementary to the anchored adapter along with a gene specific nested primer.

3.8.3.1 Reverse transcription

The procedure performed for single stranded cDNA synthesis was same as given in 3.8.2.2 explained earlier.

3.8.3.2 PCR for 3'RLM-RACE

3.8.3.2.1 Outer 3'RACE RLM-PCR

a) Following components were assembled in RNase free PCR tube on ice.

- RT reaction -1µl
- 10X PCR Buffer - 5µl
- dNTP mix - 4µl
- 3' RACE gene-specific outer primer (10µM) - 2µl
- 3' RACE Outer Primer -2 µL
- Nuclease-free water - to 50 µl
- Thermostable DNA polymerase (5U/µl) - 0.25 µl

b) Components were mixed gently and spun briefly to return the contents to the bottom of the tube.

c) PCR cycle profile set was as follows

	Stage	Reps	Temp	Time
Initial denaturation	1	1	94^0C	3 min
Amplification	2	35	94^0C 60^0C 72^0C	30 sec 30 sec 30 sec
Final extention	3	1	72^0C	7 min

3.8.3.2.2 Inner 3' RLM-RACE PCR

Outer PCR product from previous cycle was used as template (1 to 2 µl). Nested 3' RACE gene specific inner sense primer along with antisense 3' RACE inner primer was used for amplification. PCR cycling profile used was same as in inner 5' RLM-RACE PCR.

3.8.3.3 Gel purification of products

The procedure performed was same as 3.6.3 explained earlier.

3.9 Cloning

The amplicons obtained after the RACE reaction were cloned in pGEMT vector and *E. coli* cells were transformed for sequencing and storage.

3.9.1 Preparation of competent cells

Competent cells for and and transformation of 3' RACE product were prepared using Genei Competent cell preparation Kit (B) from Bangalore Genei, following the manufacturer's guideline.

Day 1

1. *Escherichia coli* JM 109 was streaked on LB agar plate from stab and incubated at 37°C for 16 to 18 hours.

Day 2

1. 100 ml SOC broth in 1000 ml conical flask was inoculated with 10 to 12 moderately sized colonies from SOC plates.
2. Overnight incubation was given at 37^0C in a shaker at 200 rpm. When the OD_{600} reached 0.3 (3 to 3.5 h only), growth was arrested by chilling. The flask was chilled for 20 min.
3. The entire culture was transferred into a 50 ml centrifuge tube and centrifuged at 3500 rpm for 15 min at 4^0C.
4. The supernatant was discarded. Keeping the tubes on ice, resuspended the bacterial pellet very gently in 33.3 ml ice cold solution A.
5. The tubes were kept on ice for 20 min and then centrifuged at 3500 rpm for 15 min at 4^0C.
6. Supernatant was discarded and pellet chilled on ice. The pellet was resuspended in 5 to 6ml of ice cold solution A.
7. The suspension was kept on ice for 10 min and aliquots of 100µl were dispensed in chilled 1.5ml Eppendorf tubes.
8. The tubes were frozen on ice for few min before storing at -70^0C.
   The competence of the cells prepared was confirmed by transformation using pUC18. The cells were plated on a plate containing LBA + 50mg/l ampicillin.

3.9.2 Ligation

The eluted product was ligated in pGEMT vector using pGEMT Easy Vector System (Promega Corporation, USA), following the manufacturer's directions.

1. Reaction mixture was prepared as described below:

2X rapid ligation buffer	-	5.0 µl
pGEMT Easy Vector (50ng)	-	1.0 µl
PCR product	-	3.0 µl
T4 DNA ligase (3 units/µl)	-	1.0 µl
		10.0 µl

2. The reaction mixture was incubated for one hour at room temperature. Then it was kept at 4^0C overnight. Next day it was used for transformation in competent cells of *E. coli.*

3.9.3 Transformation of ligated DNA into competent cells

1. The vial containing competent cells was thawed on ice.
2. The ligated product was added to the competent cells, contents mixed gently and kept on ice for 40 min.
3. The tube was taken from ice; heat shock was given at 42^0C exactly for 90 sec without shaking and placed back on ice for 5 min.
4. 250 µl of LB broth was added and the tube was inverted twice
5. The tube was incubated at 37°C for one hour with shaking.
6. 100 µl and 250 µl aliquots of the transformed cells were plated on LB/ampicillin (50 mg/l) plates layered with IPTG (6 µl) and X-gal (60 µl). (Stock: Ampicillin-5 mg/ml in water, IPTG-200 mg/ml in water, X-gal-20 mg/ml in DMSO) and incubated overnight at 37°C.
7. The recombinant clones were selected based on blue-white screening.

3.9.4 Confirmation of the presence of DNA insert by colony PCR

Colony PCR was carried out with recombinant clones to confirm the presence of inserted DNA. Recombinant bacterial colony was taken up by inoculation loop, mixed with 20µl sterile water and kept at 94°C for 2 min. After a

brief centrifugation to sediment the bacterial cell constituents, 2µl of supernatant was taken and used as a template DNA for amplification of specific DNA insert. The composition of master mix and PCR set for colony PCR is given in Appendix III.

3.10 Sequencing and Sequence analysis

The colonies were sent for double pass sequencing and the data obtained was analysed using various standard bioinformatics tools like VecScreen, EMBOSS merger tool, Blastn, Blastx, ORF finder, GenScan, EMBOSS Transeq tool, InterProScan, CDD, Pfam.

3.7.1 Vector screening

To remove the vector regions present in the sequences, vector screening was performed using VecScreen tool (www.ncbi.nlm.nih.gov/VecScreen) provided by NCBI. The vector and the adaptor sequences present were removed using Bioedit- Biological sequence alignment editor tool.

The sequence was then processed by trimseq tool (http://www.psc.edu/general/software/packages/emboss/appgroups/trimseq.html) provided by EMBOSS and ambiguous bits were trimed off from the ends of the sequence.

3.7.2 Search for Homology

The nucleotide sequence of all the sequences were compared with the sequences available in nucleotide database using BLAST tool (www.ncbi.nlm.nih.gov/Blast/; Altschul *et al.*, 1997) provided by NCBI. Nucleotide- Nucleotide sequence comparison was done using Blastn tool, while nucleotide-protein sequence comparison was done using Blastx and Blastp. The best sequence alignment results were noted and saved.

3.7.3 Detection of Open Reading Frame (ORF)

To find the open reading frame of the insert nucleotide sequence, the programmed 'ORF finder' (www.ncbi.nlm.nih.gov/gorf/gorf.html) of NCBI was used. The displayed web page showed ORF sequence in all reading frames. Open reading frames available in the entire region were noted and saved.

3.7.4 Amino acid analysis

The nucleotide sequences were translated into amino acid sequences using the tool Transeq (http://www.ebi.ac.uk/Tools/emboss/transeq/) provided by EMBOSS. The amino acid sequences obtained were analyzed to locate functional domains and active sites of protein using 'InterProScan' (www.ebi.ac.uk/InterProScan/; Zdobnov *et al.*, 2001).The conserved regions were detected using Conserved domain database (CDD) (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd. shtmlCDD) provided by NCBI. Functional motifs, domains and Hidden Markol Model (HMM) sequence were detected using Pfam tool (http://pfam.sanger.ac.uk/).

3.7.5 GENSCAN analysis

GENSCAN program was used to identify exons, promoter sites and polyA sites. The program was accessed at (http://genes.mit.edu/GENSCAN.html, Karlin, 1997).

3.7.6 Detection of repeat regions

Repeat regions and low complexity regions were detected by using RepeatMasker on-line tool.

Results

4. RESULTS

The results of the investigation conducted on the "Isolation and characterization of water stress activated protein kinase gene from black pepper (*Piper nigrum* L.) var. Kalluvally" undertaken during the period 2010-2011 at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University, Vellanikkara are presented in this chapter.

4.1 Maintenance of source plants

Potted plants of var. Kalluvally subjected to water stress have shown survival upon withholding water for one week with significant wilting and leaf drop. The plants also showed good revival and growth even after frequent water stress (Table 1a and 1b).

4.2 Selection of drought specific clones

The clones named PNK21 and PNK49 were selected from drought specific cDNA library of black pepper (*Piper nigrum* L.) var. Kalluvally maintained as glycerol culture at CPBMB.

4.3 Sequencing of the selected clones

The culture was revived on LB/ampicillin medium (Plate 2) and it yielded sufficient single colonies. Colony PCR performed with T7 and SP6 primers and the agarose gel electrophoresis confirmed the presence of the insert in the selected colonies (Plate 3). Then stabs were sent for automated sequencing. The background sequences available for the sequence enrichment are given in Appendix 1.

The sequence data obtained for PNK-21 for T7 forward and SP6 reverse sequencing was 591 bp and 603 bp respectively. Vector screening indicated vector sequence from 1 to 37 bp for forward sequencing and 1 to 61 bp for reverse sequencing. The details of results obtained for *in silico* analysis are presented in

Table 1a Water stress response of black pepper var. Kalluvally

Sl. No.	No. of plants observed	No. of days of water stress	No. of plants revived upon irrigation	Percent revival
1.	15	2	15	100
2.	15	3	15	100
3.	15	4	15	100
4.	10	5	9	90
5.	10	6	5	50
6.	10	7	2	20

Table 1b Revival of black pepper var. Kalluvally after frequent water stress

No. of plants observed	Stress Treatment (days)	Water stress interval	Interval between stress (days)	Percent revival
25	5	Once in a month	25	100
25	5	Twice in a month	10	100
25	5	Thrice in a month	5	80

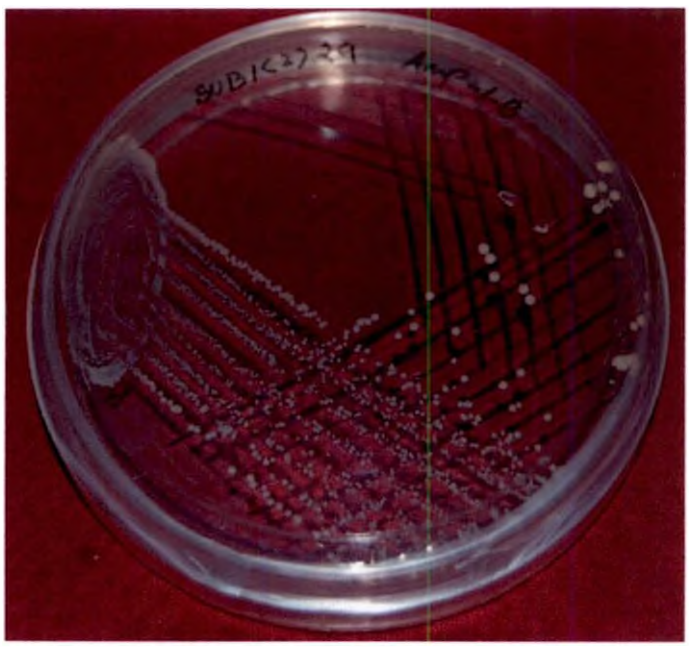
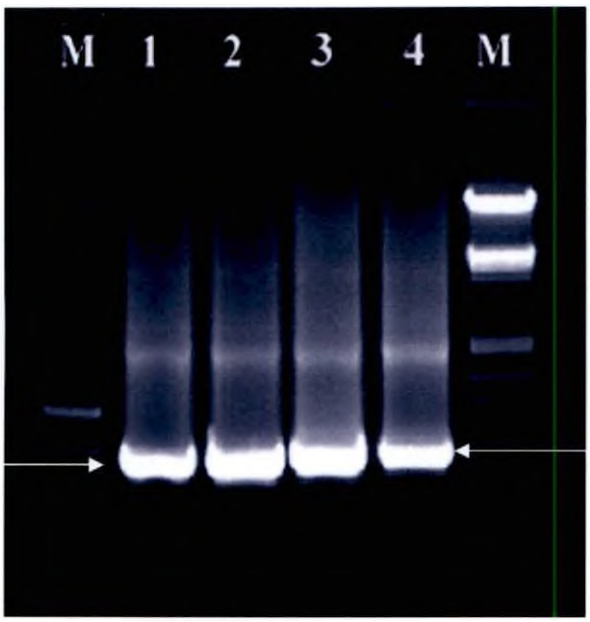


Plate 2. Single colonies of selected clone on LB/ampicillin medium



M= λDNA/*Eco*RI+*Hin*dIII marker

1, 2 = PNK21 insert

3, 4 = PNK49 insert

Plate 3. Colony PCR for PNK21 and PNK49 clones

Fig.1. After vector and adaptor screening both forward and reverse sequences gave 920 bp of sequence. This fragment was designated as PNK-21A and the sequencing data is as detailed below,

>PNK-21 A

5′GTCAGACTTTGGATTGACACACCAAGGGCCTGGTGAAGGACTTAGTCATGTCT
CAACTGTTTTTGTTGGGACTATAGGGTATGCATCACCAAAATATATTCAAACTGG
ACGTTTGACGGCTAAAAGTGATGTTTGGAGCTATGGAGTTGTCCTCTATGAACTG
ATCACTGGTAAAAAGCCAATAGACCGTACTCGTCCTAAGGGTGAACATAAACTAC
TTGAGTGGGTGAAGCCTTATTTATCAGAGAAGCGTTTTTCCATGATTCTTGATCC
AAGAATACAAAAATAGATTTCCCTGAAGTCGGCCCAAAAGCTTGCCGCTGTTGCA
AACAAATGCCTTGTACGACAGCAGAAGGCACGTCCAAAGATGAGTGAGATTTTAG
CGATTGTGAAGCAAATTGTGGAGTCATCAACAACTGGGTCTCCTCAACCTGCCAT
CAAAATCCTAGATTCAAGTGAAGTCTCCAATGAAGAACACAAAAACAGAAGCCGA
TTGAAACGAGCCATTGCTTATCTTAATATGGGTGGAGATGGCAAGAGGTTTTACT
GGTGGAAGTTCAAATCCAAGCTCAGTAAAGTTAAGTTGTGGAAGACGAAGGATAG
GACTAATGGTTTCTAGGCTAGCGTCATGATAAGAAACCAGGCATAGGAGAAGTTG
GGAAGTTTTTAAGAAGATGGCCTAAAGAATCCACCATAAAGTGTCAAAGATGTCC
TAGCCAAAACCTCAGCAGATTATGTAGGCAAATCTCTTAGTGGTGGACAACAATG
GCTAATTTAGAATCTGAACCCAATGTGTGTATCTAACCATTGTTGCTGATTTGGC
AATTGCTTTCCTATTACTGATGTTGAATTTGAGAAGATGAAGACACATGCTTTGG
ATCGCAGAGTGTTGGACAGTACCTGCCCGGGCGGCCGCTCGA 3′

Sequence information obtained for PNK49 for T7 forward and SP6 reverse sequencing was 730 bp each. Vector screening indicated vector sequence from 1 to 204 bp and 697 to 730 for forward sequencing and 1 to 16 and 491 to 730 bp for reverse sequencing. The details of results obtained for *in silico* analysis are presented in Fig. 2. After vector and adaptor screening both forward and reverse sequence gave 697 bp of sequence. The fragment was designated as PNK49A and the sequencing data is as detailed below,

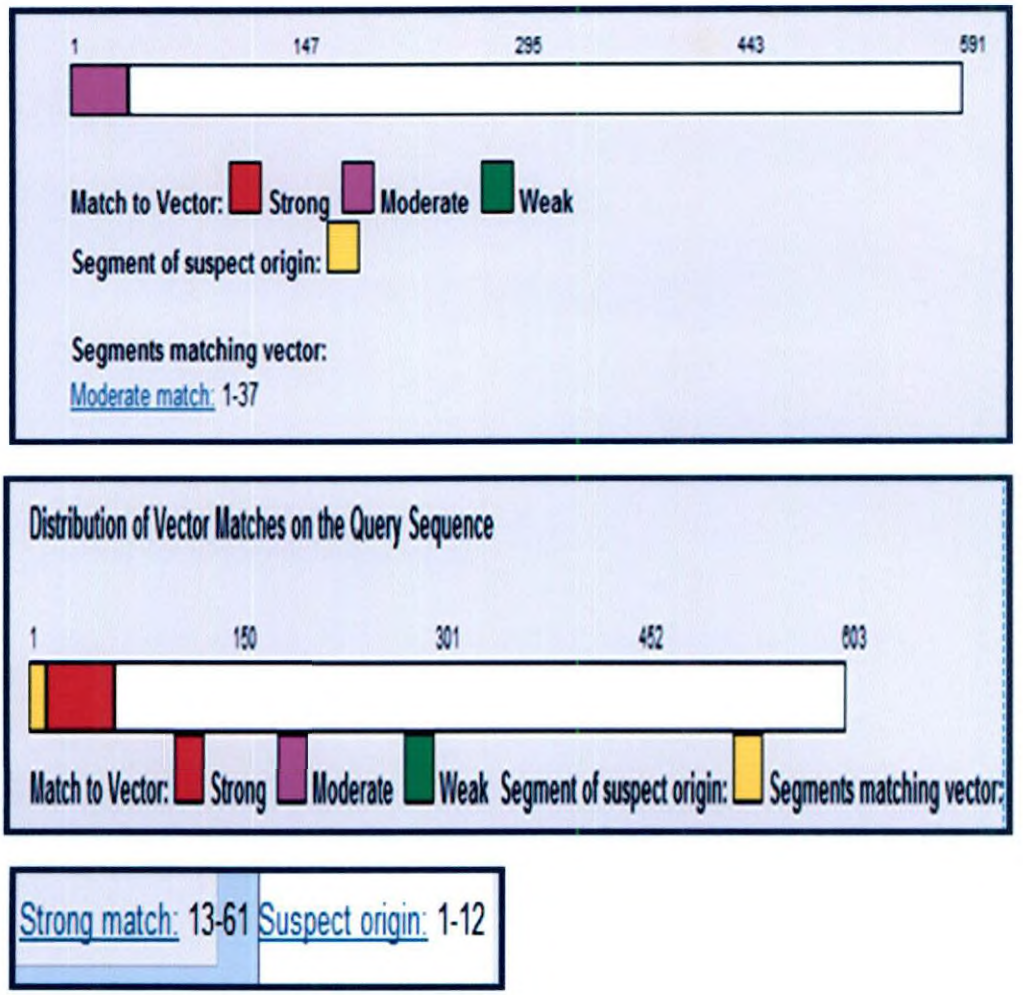


Fig. 1. Adaptor and vector screening result for PNK21 clone

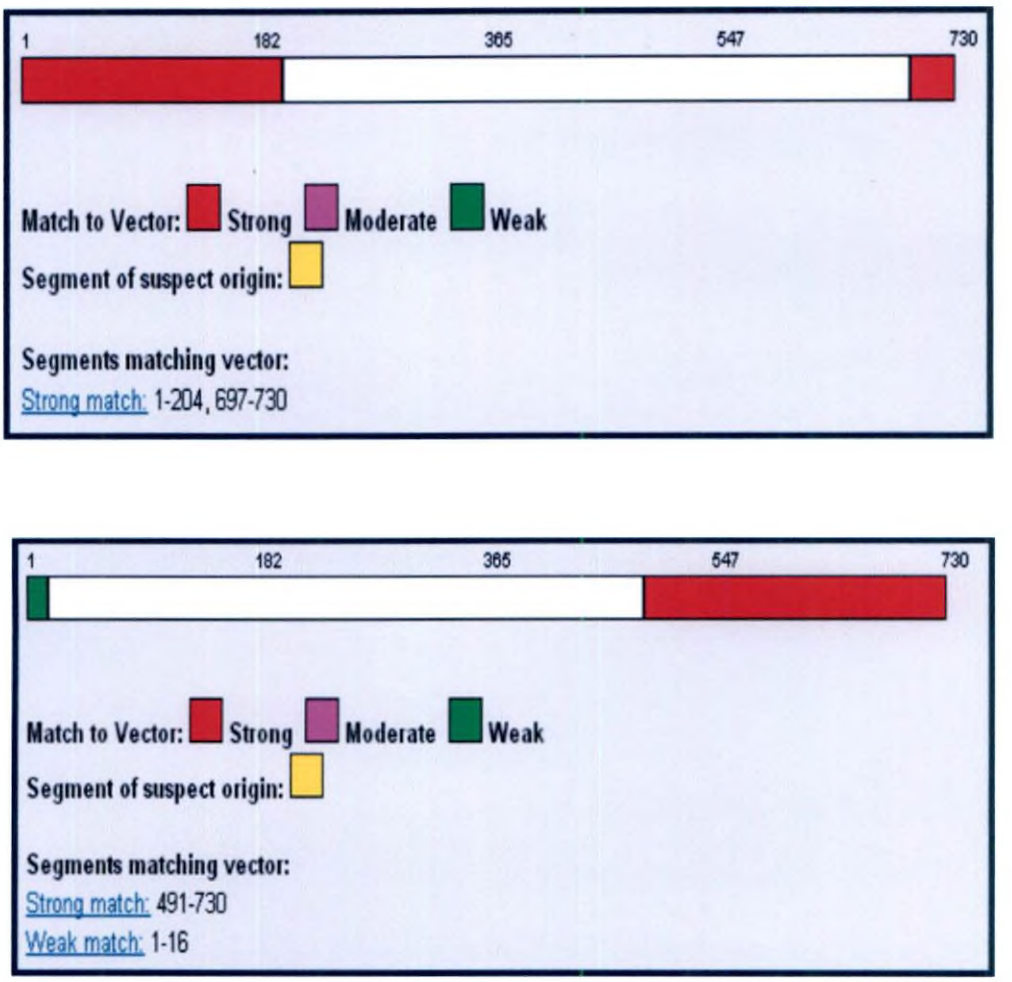


Fig. 2. Adaptor and vector screening result for PNK49 clone

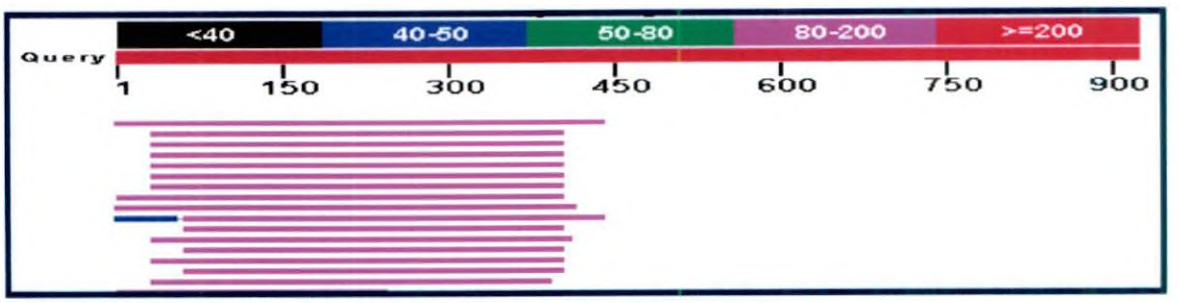
>PNK-49A

5′TCGAGCGGCCGCCCGGGCAGGTACTTCTCTGAGGTGAATTTGTTGGGGAAGAG
CAATCATGCAGCAACTTATAAAGGAATGTTGAGAGATGGTTCTTTTGTTGTTGTG
AAAAGATTTAGCAAAACCAGTTGCAAATCAGAGGAGGTGGAATTCCTGAAAGGGC
TCAAGATCCTTATGTCGTTGCAGCATGAAAACCTTGTTAGGCTTAAGGGTTTTTG
CTGCTCTAAATCTAGAGGTGAATGTTTTCTTGTATATGATTTTGTTGTTAATGGA
AATTTGCTGCAATATCTTGATTCTAGAGATGGAAGTGGGAGAATTCTTAATTGGT
CTACCAGAGTATCAATCATTCACGGCATCGCCAAAGCTATTCGCTACCTACATGC
CAGCAACCCTAGCAGGCCTTCACTTGTTCATCAGAACATCTCAGCTCAGAAGATC
CTCTTGGATAAGCATTTCAAACCATTGCTTTCAGACTCTGGCTTCCACAAGCTCC
TGGCCAACGATGCTATCTTTTCTACTATTAAATCTAGTGCTGCCATGGGGGTACC
TCGGCCGCGACCACGCCCTCCCCCTGACTCGTGGGCTCGGTCTTTCGCTGCGGCG
ACGTACCGCTCTCTCAAGGGGTAACGTTACCCAAAATCGGGTAAGCAGAAAAAAT
GGGACAAAGGCCACAAAGCCGGAACTTAAGGCCCGTCTG 3′

4.4 Blastn and Blastx analysis of PNK21A and PNK49A

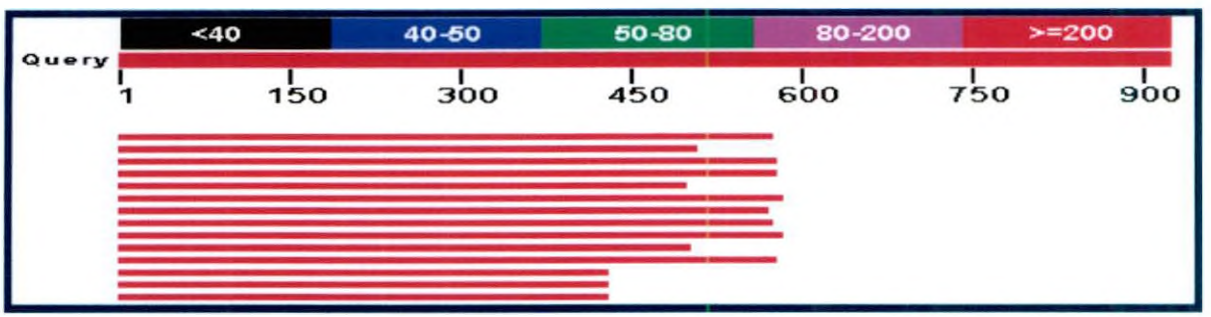
Blastn and Blastx analysis of PNK21A segment indicated homology with several kinase domain containing proteins from different plant species. In Blastx results, 55 to 62 per cent of the sequence showed over 62 per cent homology to mRNA encoding several hypothetical and known proteins from *Ricinus communis*, Arabidopsis, rice, *Vitis vinifera*, maize etc. So the complete cDNA sequences of kinase genes from various plants were used to design degenerate primers based on multiple sequence alignment to enrich the sequence information towards 5' end of mRNA transcript. No conserved domains were detected for the PNK21A sequence. The details of result obtained for Blastn and Blastx analysis for PNK21 clone are given in Fig. 3.

Blastn and Blastx analysis of PNK49A segment indicated homology with leucine rich repeat receptor kinase protein from *Ricinus communis* and *Arabidopsis thaliana.* In Blastx results, 74 to 76 per cent of the sequence showed over 69 per cent homology to mRNA encoding leucine rich repeat receptor kinase



Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
XM_002273555.1	PREDICTED: Vitis vinifera hypothetical protein LOC100254784 (LOC1002	192	192	47%	4e-45	70%	GM
NM_202541.2	Arabidopsis thaliana protein kinase domain-containing protein (AT3G098	167	167	40%	2e-37	70%	UEGM
NM_111818.3	Arabidopsis thaliana protein kinase domain-containing protein (AT3G098	167	167	40%	2e-37	70%	UEGM
AY122981.1	Arabidopsis thaliana putative protein kinase (At3g09830) mRNA, comple	167	167	40%	2e-37	70%	UGM
AY091117.1	Arabidopsis thaliana putative protein kinase (At3g09830) mRNA, comple	167	167	40%	2e-37	70%	UGM
AK317245.1	Arabidopsis thaliana AT3G09830 mRNA, complete cds, clone: RAFL22-05	163	163	40%	2e-36	70%	UM
BX823222.1	Arabidopsis thaliana Full-length cDNA Complete sequence from clone GS	163	163	40%	2e-36	70%	UGM
XM_002530889.1	Ricinus communis Protein kinase APK1B, chloroplast precursor, putative,	161	161	43%	6e-36	69%	G

Blastn



Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
XP_002273591.1	PREDICTED: hypothetical protein [Vitis vinifera]	230	230	62%	1e-65	62%	UG
XP_002530935.1	Protein kinase APK1B, chloroplast precursor, putative [Ricinus communis]	216	216	55%	2e-64	63%	G
NP_001048536.1	Os02g0819600 [Oryza sativa Japonica Group] >dbj|BAD22970.1| putativ	212	212	62%	4e-63	54%	UG
EEC74260.1	hypothetical protein OsI_09473 [Oryza sativa Indica Group]	211	211	62%	8e-63	54%	
ACM89571.1	protein kinase [Glycine max]	213	213	54%	9e-63	63%	G

Blastx

Fig. 3. Blastn and Blastx analysis result for PNK21A sequence

protein from *Ricinus communis*, Arabidopsis etc. So the complete cDNA sequences of kinase genes from various plants were used to design degenerate primers based on multiple sequence alignment to enrich the sequence information towards 5' end of mRNA transcript. Protein kinase conserved domain was found for the PNK49A sequence. The details of result obtained for Blastn and Blastx analysis for PNK49 clone are given in Fig. 4.

4.5 Primer designing for enriching the available sequences

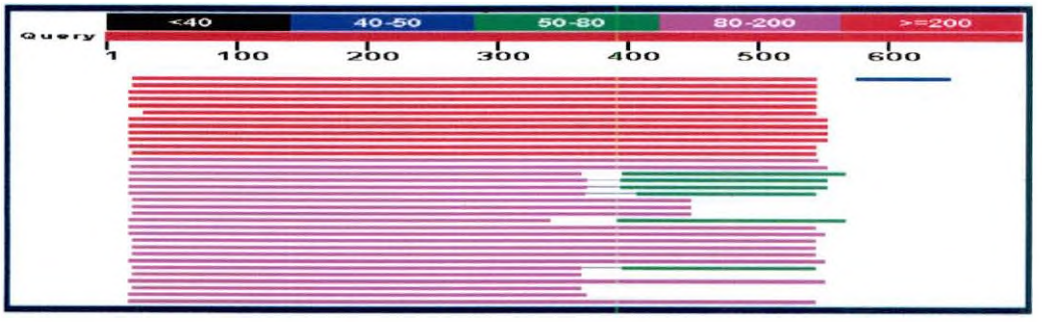
To enrich the protein kinase gene fragment from *Piper nigrum* L. by using RT-PCR, several complete cDNA sequences (cds) of protein kinases previously identified from various crop plants were used along with PNK21A and PNK49A. Most conserved regions were used to design degenerate oligonucleotide primers for RT-PCR. Maximum homology in the short and highly conserved region at the 5' end among protein kinase cDNAs was used to design degenerate primers. Details of the primer designed for above two clones are provided in Table 3 and 4.

With reference to other kinase cDNAs, the putative full-length gene for PNK21A was considered to be around 1400 bp. Thus, it was expected that there still remained at least 400 bp towards the 5' end and around 150 bp at the 3' end of the mRNA to be retrieved.

PNK49A full length cDNA was considered to be around 2000 bp. Thus, it was expected that there still remained at least 900 bp towards the 5' end and around 200 bp at the 3' end of the gene to be retrieved.

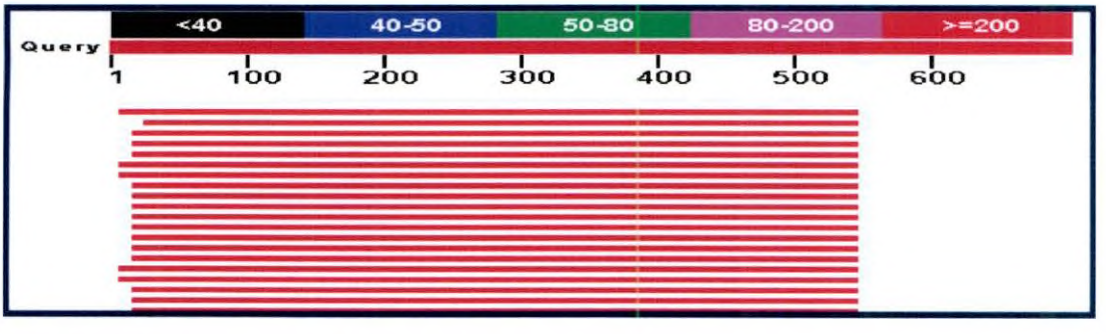
4.6 Isolation of total RNA from water stressed Kalluvally

The total RNA sample isolated from stressed plant, after agarose gel electrophoresis is presented in Plate 4. Three distinct bands were observed to represent 28S rRNA, 18S rRNA and small RNAs. Size of 28S rRNA band was observed to be more or less twice that of the 18S rRNA band.



Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
XM_002522365.1	Ricinus communis leucine rich repeat receptor kinase, putative, mRNA	255	255	74%	3e-64	70%	G
XM_002511650.1	Ricinus communis leucine rich repeat receptor kinase, putative, mRNA	244	244	74%	5e-61	70%	G
XM_002279962.1	PREDICTED: Vitis vinifera hypothetical protein LOC100247285 (LOC1002	239	239	74%	2e-59	70%	UGM
XM_002320022.1	Populus trichocarpa predicted protein, mRNA	230	230	74%	1e-56	69%	UG
XM_002510871.1	Ricinus communis leucine rich repeat receptor kinase, putative, mRNA	224	224	74%	5e-55	69%	G
XM_002301246.1	Populus trichocarpa predicted protein, mRNA	221	221	73%	6e-54	69%	G
FJ708800.1	Arabidopsis thaliana leucine-rich repeat receptor-like protein kinase (LR	219	219	76%	2e-53	69%	UGM

Blastn



Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
XP_002511696.1	leucine rich repeat receptor kinase, putative [Ricinus communis] >gb|EEI	256	256	76%	3e-82	69%	G
XP_002301282.1	predicted protein [Populus trichocarpa] >gb|EEE80555.1| predicted prot	251	251	74%	1e-81	68%	UG
XP_002279998.1	PREDICTED: hypothetical protein [Vitis vinifera]	259	259	75%	5e-80	69%	UG
CAN72186.1	hypothetical protein VITISV_012898 [Vitis vinifera]	259	259	75%	8e-80	69%	
XP_002510917.1	leucine rich repeat receptor kinase, putative [Ricinus communis] >gb|EEI	256	256	75%	2e-79	68%	G
XP_002320058.1	predicted protein [Populus trichocarpa] >gb|EEE98373.1| predicted prot	256	256	76%	3e-79	67%	UG
XP_002522411.1	leucine rich repeat receptor kinase, putative [Ricinus communis] >gb|EEI	256	256	76%	4e-79	69%	G
AAM62629.1	receptor-like protein kinase [Arabidopsis thaliana]	256	256	75%	8e-79	68%	

Blastx

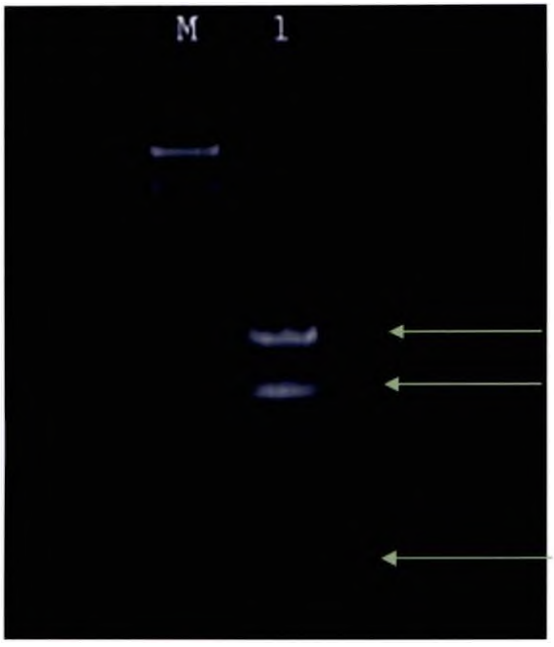
Fig. 4. Blastx and Blastn analysis result for PNK49A sequence

Table 2 Details of different accessions used for designing internal primers

Clone ID	Details of complete cDNA sequence		(cds) used		Primer details	
	Accession No.	**Plant species**	**Size of cds (bp)**	**Percent identity**	**Primer/ Strand**	**Primer length**
PNK21 A	XM_002519466.1	*Ricinus communis*	1269	71	N1 forward (+)	20 bp
	AY122981.1	*Arabidopsis thaliana*	1288	71		
	AY091117.1	*Arabidopsis thaliana*	1930	71		
	XM 002273555.1	*Vitis vinifera*	6151	72		
	XM_002530889.1	*Ricinus communis*	1473	71		
PNK49 A	XM_002522365.1	*Ricinus communis*	2000	74	N5 forward (+)	19 bp
	XM_002279962.1	*Vitis vinifera*	2067	74		
	XM_002320022.1	*Populus trichocarpa*	1950	74	N8 reverse (-)	16 bp
	AY136341.1	*Arabidopsis thaliana*	1675	45		
	XM_002510871.1	*Ricinus communis*	1811	74		

Table 3 Details of degenerate primers used in RT PCR for amplifying internal sequences

Clone ID	Primer/ Strand	Primer Sequence (5' to 3')	Source	Length	T_m
PNK21	N1 (+)	GCT/CCAAGATGCA/TGCTCGT/CGG	CDS	20 bp	63°C
	N2 (-)	TTGCCATCTCCACCCACCCATATT	PNK21	20 bp	63°C
PNK49	N5 (+)	TG/TCAAGTTT/CTGCAGCTC/T/GTG	CDS	19 bp	56°C
	N6 (-)	TGCTCTTCCCCAACAAATTC	PNK49	20 bp	62°C
	N7 (+)	AGGCCTTCACTTGTTCAT	PNK49	18 bp	52°C
	N8 (-)	GGAGT/CTCATGAG/TTGCA	CDS	16 bp	50°C



28S rRNA
18S rRNA

5S rRNA
+small RNAs

M= λDNA/*Eco*RI+*Hin*dIII Marker

1= Total RNA

Plate 4. Total RNA isolated from water stressed Kalluvally

The values for spectrophotometric analysis of total RNA isolated showed absorbance ratio of 260/280 ranged between 1.90 and 2.0, while 260/230 ranged between 1.5 and 2.0, which indicated good quality of RNA. The ratio of the peak areas (28S/18S) corresponding to 28S and 18S was used as a reference for RNA degradation as in an intact RNA sample 28S peak area should be double that of the 18S. The quantity of total RNA ranged between 400 ng/µl and 900 ng/µl. The total RNA isolated was good enough for cDNA synthesis.

4.6.1 cDNA synthesis and RT-PCR for internal sequence amplification

Single stranded cDNA was synthesized from total RNA sample and it was used for PCR amplification. Various conditions including different concentration of template, primers and temperature were attempted with each set of primers in degenerate RT-PCR reactions. Details of procedures followed and optimization is given in Table 5.

Degenerate RT-PCR for PNK21A produced a 600 bp fragment as shown in Plate 5. The expected PCR product was gel purified and sent for sequencing.

RT-PCR for PNK49A amplified a 600 bp fragment (Plate 5) against the expected amplicon of 800 bp, but could not produce consistent results even after reamplification.

The amplicon of expected size for PNK21 was gel purified with the concentration of the purified DNA 21.6 ng/µl. PNK21A forward and reverse sequencing result is as follows.

>PNK-21A_FWD

GGGATTTTCAGATTATATTTAGAGATTTTAAGACGTCAAATATACTTTTAGACAA
AGATTGGAATGCCAAGTTGTCAGACTTTGGATTGGCTCGCCAAGGGCCTGGTGAA
GGACTTAAACATGTCTCAACTGCAGTTGTTGGGACTA

Table 4 Details of the optimization conditions used for RT PCR

Sl. No.	Clone ID	Treatment	Amplicon expected	Presence of amplicon	Amplicon obtained
1.	PNK21A	$D_3 P_2 T_3 C_3$	600 bp	Yes	600 bp
		$D_3 P_2 T C_3$	600 bp	No	-
		$D_1 P_2 T C_1$	600 bp	No	-
		$D_2 P_2 T C_4$	600 bp	No	
3.	PNK49A	$D_3 P_2 T_4 C_3$	800 bp	Yes	600 bp
		$D_2 P_2 T C_4$	800 bp	No	-
		$D_1 P_3 T C_1$	800 bp	No	-
		$D_3 P_2 T C_3$	200 bp	No	-

PCR set used:

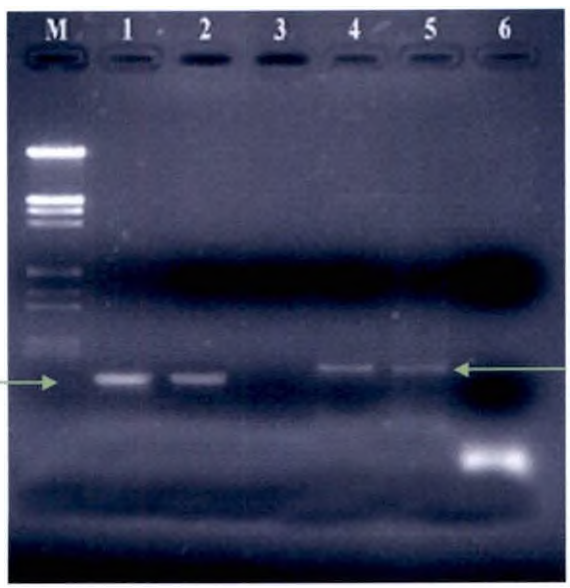
- Template (cDNA) (µl) (D)
- Primer concentration (pM) (P)
- Annealing temp (T)
- No of cycles (C)

$\mathbf{D_1}= 2$, $\mathbf{D_2}= 3$, $\mathbf{D_3}= 4$

$\mathbf{P_1}= 5$, $\mathbf{P_2}= 10$, $\mathbf{P_3}= 20$

$\mathbf{T_1}= T_m-2$, $\mathbf{T_2}= T_m- 3$, $\mathbf{T_3}= T_m-4$, $\mathbf{T_4}=T_m-5$

$\mathbf{C_1}= 45$, $\mathbf{C_2}= 37$, $\mathbf{C_3}= 35$, $\mathbf{C_4}= 30$



M= λDNA/*Eco*RI+*Hin*dIII Marker

1,2, 3 = PNK21 gene fragment

4, 5 = PNK49 gene fragment

6 = Negative control

Plate 5. RT-PCR product amplified with gene specific primer

>PNK-21A_REV

GGCTGGGTTTCACCGGGGCTTCTGTTTTTGTGTTCTTCATTGGAGACTTCACTTG
AATCTAGGATTTTGATGGCAGGTTGAGGAGACCCAGTTGTTGATGACTCCACAAT
TTGCTTCACAATCGCTAAAATCTCACTCATCTTTGGACGTGCCTTCTGCTGTCGT
ACAAGGCATTTGTTTGCAACAGCAGCAAGCTTTTGGGCCGACTTCAGGGAATCTG
TTCTTGTATTCTTGGATCA

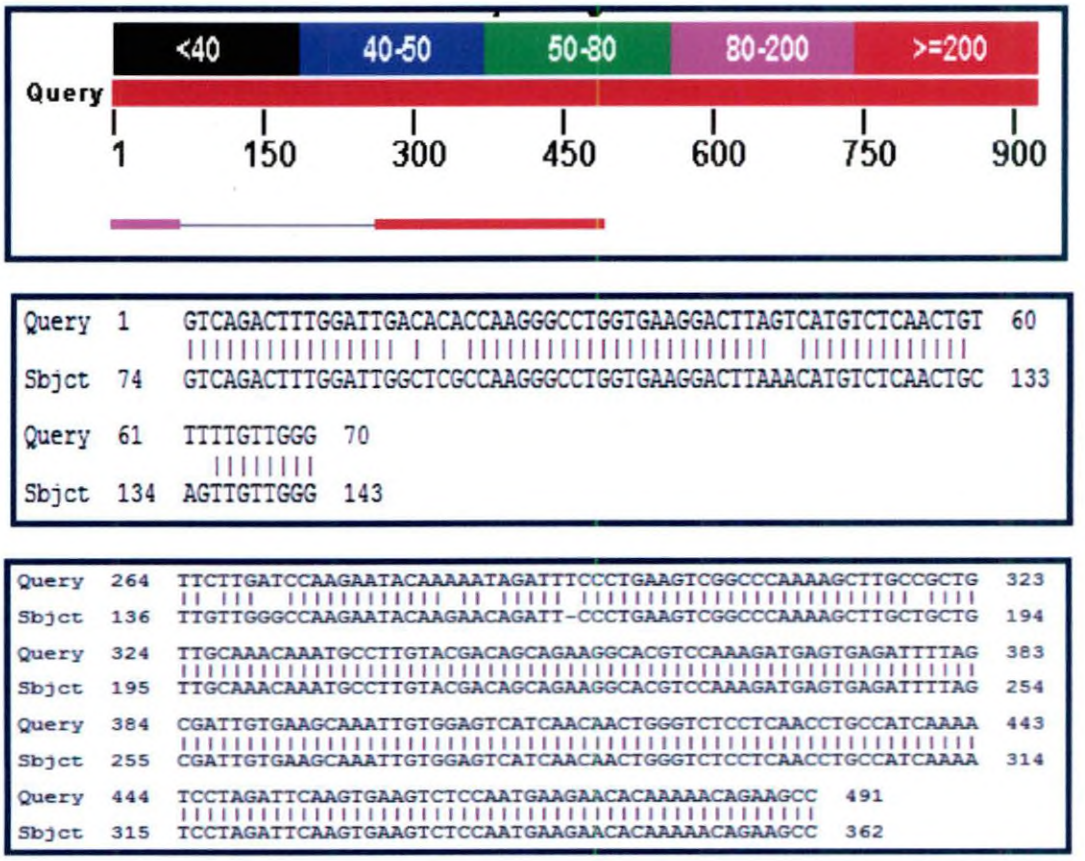
Both the sequences were subjected to vector and adaptor screening. Only forward sequence data showed sequence enrichment towards 5' end whereas reverse sequence data showed 100 per cent overlap with background sequence.

The overlapping between forward sequence (147 bp) obtained towards 5' end with PNK21A is as detailed below,

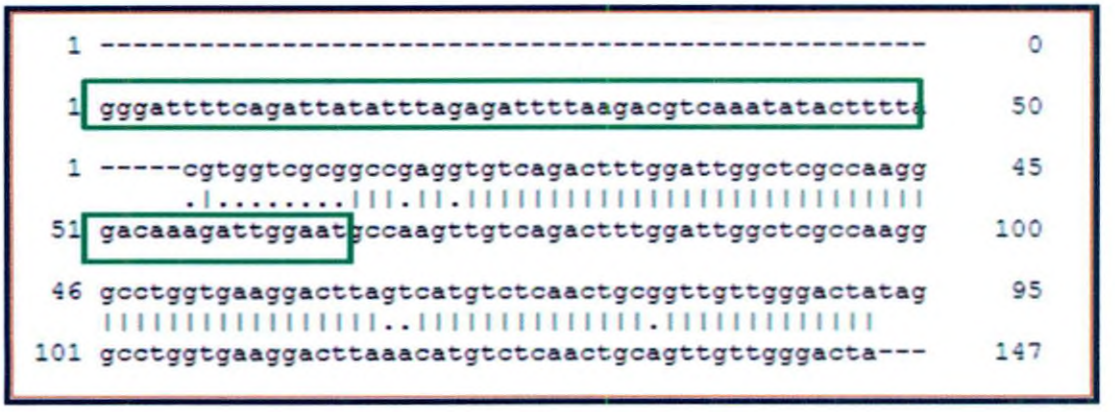
```
  1 ---------------------------------------------------      0

  1 gggattttcagattatatttagagattttaagacgtcaaatatactttta     50

  1 -----cgtggtcgcggccgaggtgtcagactttggattggctcgccaagg     45
        .|........|||.||.||||||||||||||||||||||||||||
 51 gacaaagattggaatgccaagttgtcagactttggattggctcgccaagg    100

 46 gcctggtgaaggacttagtcatgtctcaactgcggttgttgggactatag     95
    ||||||||||||||||..|||||||||||||.|||||||||||||
101 gcctggtgaaggacttaaacatgtctcaactgcagttgttgggacta---    147
```

Forward and reverse sequence data has shown an overlapping of 91 bp with the PNK21A at 5' end. So only 66 bp sequence out of 147bp was merged. The details of vector and adaptor screening of RT PCR sequencing result is given in Fig. 5.

Before proceeding to the RLM-RACE PCR, the gene fragment available was 990 bp (designated as PNK21B) which was obtained by merging RT-PCR forward sequence and PNK21A as given below,



Blastn between forward and reverse RT sequence with PNK21A



Blast2n between forward RT sequence and PNK21A

Fig. 5 RT-PCR sequence analysis for PNK21 clone

5′GGGATTTTCAGATTATATTTAGAGATTTTAAGACGTCAAATATACTTTTAGAC
AACGTGGTCGCGGCCGAGGTGTCAGACTTTGGATTGGCTCGCCAAGGGCCTGGTG
AAGGACTTAGTCATGTCTCAACTGCGGTTGTTGGGACTATAGGGTATGCAGCACC
AGAATATATTCAAACTGGACGTTTGACGGCTAAAAGTGATGTTTGGAGCTATGGA
GTTGTCCTCTATGAACTGATCACTGGTAGAAAGCCAATAGACCGTAATCGTCCTA
AGGGTGAACATAAACTACTTGAGTGGGTGAAGCCTTATTTATCAGAGAAGCGTTT
TTCCATGATTCTTGATCCAAGAATACAAGAATAGATTTCCCTGAAGTCGGCCCAA
AAGCTTGCCGCTGTTGCAAACAAATGCCTTGTACGACAGCAGAAGGCACGTCCAA
AGATGAGTGAGATTTTAGCGATTGTGAAGCAAATTGTGGAGTCATCAACAACTGG
GTCTCCTCAACCTGCCATCAAAATCCTAGATTCAAGTGAAGTCTCCAATGAAGAA
CACAAAAACAGAAGCCGATTGAAACGAGCCATTGCTTATCTTAATATGGGTGGAG
ATGGCAAGAGGTTTTACTGGTGGAAGTTCAAATCCAAGCTCAGTAAAGTTAAGTT
GTGGAAGACGAAGATAGGACTAATGGTTTCTAGGCTAGCGTCATGATAAGAAACC
AGGCATAGGAGAAGTTGGGAAGTTTTTAGAGATGGCCTAAAGAATCCACCATAAA
GTGTCAAAGATGTCCTAGCCAAAACCTCAGCAGATTATGTAGGCAAATCTCTTAG
TGGTGGACAACAATGGCTAATTTAGAATCTGAACCCAATGTGTGTATCTAACCAT
TGTTGCTGATTTGGCAATTGCTTTCCTATTACTGATGTTGAATTTGAGAAGATGA
AGACACATGCTTTGGATCGCAGAGTGTTGGACAGTACCTGCCCGGGCGGCCGCTC
GA3′

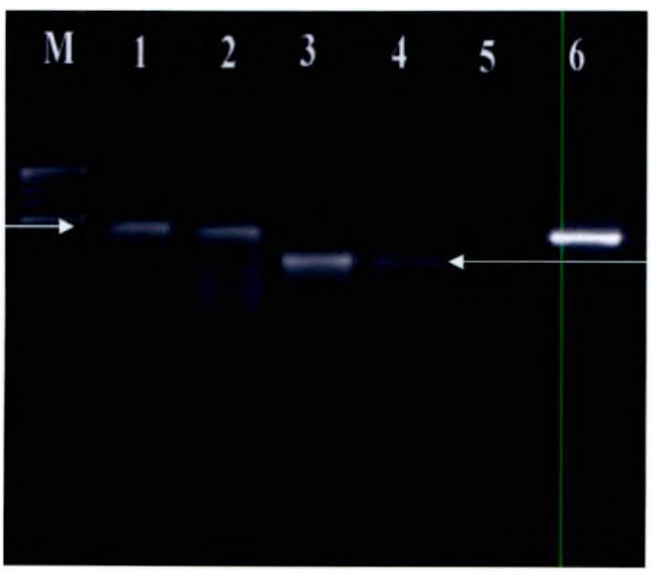
4.7 Rapid amplification of the cDNA ends (RACE) for full length cDNA cloning

To retrieve the missing 5' and 3' ends of the protein kinase gene fragments, specific primers were designed from the internal fragment (PNK21B) (Table 6).

The 3' RACE reaction was carried out to amplify and clone 3' end of the mRNA transcript. The first strand cDNA was synthesized from highly pure total RNA, using the supplied 3'RACE adapter. The cDNA was then subjected to PCR using F1 gene specific forward primers and the reverse adapter primer (3' RACE Adapter) generated only faint bands. Therefore, the nested 3' RACE reactions were performed using the initial 3' RACE PCR products as templates. Both the nested reactions produced observable bands (Plate 6). The nested PCR product was gel purified and cloned into the TA cloning vector for sequence analysis.

Table 5 Details of RACE primers designed for PNK21 clone

Sl. no.	Reaction type	Primer	Primer sequence (5' to 3')	Length	T_m
1.	3' RACE	F1	GATGTCCTAGCCAAAACCTCAG	22 bp	60.1°C
2.		F2	GGCAAATCTCTTAGTGGTGGAC	22 bp	60°C
3.		F3	GTTGTGGAAGACGAAGGATAGG	22 bp	60°C
4.		F4	GATCGCAGAGTGTTGGACAGTA	22 bp	60.3°C
5.	5'RACE	R1	CGGTCTATTGGCTTTTTACCAGTG	24 bp	63.04°C
6.		R2	AGGACAACTCCATAGCTCCAAACA	24 bp	63.34°C
7.		R3	GGACAACTCCATAGCTCCAAAC	22 bp	60°C
8.		R4	CCTTAGGACGAGTACGGTCTATTG	24 bp	60.41°C



M = 100 bp ladder

1, 2= F1 primer pair

3, 4 = F2 primer pair

5 = Negative control

6 = Positive control

Plate 6. 3' RACE reaction with nested primers

The recombinant clones containing insert were selected by blue-white screening and the details of the result obtained for blue- white screening is given in Plate 7.

Colony PCR was performed with T7 and SP6 primers for both white and blue colonies and the agarose gel electrophoresis confirmed the presence of the insert in the selected colonies (Plate 8). Then stabs were sent for automated sequencing.

A 353 bp sequence was isolated which was sharing overlapping region with PNK21B. The sequencing results indicated, the 3' termini of the protein kinase mRNA was successfully isolated.

The first 5' RACE reactions were carried out initially with the gene specific reverse primers R1 and R2 with the forward 5' RACE outer primer, which generated neither faint nor distorted bands. To obtain the desirable RACE products, nested PCR reactions were performed using the initial RACE products as the templates and their respective nested reverse primers, R3 and R4, and the forward 5' RACE inner primer, which was expected to produce the final RACE products with the expected size (Plate 9).

The forward and reverse sequencing result of 3'RACE is given as below.

>3' RACE T7 (forward)

GCCGCCATGGCCGCGGGATTGATGTCCTAGCCAAAACCTCAGCAGATTATGTAGG
CAAATCTCTTAGTGGTGGACAACAATGGCTAATTTAGAATCTGAACCCAACGTGT
GTATCTAACCATTGTTGCTGATTTGGCAATTGCTTTCCTATTACTGATGTTGAAT
TTGAGAAGATGAAGACACATGCTTTGGATCGCAGAGTGTTGGACAGTACTAGCAA
CATATTATCTGATAGGTTACGAGCTGATCATAATTGGTAAGTTTGTAAGTTATGC
AATTTTGTATATCTTAAATTGAGAAAGCATCATACATTCAACAGACACCAATGGA
AAAATGTTGTATTATGTTTTTTGTCAAAAGCTATAAATGAACTAAAAAAAAAAAA
CCTATAGTGAGTCGTATTAATTCTGTGCTCGCAATCACTAGTGCGGCCGCCTGCA

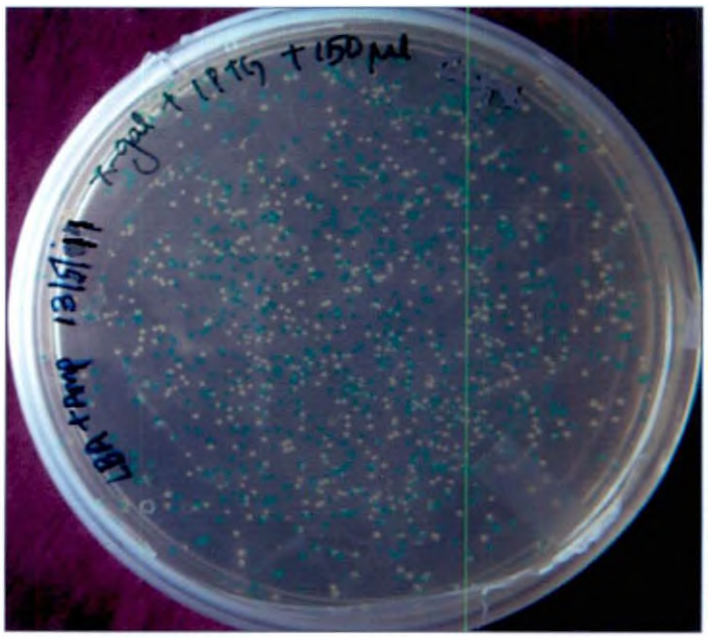
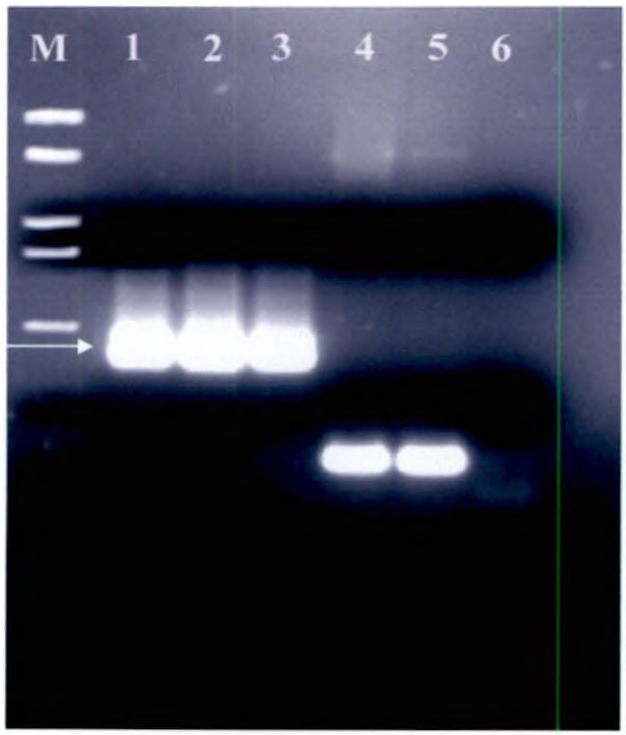


Plate 7. Blue- white screening for transformed cells on LB/ampicillin agar plate

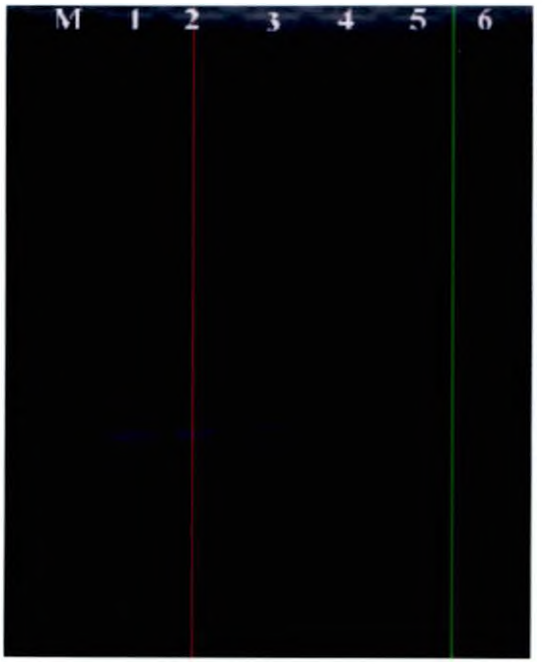


M – λDNA/*Eco*RI+*Hin*dIII marker

1, 2 & 3 – White colony

4, 5 – Blue colony

Plate 8. Colony PCR for 3' RACE clone



M = 100 bp ladder

1, 2 = R1 primer

3, 4 = R2 primer

5 = P3 primer

6 = Negative control

Plate 9. 5' RACE reaction amplified with three sets of primers

(No amplification)

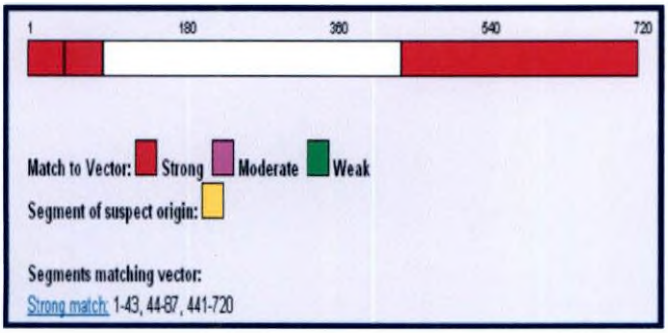
GGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTA
TAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAA
ATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAA
AGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTG
CCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAAC
GCGCGGGGAGAGGCG

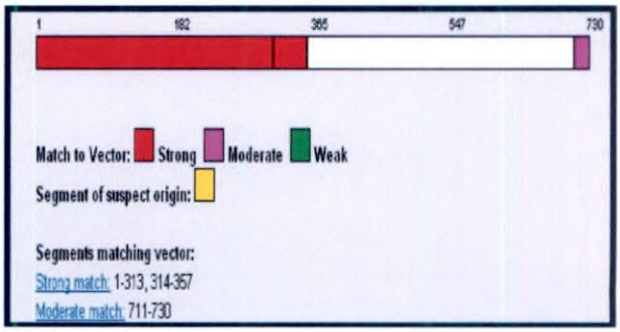
>3-RACE_SP6 (reverse)

GCTCTCCCATATGGTCGACCTGCAGGCGGCCGCACTAGTGATTGCGAGCACAGAA
TTAATACGACTCACTATAGGTTTTTTTTTTTTAGTTCATTTATAGCTTTTGACAA
AAAACATAATACAACATTTTTCCATTGGTGTCTGTTGAATGTATGATGCTTTCTC
AATTTAAGATATACAAAATTGCATAACTTACAAACTTACCAATTATGATCAGCTC
GTAACCTATCAGATAATATGTTGCTAGTACTGTCCAACACTCTGCGATCCAAAGC
ATGTGTCTTCATCTTCTCAAATTCAACATCAGTAATAGGAAAGCAATTGCCAAAT
CAGCAACAATGGTTAGATACACACGTTGGGTTCAGATTCTAAATTAGCCATTGTT
GTCCACCACTAAGAGATTTGCCTACATAATCTGCTGAGGTTTTGGCTAGGACATC
AATCCCGCGGCCATGGCGGCCGGGAGCATGCGACGTCGGGCCCAATTCGCCCTAT
AGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAA
ACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTG
GCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTG
AATGGCGAATGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGT
TACGC

The sequence obtained for the 3' RACE reaction was 720 bp for both forward and reverse sequencing. The details of sequence analysed to remove vector and adaptor region are presented in Fig. 6. After vector and adaptor screening, the total sequence obtained was found to be 365 bp as detailed below,

5′GATGTCCTAGCCAAAACCTCAGCAGATTATGTAGGCAAATCTCTTAGT
GGTGGACAACAATGGCTAATTTAGAATCTGAACCCAACGTGTGTATCTAACCATT
GTTGCTGATTTGGCAATTGCTTTCCTATTACTGATGTTGAATTTGAGAAGATGAA
GACACATGCTTTGGATCGCAGAGTGTTGGACAGTACTAGCAACATATTATCTGAT





Vector and Adapter screening

GCCGCCATGGCCGCGGGATTGATGTCCTAGCCAAAACCTCAG AGA
TTATGTAGGCAAATCTCTTAGTGGTGGACAACAATGGCTAATTTAGA
ATCTGAACCCAACGTGTGTATCTAACCATTGTTGCTGATTTGGCAAT
TGCTTTCCTATTACTGATGTTGAATTTGAGAAGATGAAGACACATGC
TTTGGATCGCAGAGTGTTGGACAGTACTAGCAACATATTATCTGATA
GGTTACGAGCTGATCATAATTGGTAAGTTTGTAAGTTATGCAATTTT
GTATATCTTAAATTGAGAAAGCATCATACATTCAACAGACACCAATG
GAAAAATGTTGTATTATGTTTTTTGTCAAAAGCTATAAATGAACTAA
AAAAAAAAAACCTATAGTGAGTGAACTAAAAAAAAAAAAACCTATA
GTGAGTCGTATTAATTCTGTGCTCGCAATCACTAGTGCGGCCGCCTG
CAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCT
TGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCAT
AGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAAC
ATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGA
GTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCA
GTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGC
GCGGGGAGAGGCG

Blue- forward primer
Green-3' RACE sequence
Black- PolyA tail

Red- Adapter complement
Purple- Vector sequence

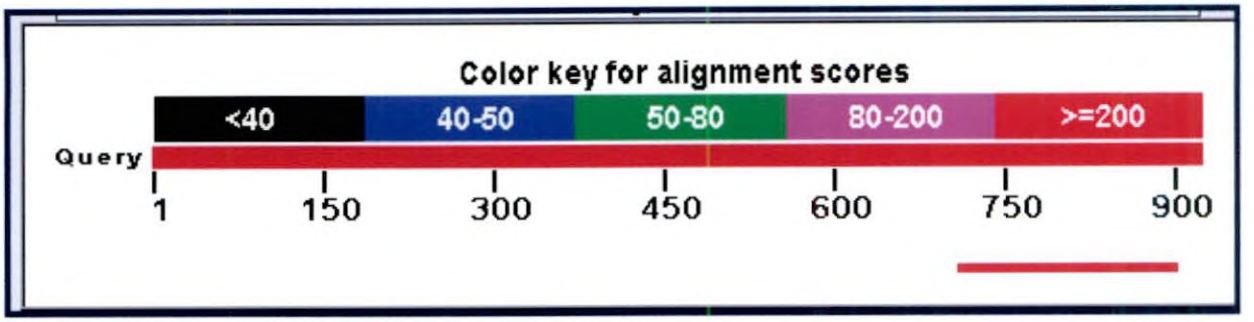
Fig. 6. Vector and Adapter screening for 3' RACE sequence

AGGTTACGAGCTGATCATAATTGGTAAGTTTGTAAGTTATGCAATTTTGTATATC
TTAAATTGAGAAAGCATCATACATTCAACAGACACCAATGGAAAAATGTTGTATT
ATGTTTTTTGTCAAAAGCTATAAATGAACTAAAAAAAAAAAA3′

3'RACE sequence and PNK21B had shown an overlapping region of 194 bp. So remaining 171 bp sequence of 3'RACE was merged with PNK21B. After merging both the sequences the total gene fragment enriched was 1141 bp. The details of overlapping between 3' RACE sequence data and PNK21B is given in Fig. 7.

PNK21C sequence subjected to Trimseq processing had shown no ambiguous bases and the output is given as below,

5′GGGATTTTCAGATTATATTTAGAGATTTTAAGACGTCAAATATACTTTTAGAC
AACGTGGTCGCGGCCGAGGTGTCAGACTTTGGATTGGCTCGCCAAGGGCCTGGTG
AAGGACTTAGTCATGTCTCAACTGCGGTTGTTGGGACTATAGGGTATGCAGCACC
AGAATATATTCAAACTGGACGTTTGACGGCTAAAAGTGATGTTTGGAGCTATGGA
GTTGTCCTCTATGAACTGATCACTGGTAGAAAGCCAATAGACCGTAATCGTCCTA
AGGGTGAACATAAACTACTTGAGTGGGTGAAGCCTTATTTATCAGAGAAGCGTTT
TTCCATGATTCTTGATCCAAGAATACAAGAATAGATTTCCCTGAAGTCGGCCCAA
AAGCTTGCCGCTGTTGCAAACAAATGCCTTGTACGACAGCAGAAGGCACGTCCAA
AGATGAGTGAGATTTTAGCGATTGTGAAGCAAATTGTGGAGTCATCAACAACTGG
GTCTCCTCAACCTGCCATCAAAATCCTAGATTCAAGTGAAGTCTCCAATGAAGAA
CACAAAAACAGAAGCCGATTGAAACGAGCCATTGCTTATCTTAATATGGGTGGAG
ATGGCAAGAGGTTTTACTGGTGGAAGTTCAAATCCAAGCTCAGTAAAGTTAAGTT
GTGGAAGACGAAGATAGGACTAATGGTTTCTAGGCTAGCGTCATGATAAGAAACC
AGGCATAGGAGAAGTTGGGAAGTTTTTAGAGATGGCCTAAAGAATCCACCATAAA
GTGTCAAAGATGTCCTAGCCAAAACCTCAGCAGATTATGTAGGCAAATCTCTTAG
TGGTGGACAACAATGGCTAATTTAGAATCTGAACCCAATGTGTGTATCTAACCAT
TGTTGCTGATTTGGCAATTGCTTTCCTATTACTGATGTTGAATTTGAGAAGATGA
AGACACATGCTTTGGATCGCAGAGTGTTGGACAGTACCAGCAACATATTATCTGA
TAGGTTACGAGCTGATCATAATTGGTAAGTTTGTAAGTTATGCAATTTTGTATAT




```
>lcl|49693
Length=353

 Score =  345 bits (382),  Expect = 3e-99
 Identities = 193/194 (99%), Gaps = 0/194 (0%)
 Strand=Plus/Plus

Query  707  GATGTCCTAGCCAAAACCTCAGCAGATTATGTAGGCAAATCTCTTAGTGGTGGACAACAA  766
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    GATGTCCTAGCCAAAACCTCAGCAGATTATGTAGGCAAATCTCTTAGTGGTGGACAACAA  60

Query  767  TGGCTAATTTAGAATCTGAACCCAATGTGTGTATCTAACCATTGTTGCTGATTTGGCAAT  826
            |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  61   TGGCTAATTTAGAATCTGAACCCAACGTGTGTATCTAACCATTGTTGCTGATTTGGCAAT  120

Query  827  TGCTTTCCTATTACTGATGTTGAATTTGAGAAGATGAAGACACATGCTTTGGATCGCAGA  886
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  TGCTTTCCTATTACTGATGTTGAATTTGAGAAGATGAAGACACATGCTTTGGATCGCAGA  180

Query  887  GTGTTGGACAGTAC  900
            ||||||||||||||
Sbjct  181  GTGTTGGACAGTAC  194
```

Fig. 7. Overlapping between 3'RACE product and PNK21B

CTTAAATTGAGAAAGCATCATACATTCAACAGACACCAATGGAAAAATGTTGTAT
TATGTTTTTTGTCAAAAGCTATAAATGAACT3′

4.8 Cloning of the 3' end of the protein kinase gene

It was anticipated that with the successful retrieval of both 3' and 5' end of protein kinase gene, the full-length fragment could then be assembled using the RACE products and the central EST fragment enriched by degenerate RT-PCR. But 5' end of cDNA was not retrieved fully. Sequence assembly revealed that the cDNA of the protein kinase was 1141 bp in length.

4.9 Nucleotide sequence analysis

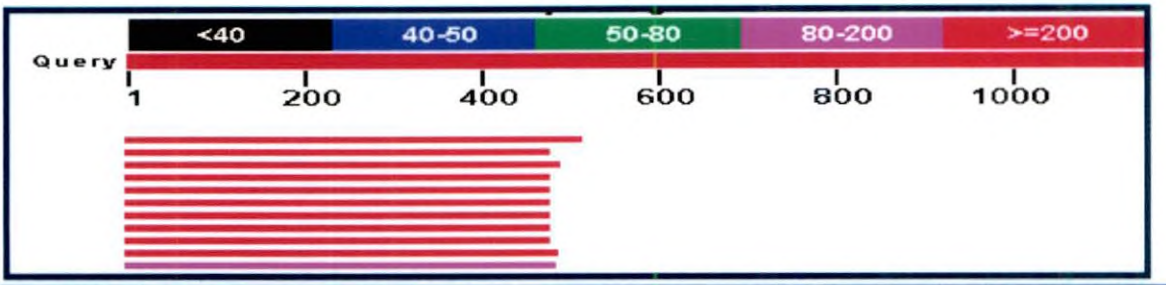
PNK21C had shown a polyadenylation signal sequence of TATAAA just upstream to the polyA tail at 3' end of the sequence.

4.9.1 Blastn and Blastx analysis

Blastn and Blastx analysis of PNK21C segment indicated homology with APK1B protein kinase of *Ricinus communis* and hypothetical protein from *Vitis vinifera*. In Blastx results, 56 per cent of the sequence showed over 66 per cent identity to mRNA encoding *Vitis* hypothetical protein and 50 per cent identity with APK1B protein from *Ricinus communis*. Blastn result had shown over 40 to 44 per cent homology with hypothetical *Vitis* protein and with different kinase like APK1B, APK1A and kinase domain containing proteins from different crops like *Ricinus communis*, *Arabidopsis thaliana* with 70 per cent identity. The details of result obtained for Blastn and Blastx analysis for PNK21C clone are given in Fig. 8.

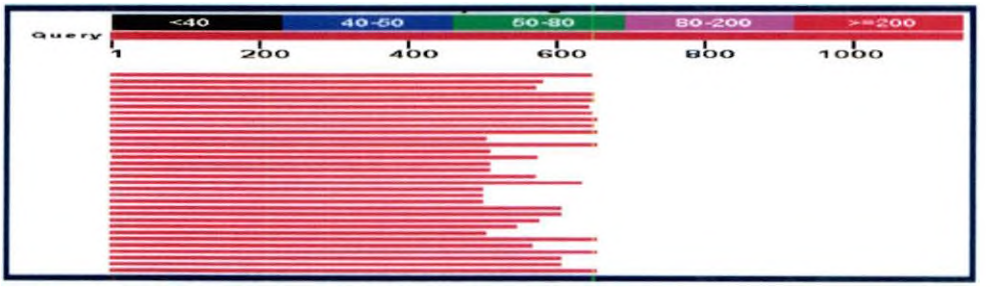
4.9.1 Open reading frame analysis

The sequence was found to have ORF with the 264 bp in length. It encoded a peptide of 87 amino acids. The sequence did not have any conserved domain. The details of result obtained for ORF prediction is shown in Fig. 9.



Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
XM_002273555.1	PREDICTED: Vitis vinifera hypothetical protein LOC100254784 (LOC100254784), mRNA	259	259	44%	4e-65	71%	GM
XM_002530899.1	Ricinus communis Protein kinase APK1B, chloroplast precursor, putative, mRNA	235	235	41%	4e-58	71%	G
XM_002519466.1	Ricinus communis Protein kinase APK1A, chloroplast precursor, putative, mRNA	217	217	42%	1e-52	70%	G
NM_202541.2	Arabidopsis thaliana protein kinase domain-containing protein (AT3G09830) mRNA, cor	215	215	41%	4e-52	70%	UEGM
NM_111818.3	Arabidopsis thaliana protein kinase domain-containing protein (AT3G09830) mRNA, cor	215	215	41%	4e-52	70%	UEGM
AY122961.1	Arabidopsis thaliana putative protein kinase (At3g09830) mRNA, complete cds	215	215	41%	4e-52	70%	UGM
AY091117.1	Arabidopsis thaliana putative protein kinase (At3g09830) mRNA, complete cds	215	215	41%	4e-52	70%	UGM
AK317246.1	Arabidopsis thaliana AT3G09830 mRNA, complete cds, clone: RAFL22-05-E06	210	210	41%	2e-50	70%	UM
BX823222.1	Arabidopsis thaliana Full-length cDNA Complete sequence from clone GSLTLS12ZF01 c	210	210	41%	2e-50	70%	UGM
FJ014818.1	Glycine max clone cw136 protein kinase mRNA, complete cds	208	208	42%	6e-50	69%	UG

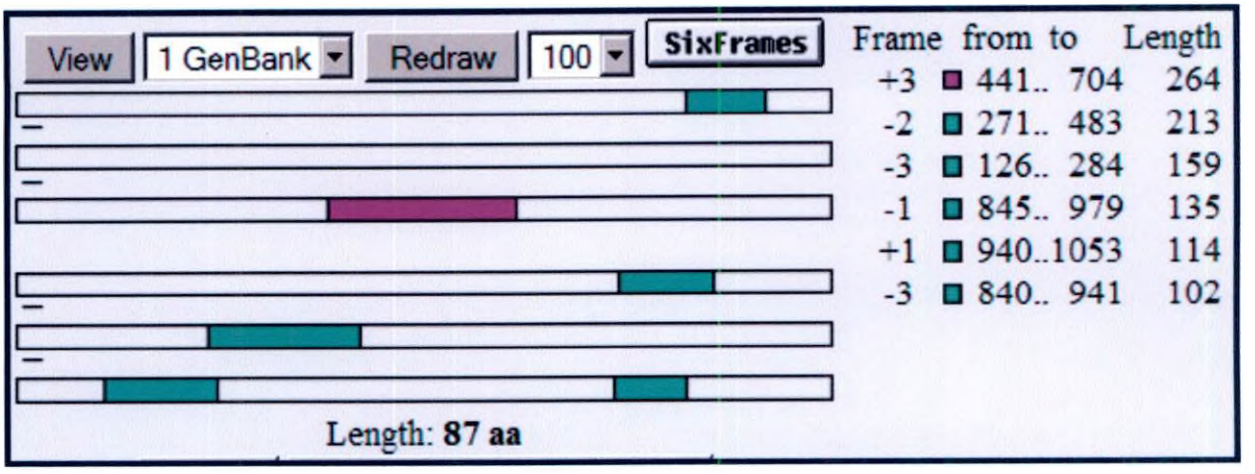
Blastn



Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
XP_002273591.1	PREDICTED: hypothetical protein [Vitis vinifera]	253	253	56%	1e-72	66%	UG
XP_002530935.1	Protein kinase APK1B, chloroplast precursor, putative [Ricinus communis] >gb|EEF3145	238	238	50%	7e-72	68%	G
ACM89571.1	protein kinase [Glycine max]	238	238	49%	2e-71	68%	G
NP_001048536.1	Os02g0819600 [Oryza sativa Japonica Group] >dbj|BAD22970.1| putative protein serin	234	234	56%	9e-71	58%	UG
EEC74260.1	hypothetical protein OsI_09473 [Oryza sativa Indica Group]	233	233	56%	2e-70	58%	
XP_002276673.1	PREDICTED: hypothetical protein [Vitis vinifera]	232	232	56%	5e-70	62%	UG
CBI32759.3	unnamed protein product [Vitis vinifera]	233	233	56%	9e-70	62%	
BAK01480.1	predicted protein [Hordeum vulgare subsp. vulgare]	229	229	57%	1e-68	59%	
BAJ91599.1	predicted protein [Hordeum vulgare subsp. vulgare] >dbj|BAJ96520.1| predicted protei	229	229	56%	2e-68	58%	
XP_002315317.1	predicted protein [Populus trichocarpa] >gb|EEF01488.1| predicted protein [Populus tr	228	228	57%	4e-68	58%	UG
ACJ85098.1	unknown [Medicago truncatula]	225	225	44%	8e-68	72%	
AAF43496.1	protein serine/threonine kinase [Lophopyrum elongatum] >gb|AAK11674.1|AF339747_1	226	226	57%	1e-67	58%	

Blastx

Fig. 8. Blastn and Blastx analysis of PNK21C




```
441 atgagtgagattttagcgattgtgaagcaaattgtggagtcatca
    M  S  E  I  L  A  I  V  K  Q  I  V  E  S  S
486 acaactgggtctcctcaacctgccatcaaaatcctagattcaagt
    T  T  G  S  P  Q  P  A  I  K  I  L  D  S  S
531 gaagtctccaatgaagaacacaaaaacagaagccgattgaaacga
    E  V  S  N  E  E  H  K  N  R  S  R  L  K  R
576 gccattgcttatcttaatatgggtggagatggcaagaggttttac
    A  I  A  Y  L  N  M  G  G  D  G  K  R  F  Y
621 tggtggaagttcaaatccaagctcagtaaagttaagttgtggaag
    W  W  K  F  K  S  K  L  S  K  V  K  L  W  K
666 acgaagataggactaatggtttctaggctagcgtcatga 704
    T  K  I  G  L  M  V  S  R  L  A  S  *
```

Fig. 9. Result of ORF analysis for PNK21C showing longest frame

Blastp result for the longest ORF had shown significant alignment with protein kinase from *Glycine max* and *Zea mays* with ~94 per cent query coverage. The details of result obtained for Blastp is shown in Fig. 10.

4.9.2 GeneScan analysis

The GenScan results showed that the query nucleotide sequence contained single gene/exon of length 311 base pairs. Blastp result for this exon was showing *Glycine max* protein kinase with 94 per cent query coverage. Coding region score of 323 and exon score of 17.64 and the polyA tail from 1130 to 1135 bp. The results are shown in Fig. 11.

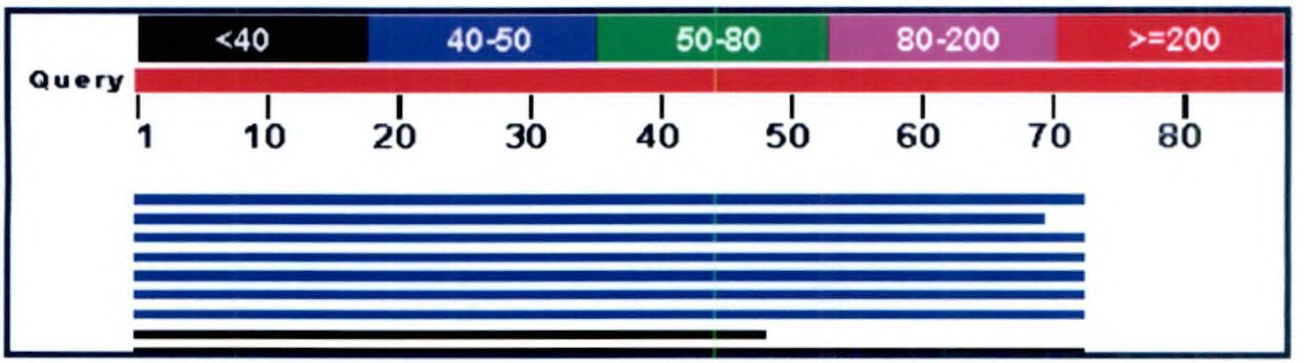
4.9.3 Nucleotide translation to amino acid

EMBOSS Transeq tool has shown six different frames of which third frame (+3) has been found to be significant having 369 aa sequence as given below,

DFQIIFRDFKTSNILLDNVVAAEVSDFGLARQGPGEGLSHVSTAVVGTIGYAAPE
YIQTGRLTAKSDVWSYGVVLYELITGRKPIDRNRPKGEHKLLEWVKPYLSEKRFS
MILDPRIQE*ISLKSAQKLAAVANKCLVRQQKARPKMSEILAIVKQIVESSTTGS
PQPAIKILDSSEVSNEEHKNRSRLKRAIAYLNMGGDGKRFYWWKFKSKLSKVKLW
KTKIGLMVSRLAS**ETRHRRSWEVFRDGLKNPP*SVKDVLAKTSADYVGKSLSG
GQQWLI*NLNPMCVSNHCC*FGNCFPITDVEFEKMKTHALDRRVLDSTSNILSDR
LRADHNW*VCKLCNFVYLKLRKHHTFNRHQWKNVVLCFLSKAINEL

Blastp result for Transeq predicted amino acid sequence indicated homology with proteins from several crop plants like *Vitis vinifera, Ricinus communis, Glycine max, Oriza sativa, Arabidopsis thaliana* etc with around 55 to 60 per cent query coverage and maximum score up to 280.

The Interproscan results indicated the presence of protein kinase-catalytic domain, serine/threonine-protein kinase domain, protein kinase-like domain and serine/threonine-protein kinase-like domain.



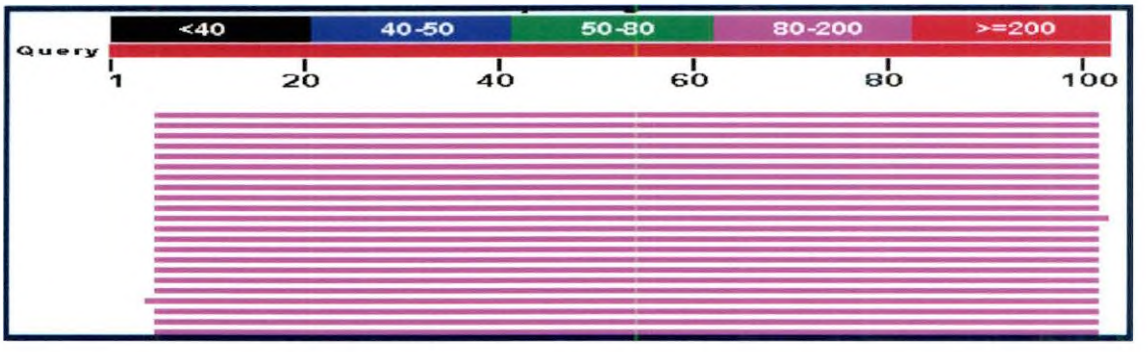
Accession	Description	Max score	Total score	Query coverage	E value	Links
NP_001064700.2	Os10g0442800 [Oryza sativa Japonica Group] >dbj|BAF26614.2| Os10g0442800 [Oryza	47.0	47.0	82%	3e-05	UG
XP_002273591.1	PREDICTED: hypothetical protein [Vitis vinifera]	47.0	47.0	79%	6e-05	UG
ABG66097.1	Protein kinase domain containing protein, expressed [Oryza sativa Japonica Group]	46.6	46.6	82%	8e-05	
AAP53976.2	Protein kinase domain containing protein, expressed [Oryza sativa Japonica Group] >dbj|	46.6	46.6	82%	8e-05	
EEC67045.1	hypothetical protein OsI_33788 [Oryza sativa Indica Group]	46.2	46.2	82%	9e-05	
BAK01480.1	predicted protein [Hordeum vulgare subsp. vulgare]	45.4	45.4	82%	2e-04	
AAF43496.1	protein serine/threonine kinase [Lophopyrum elongatum] >gb|AAK11674.1|AF339747_1	43.5	43.5	82%	8e-04	
XP_002530935.1	Protein kinase APK1B, chloroplast precursor, putative [Ricinus communis] >gb|EEF31450.	35.4	35.4	55%	0.70	G

Fig. 10. Blastp analysis for longest ORF

```
Gn.Ex Type S .Begin ...End .Len Fr Ph I/Ac Do/T CodRg P.... Tscr..
----- ---- - ------ ------ ---- -- -- ---- ---- ----- ----- ------

 1.01 Term +     52     362  311  2  2    4   33   323 0.900  12.64
 1.02 PlyA +   1130    1135    6                              -0.45
```

```
>/tmp/07_22_11-02:11:01.fasta|GENSCAN_predicted_peptide_1|103_aa
XNVVAAEVSDFGLARQGPGEGLSHVSTAVVGTIGYAAPEYIQTGRLTAKSDVWSYGVVLY
ELITGRKPIDRNRPKGEHKLLEWVKPYLSEKRFSMILDPRIQE
```

GENSCAN output for PNK21C



Accession	Description	Max score	Total score	Query coverage	E value	Link
ACM89571.1	protein kinase [Glycine max]	172	172	94%	2e-50	G
NP_001048536.1	Os02g0819600 [Oryza sativa Japonica Group] >dbj|BAD22970.1| putative prot	170	170	94%	3e-50	UG
EEC74260.1	hypothetical protein OsI_09473 [Oryza sativa Indica Group]	170	170	94%	3e-50	
NP_001146659.1	hypothetical protein LOC100280259 [Zea mays] >gb|ACL54482.1| unknown [Z	169	169	94%	1e-49	UG
ACG27469.1	protein kinase APK1A [Zea mays]	169	169	94%	1e-49	

Fig. 11. GENSCAN result and Blastp analysis for amino acid sequence predicted by GENSCAN

The sequence had PKc_like superfamily conserved domain in CDD imported from PRK database. Pfam database showed multi-domains like Pkinase and Pkinase_Tyr. The details of results obtained for Transeq tool analysis is presented in Fig. 12. The results obtained for conserved domain analysis, functional domains detection and Blastp are shown in Fig. 13 and 14.

No repeats were found in the PNK21C sequence. The RepeatMasker analysis result is shown in Fig. 15.

The assembled sequence was searched for uniqueness in the gene databases available at URL www.ncbi.nlm.nih.gov. using BLAST. It may be appreciated from the results that for the assembled sequence, out of 1141 bases the best query coverage was for first 550 bases and also the maximum identity was 70 per cent. Such a low identity in sequence with the known sequences confers novelty to the cloned sequence.

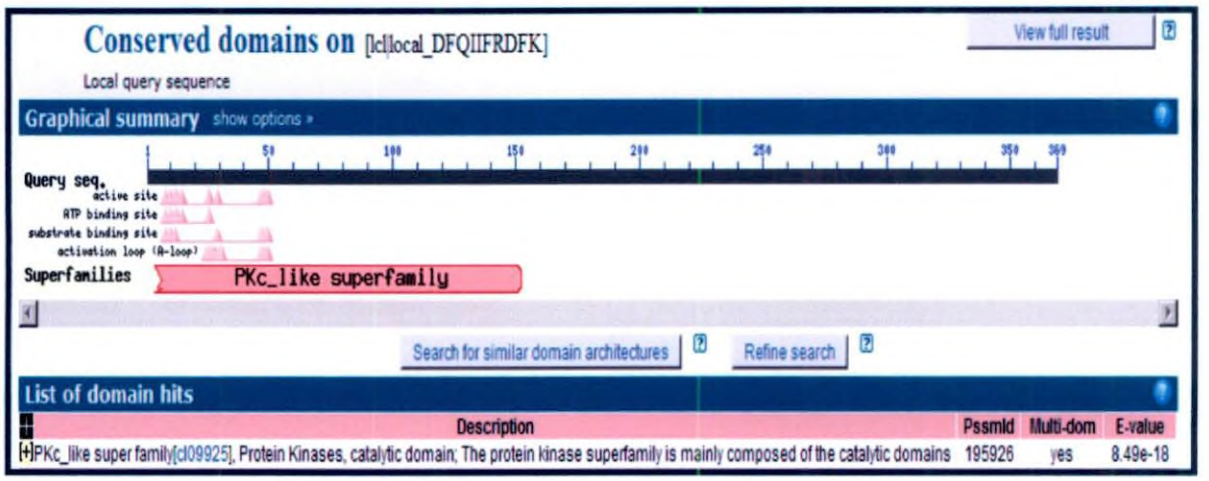
EMBOSS Transeq Results

	Transeq Results
Frame	3
Translation table	Standard (0)
Regions	START-END
Trim	no
Reverse	no
Color	no
Transeq output	transeq-20110625-0548415084.output

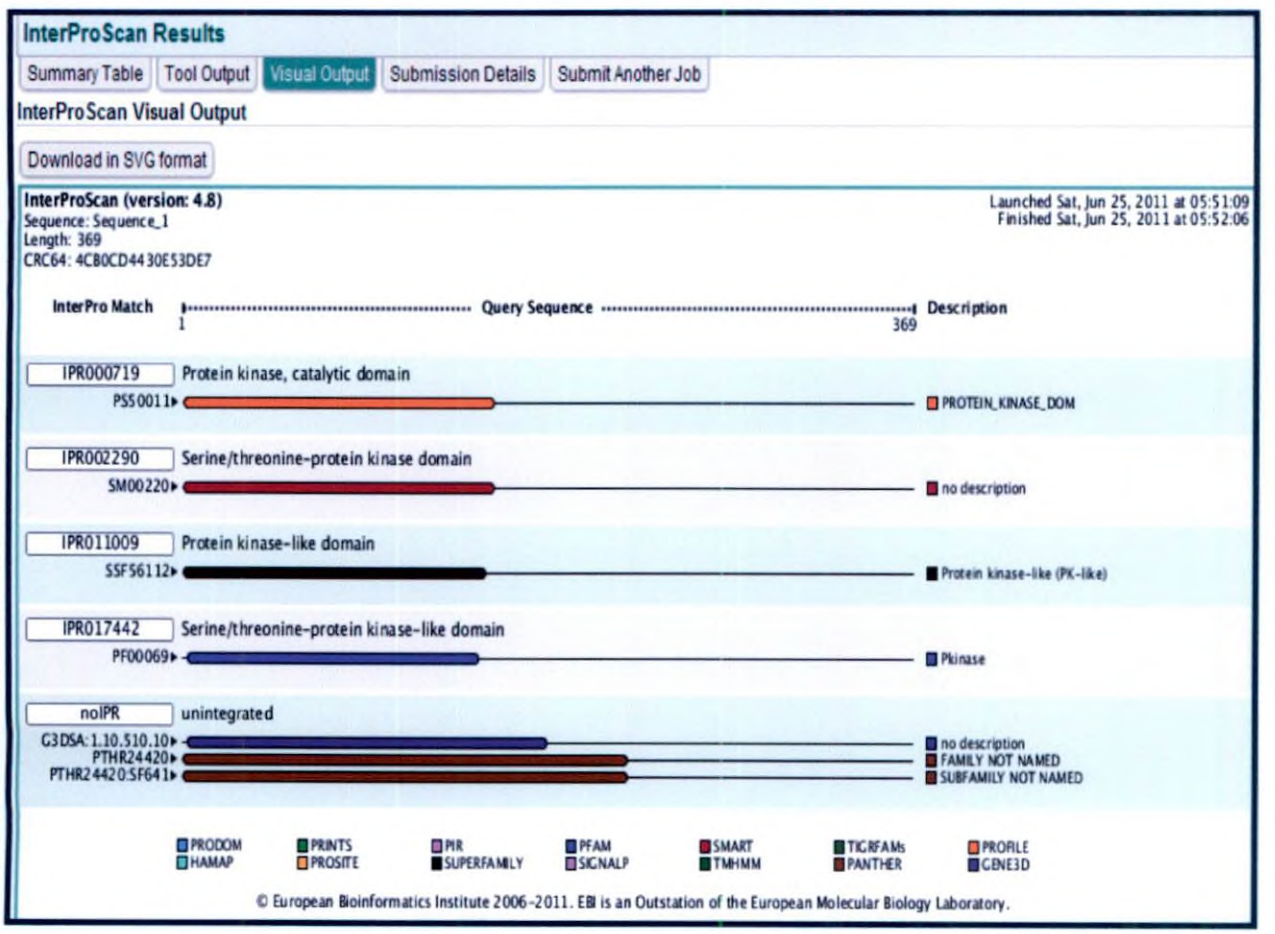
SUBMIT ANOTHER JOB

```
>EMBOSS_001_3
DFQIIFRDFKTSNILLDNVVAAEVSDFGLARQGPGEGLSHVSTAVVGTIGYAAPEYIQTG
RLTAKSDVWSYGVVLYELITGRKPIDRNRPKGEHKLLEWVKPYLSEKRFSMILDPRIQE*
ISLKSAQKLAAVANKCLVRQQKARPKMSEILAIVKQIVESSTTGSPQPAIKILDSSEVSN
EEHKNRSRLKRAIAYLNMGGDGKRFYWWKFKSKLSKVKLWKTKIGLMVSRLAS**ETRHR
RSWEVFRDGLKNPP*SVKDVLAKTSADYVGKSLSGGQQWLI*NLNPMCVSNHCC*FGNCF
PITDVEFEKMKTHALDRRVLDSTSNILSDRLRADHNW*VCKLCNFVYLKLRKHHTFNRHQ
WKNVVLCFLSKAINEL
```

Fig. 12. PNK21C nucleotide translation to amino acid sequence

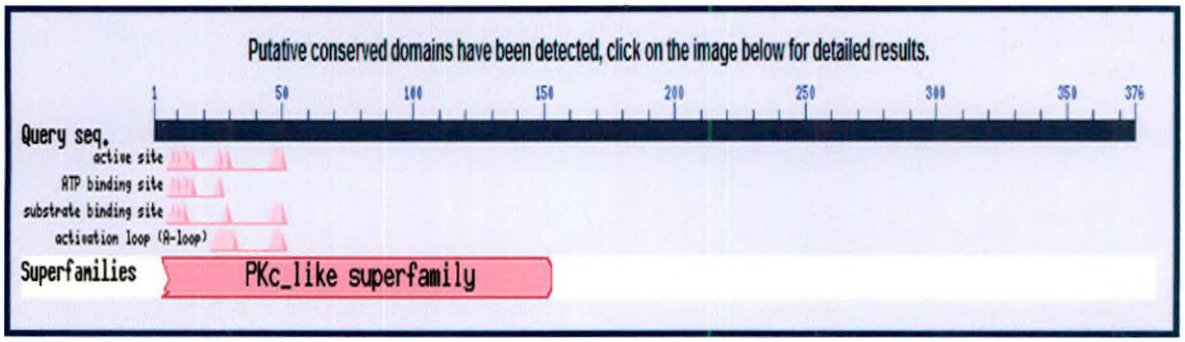


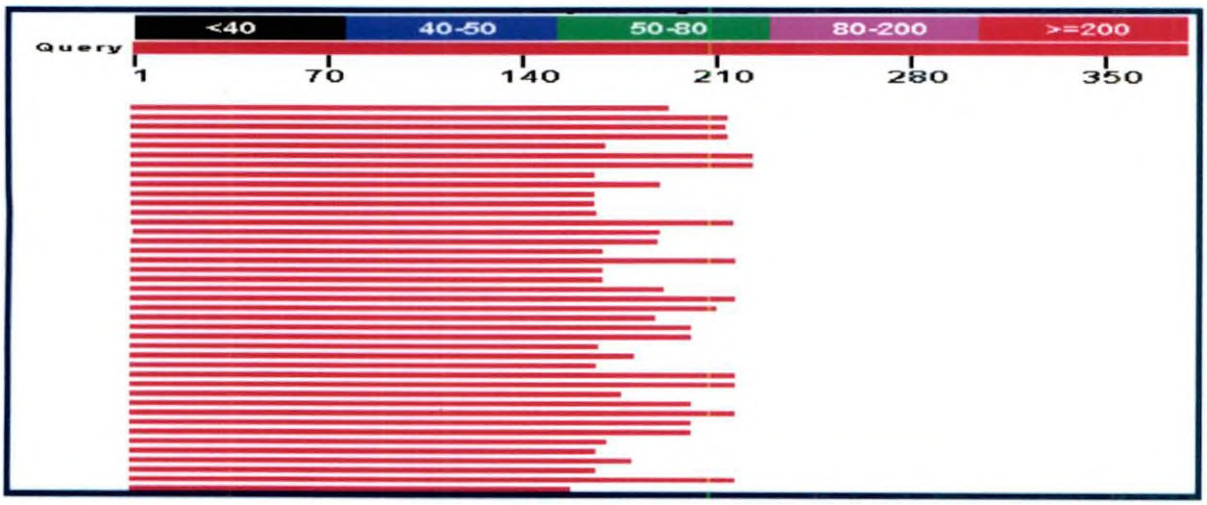
Conserved domain analysis result



Functional domains analysis by InterProScan

Fig. 13. Result of CDD and InterProScan analysis for Transeq coded sequence





Accession	Description	Max score	Total score	Query coverage	E value	Links
XP_002530935.1	Protein kinase APK1B, chloroplast precursor, putative [Ricinus communis] >gb|EEF31450.1| Prc	269	269	51%	5e-84	G
XP_002273591.1	PREDICTED: hypothetical protein [Vitis vinifera]	280	280	56%	8e-82	UG
XP_002276673.1	PREDICTED: hypothetical protein [Vitis vinifera]	260	260	56%	8e-81	UG
CBI32759.3	unnamed protein product [Vitis vinifera]	260	260	56%	2e-80	
ACM95571.1	protein kinase [Glycine max]	261	261	45%	2e-80	G

Fig. 14. Blastp result for Transeq coded sequence

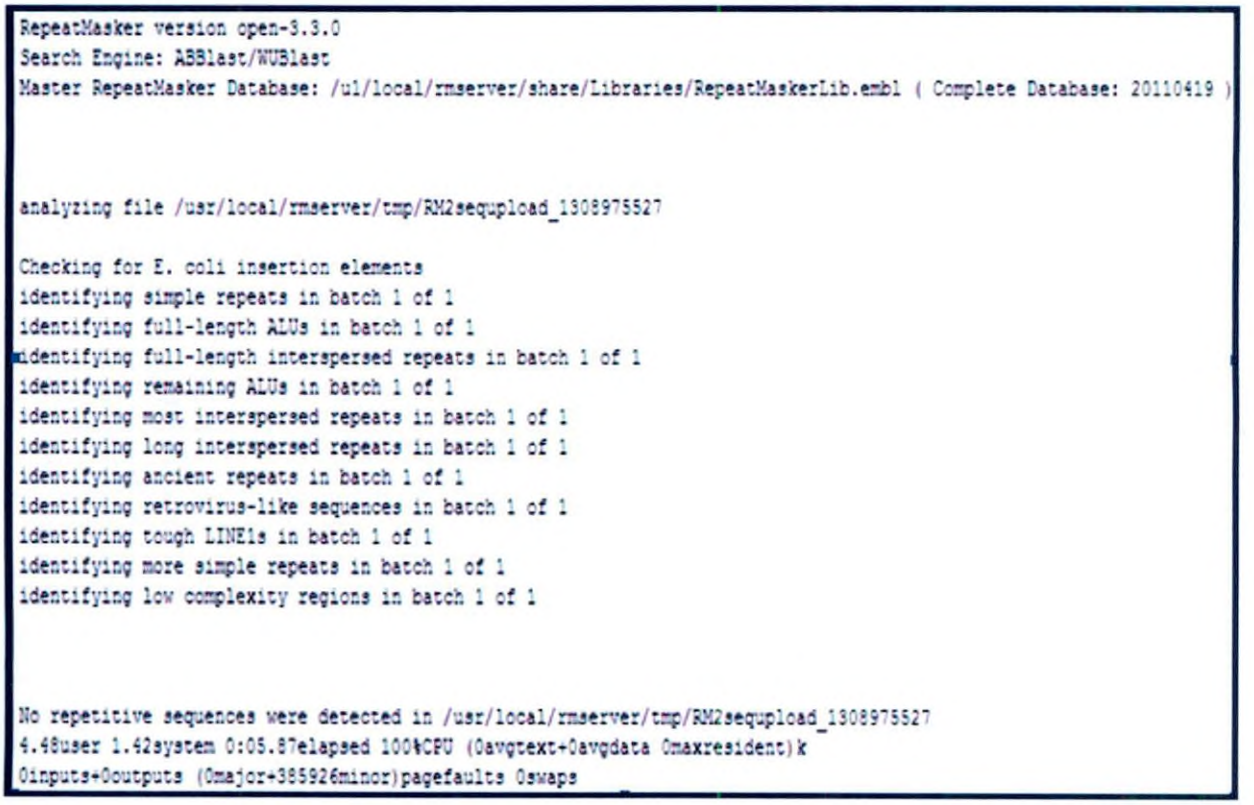


Fig. 15. RepeatMasker analysis result

Discussion

5. Discussion

Abiotic stresses adversely affect agriculture and result in crop deterioration. Due to widespread occurrence of these stresses, plants are affected in many ways, which ultimately lead to economic losses in agriculture worldwide.

Kalluvally is a drought tolerant genotype of black pepper characterized by physiological and biochemical parameters. Drought tolerance is the mechanism causing minimum loss of yield in a water deficit environment relative to the maximum yield in a water constraint free management of the crop. The effect of stress on growth and yield parameters is through its effects on various physiological and developmental processes. Understanding of effects of drought at genomic level is absolutely essential. Since coding sequences occupy just a small fraction of the plant genome, it is not an easy task to distinguish all the coding sequences from intergenic sequences and to partition coding sequences accurately into units of transcription. For these reasons, genome annotations rely upon transcript derived sequence to relative gene models and improve sequence annotation.

This work represents an effort in the isolation and characterization of full length protein kinase gene from black pepper (*Piper nigrum* L.) var. Kalluvally. Based on high sequence conservation among various protein kinase genes from different plant species, isolation of full length fragment of protein kinase gene from black pepper was attempted in the present study. Results obtained in the present study are discussed in this chapter.

5.1 Selection of protein kinase clones

An estimated 1 to 3 per cent of functional eukaryotic genes encode protein kinases, suggesting that they are involved in many aspects of cellular regulation and metabolism (Stone and Walker, 1995) and signal transduction is one of the current molecular approaches in the management of drought stress (Muoma *et al.*, 2010).

The present study involved the selection of protein kinase clones for sequence enrichment. Candidate clones were chosen based on EST sequences. In the previous study by Osakabe *et al.* (2010) it was found that the overproduction of the membrane-bound receptor-like protein kinase1, RPK1, enhances abiotic stress tolerance in *Arabidopsis*. PNK21 was the clone showing protein kinase domain and PNK49 clone shared maximum identity with the receptor-like protein kinase genes in the EST library developed for drought tolerance in black pepper at the centre and hence, were selected for the study.

5.2 Sequencing of cDNA clones

The glycerol cultures maintained for long-term storage were revived on LB/ampicillin medium. Colony PCR was performed to confirm the presence and size of insert. The colonies were then fully sequenced to high accuracy and analyzed. This information led to the designing of further experiments to deduce the sequences towards both the ends. The sequence data obtained for both the clones were designated as PNK21A and PNK49A.

5.3 *In Silico* analysis of PNK21A and PNK49A clones

When the PNK21A sequence was subjected to Blastn and Blastx homology search, it revealed 62 per cent identity with protein kinase APK1B protein from *Ricinus communis*, a hypothetical protein of *Vitis* and protein kinase protein from *Glycine max.*

PNK49A sequence on Blastn and Blastx homology search revealed the 69 per cent identity with leucine rich repeat receptor kinase of *Ricinus communis* and, with a hypothetical protein of *Vitis. Ricinus communis* is a drought tolerant species and *Vitis vinifera* is a perennial climber. Homology of black pepper sequences with these unique species adds much impact to the metabolomics of the study.

The sequence data obtained was not full length hence remaining sequence information both towards 5' end and 3' end was attempted by reverse transcriptase polymerase chain reaction by designing degenerate primers.

5.4 Primer designing

The success of the PCR strategy is highly dependent on the small synthetic oligonucleotides that hybridize to the complementary DNA sequence. These short nucleotides function in pairs known as the forward and reverse primers, which amplify a specific DNA sequence and, more importantly, anneal exclusively to that DNA target locus (Lexa, 2001).

Selected clones were the members of a large protein kinase gene superfamily. Model plants most suitable for genetic analysis like Arabidopsis, rice showed around 400 members of this large super family with around a thousand of transcripts. So the homology based approach was used to amplify the mRNAs of protein kinase genes.

Blastn and Blastx results showing homology were exploited for primer designing. In order to amplify the protein coding sequence of selected clones, complete cDNA sequences showing most significant hits with each clone (viz. PNK21 and PNK49) were analysed to get open reading frames. These ORFs were aligned by ClustalW software and most conserved region was selected as degenerate forward primer to amplify 5' end of the given three clones. The reverse primers were designed for each clone based on available sequence information using Primer3 software. All the physical parameters were taken into consideration while designing reverse primers for amplification towards 5' end.

Similarly, for amplification towards 3' end of cDNA, degenerate primers designed from conserved region served as reverse primer and forward primer was designed from available sequences.

The primer pairs were designed and selected so that they extend towards each other, polymerizing the complementary DNA sequence to the extent that the target region is covered in each cycle of the PCR.

5.5 Water stress treatment

Kalluvally is a drought tolerant genotype of black pepper (*Piper nigrum* L.). This rainfed crop suffers due to severe soil moisture shortage during summer months. So water stress treatment and collection of leaf sample for RNA isolation was planned during Jan to May months. The potted plants even under frequent water stress showed good tolerance. Eighty per cent of the plants survived even after imposing five days water stress thrice in a month (Table 1b).

5.6 Up-regulation of protein kinases during water stress

The cloning and characterization of a cDNA encoding protein kinase that had sequence homology to members of the mitogen-activated protein kinase (MAPK) kinase family was found to be induced by water stress in *Arabidopsis thaliana* (Mizoguchi *et al.*, 1996).

Differentially expressed transcripts of protein kinase genes were found to be up-regulated and characterized during the previous studies by Kushwah (2008); Mali (2010). Hence, it was expected that protein kinase genes under study were expressed on water stress.

5.7 Total RNA isolation from stressed plants

Dehydrated leaves accumulate polysaccharides and RNases. Poor yields and low-quality degraded RNA may result from inefficient cell lysis or high levels of nuclease activity. A greater challenge in isolating high-quality RNA is the aqueous byproducts of secondary metabolism that may accumulate in many of higher plants, especially in mature tissues.

RNA was isolated from black pepper leaves for preparation of cDNA. RNA isolation presents more challenges than DNA isolation because of the

susceptibility of RNA to degradation by ribonucleases (RNases). RNases are very stable and ubiquitous enzymes which do not generally require cofactors to function. Since, only minute amounts of RNases are sufficient to cleave RNA; extreme caution is needed for an effective RNA isolation procedure.

In the present study, to avoid RNA degradation all the glassware, mortar and pestle, microcentrifuge tubes and microtips were treated with autoclaved DEPC treated water. DEPC (Diethyl pyrocarbonate) alkylates histidine molecules present in the active site of RNase enzyme thus inhibiting its activity (Fersht, 1977). DEPC treated water was autoclaved in order to remove traces of DEPC that might otherwise modify purine residues in RNA by carboxymethylation and thus affecting its translation. Gloves were worn throughout the experiment, to avoid RNA degradation from RNases that are present on the bare hands.

Another problem in isolating high quality RNA is the interference with aqueous metabolites like phenols and polysaccharides which accumulate in tissues and may be purified along with nucleic acids (Loomiz, 1974; Stokes *et al.*, 1990). In the present study water stressed leaves were taken for isolating RNA. In water stressed leaves, dehydration leads to an increase in solute concentration as the protoplast volume shrinks. Low yields of RNA may also be due to the high levels of polysaccharides and phenols that accumulate after dehydration (Hopking, 1999) and bind to RNA during the extraction process (Chang *et al.*, 1993). Therefore, to isolate high quality RNA, the procedure described by Chomczynski and Sacchi (1987) with some modifications was adopted.

Young, tender leaves from water stressed plants were used for isolation of total RNA, since nucleic acid yields from young tissues are often higher than old tissue. This is because young tissue generally contains actively dividing cells. In addition, young tissue may have fewer metabolites which can affect the performance of downstream application if not completely removed during nucleic acid purification (Chang, 1993).

Mortar-homogenization-maceration was used every time during the extraction procedure. Plant tissues were directly placed in liquid nitrogen in a precooled mortar. RNA isolation reagent was then added, resulting in both tissue and reagent freezing. Homogenization with a precooled pestle was continued at 25^0C and tissue gradually defrosted. This homogenization maceration step was stopped when solution changed from a thick paste to liquid, which took 4 to 6 minutes. Thus, tissue maceration started while tissue and reagent were defrosting. The homogenate was then transferred to an Eppendorf tube for further extraction procedure. This method was adopted because the RNA extraction efficiency was significantly more than in pulverization (Portillo *et al.*, 2006).

In the present study, total RNA was isolated using Trizol reagent. Trizol combines phenol and Guanidine thiocyanate in a monophase solution that facilitates the immediate inhibition of RNase activity. The homogenate was incubated at room temperature which allows nucleoprotein complexes to completely dissociate. Chloroform was added to remove chlorophyll and proteins. Even after chloroform extraction, there was formation of a flocculent white precipitate in the aqueous phase. This was likely to be due to precipitation of insoluble lipids and thus was remedied by adding additional chloroform extraction.

Isopropanol was added to precipitate RNA which was then followed by ethanol (75%) wash. It solubilizes salts but not nucleic acids. The pellet was dissolved in autoclaved DEPC treated water.

The modified procedure allowed the recovery of intact, high quality RNA from normal as well as water stressed leaves. Three distinct rRNA bands corresponding to 28S, 18S and 5S + tRNA were apparent in the sample when analyzed on agarose gel (Plate 4). In addition, the samples were quantified spectrophotometrically and their quality and quantity was assessed. The ratio for OD_{260}/OD_{280} was approximately 2.0 indicated pure RNA. The ratio for

OD_{260}/OD_{230} greater than 1.8 indicates pure RNA free from polysaccharides and polyphenols.

5.8 Reverse transcription and cDNA amplification

Messenger RNA (mRNA) cannot be itself amplified by polymerase chain reaction. Hence, mRNA was converted to DNA by complementary DNA (cDNA) synthesis. Enzyme reverse transcriptase which was RNA dependent DNA polymerase synthesizes a DNA polynucleotide strand complementarity to an existing RNA strand.

cDNA synthesis markedly affects the efficiency of PCR amplification and was optimized for increased PCR detection of RNA transcripts. Although the optimization protocol may vary depending on the specific transcript being analysed, the method of cDNA priming and other specific conditions, the initial preparation of cDNA was carefully analyzed. Both too little or too much RNA produced less cDNA than optimal for PCR amplification. For low concentrations of RNA, cDNA of better quality may be produced with higher amounts of dNTPs, thus enhancing detection for specific transcripts. Since, the expression level for both the genes was not known, the quantity of total RNA used for reverse transcription was optimized.

DNA amplification utilizing the polymerase chain reaction (PCR) has greatly enhanced the ability to detect the presence of rare mRNA species in cells by employing specific sense and antisense oligonucleotide primers with thermostable DNA polymerase, PCR allows amplification of target sequences by several log orders of magnitude (Mullis *et al.*, 1986; Mullis and Faloona, 1987). This technique is being used increasingly to quantify rare mRNA species from cells (Wang *et al.*, 1989). In these studies, it was critical that the initial cDNA synthesis step be well controlled and optimized to produce a uniform substrate for amplification. We have examined RNA concentration in the initial cDNA synthesis reaction to optimize it for PCR amplification of high or low abundance messages.

Degenerate primers were designed for the amplification of 5' end of the gene. The forward primer was designed from a homologous region selected by alignment of the sequences of complete cDNA sequences of protein kinase from different crop species, using clustalW megalign software of NCBI. Similarly, a reverse primer was designed from the available EST sequence.

PNK21 cDNA was successfully amplified towards 5' end with the degenerate primers designed. PCR product eluted from agarose gel slice was not enough for cloning even after reamplification; so it was directly sequenced and sequence obtained had shown overlapping region with sequence available. The problem was encountered during the direct sequencing which had given partial reads. The sequence obtained after sequencing the amplified fragment, was about 400 bp with overlapping homology of 330 bases with EST available. The fragment obtained was merged with PNK21A and designated as PNK21B for clarity during discussion.

PNK49 clone had given a band of around 600 bp against the expected size of 800 bp. The results obtained for PNK49 were not consistent throughout study and the reason for such a non-specific amplification may be the difference between the melting temperatures between reverse and forward primer which was $\pm 6^0$C.

5.9 Rapid amplification of cDNA ends

5.9.1 Molecular basis of RACE

High quality total RNA prepared with TRI® Reagent was used for RLM-RACE. Purity was further confirmed by looking for a 28S ribosomal RNA band that was twice the intensity of 18S band and both the bands were descrete, with no smearing.

Total selected RNA was treated with calf intestine alkaline phosphatase (CIP) to remove free 5'-phosphates from molecules such as ribosomal RNA,

fragmented mRNA, tRNA and contaminating genomic DNA. The cap structure found on intact 5' ends of mRNA was not affected by CIP. Ammonium acetate treatment was followed to stop the activity of CIP enzyme.

A phenol/chloroform extraction and ethanol precipitation following the CIP treatment was included in the protocol because it was critical to remove all traces of CIP enzyme. The reaction mix was thoroughly homogenized with phenol: chloroform by vigorous vortexing. Acid phenol was used in the extraction.

The RNA was then treated with tobacco acid pyrophosphatase (TAP) to remove the cap structure from full-length mRNA, leaving a 5'-monophosphate. A 45 base RNA adapter oligonucleotide was ligated to the RNA population using T4 RNA ligase. The adapter cannot ligate to dephosphorylated RNA because these molecules lack the 5'-phosphate necessary for ligation. During the ligation reaction, the majority of the full length, decapped mRNA acquires the adapter sequence as its 5' end.

A random-primed reverse transcription reaction and nested PCR then amplified the 5' end of a specific transcript. Two nested primers corresponding to the 5' RACE adapter sequence and the gene specific two sets of nested antisense primers specific to the protein kinase gene were used for amplification.

First strand cDNA for 3' RACE was synthesized from highly pure total RNA, using the supplied 3' RACE Adapter. The cDNA was then subjected to PCR using the 3' RACE primers which was complimentary to the anchored adapter and gene specific reverse primer designed.

5.9.2 Control reaction

An upstream 3' RACE control primer for mouse ß-actin was included in with the RLM RACE kit to perform 3' RACE on the Mouse thymus RNA to confirm functioning of the kit. This primer was used in conjunction with the 3'

RACE outer primer to amplify the 3' end of the G-coupled chemokine receptor gene and a predominant 513 bp product from the PCR was obtained as expected.

5.9.3 Standard reaction

Standard reaction with 10 μg of starting total RNA was performed. As compared to the small reaction which uses 1 μg or less total RNA, the extra material in case of standard reaction provided advantage during partial sample loss and repetition of downstream reaction of extraction with acid phenol: chloroform.

From the PNK21B sequence, four forward primers (F1, F2, F3 and F4) were designed to be used for nested PCR reaction using 3'RACE PCR. Since mRNA possesses a polyA tail at 3' end, oligo dT was used as a reverse primer. The sequence towards 3' end of the PNK21B sequence was thereby determined. F2 primer was located 40 bases downstream from the F1 primer. Therefore, the fragment which was obtained by amplifying with F1 primer was nested within the sequence amplified by using F2 as a primer.

5' RACE was performed using four reverse primers (R1, R2, R3 and R4) designed from PNK21B. Nested PCR was performed with outer 5' adapter specific and inner 5' adapter specific primers and four designed primers. 5' RACE reaction even after reamplification showed no detectable band.

5.10 Bacterial transformation

Competence of JM109 *E. coli* cells was confirmed by transforming the cells with plasmid (pUC18) having ampicillin resistance. *E. coli* cells alone could not grow on ampicillin containing media, as they lack the gene for ampicillin resistance. But the competent cells harbouring the plasmid could grow in the media. In the present study, large number of blue colonies were observed on

LB/ampicillin plate after overnight incubation at 37°C, confirming the competence of *E. coli* (JM109) cells for transformation.

For cloning of cDNA fragments, pGEMT vector of approx. 3kb size was used. It contains T7 and SP6 RNA polymerase promoters that flank a multiple cloning region within the α peptide–coding region of the enzyme β-galactosidase. Thus, due to insertional inactivation of the α peptide region, the recombinants can be directly identified by the blue-white screening of indicator plates. The vector contains multiple restriction stress within the multiple cloning regions thus facilitating easy ligation of insert and its release by digestion with restriction enzyme.

In the present study, the amplified fragment of cDNA with gene specific primers were used to transform the competent cells. The white colonies could be easily distinguished and picked up from the selection media containing 5-bromo-4-chloro-3-indolyl β-D galactoside (X-gal) and isopropyl thiogalactoside (IPTG).

pGEMT vector contained polycloning sites inside a gene encoding for β-galactosidase. Thus, insertion of a new sequence would disrupt the reading frame of galactosidase encoding gene. As a result of α-complementation, the bacterial cell and vector together provided the complete protein, because one part of the gene was present in bacteria and the other in vector (Ullmann *et al.*, 1967). The colonies which have not taken up the plasmid further utilized the substrate and appeared as blue colonies on chromogenic substrate, X-gal (Horwitz *et al.*, 1964). Due to the disruption of α-complementation, all the transformed colonies harbouring the recombinant plasmid appeared as white.

The plasmid DNA isolated from white and blue colonies gave bands with different molecular weights. Most of the plasmids from white colonies had higher molecular weight than the plasmid of blue colonies due to the presence of insert.

The presence of the insert was further confirmed by PCR amplification of the plasmids with T7 and SP6 primers. Amplicons for blue and white colonies

differed in their size. The PCR product of blue colony plasmid had lower molecular weight since it lacked the insert and only T7 and SP6 regions present in it could get amplified, while the PCR product of white colonies had higher molecular weight as the insert was also amplified along with T7 and SP6 regions of the vector.

5.11 Sequencing of cloned fragments

The DNA sequences that were obtained by forward and reverse sequencing were merged together after removing vector and adaptor screening. The presence of overlapping region with PNK21B has indicated the continuous sequence towards the 3' end.

Towards the 3' end an untranslated region (UTR) was observed followed by polyadenylation signal but the exact length of 3'UTR could not be determined due to lack of information on exact stop codon. A polyA tail was present at extreme right of the sequence obtained. This fragment was found to overlap with the PNK21B sequence, starting from the F2 primer region. The presence of overlap indicated that amplification was accurate and the sequence obtained was continuous with the sequence of PNK21B. Evaluation of the position of the 3' RACE sequence relative to the PNK21B sequence indicated that this fragment was located towards the 3' end and was 365 bp long.

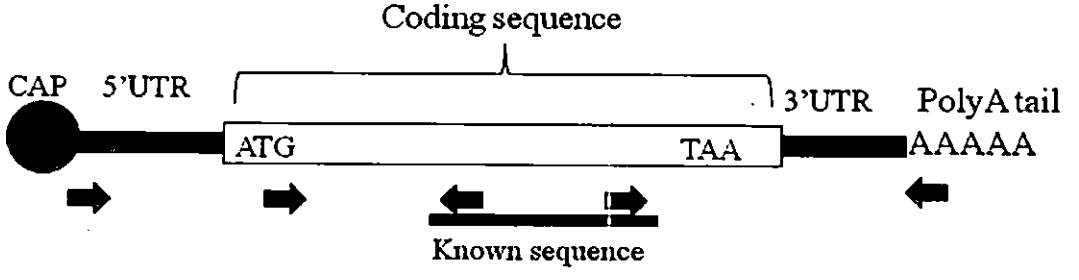


Fig. 16. Full length mRNA structure

The sequence obtained after merging 3' RACE amplified sequence and PNK21B was designated as PNK21C and the length of the fragment was 1141bp.

After determining the position of the 3' fragment, an attempt was made to amplify the remaining region towards the 5' end.

RACE PCR approach was used for full coding sequence amplification. In the present study, full length cDNA clone amplification could not be achieved as the 5' end sequence was not unraveled. Failure of 5' RACE reaction might be due to the very large size of the mRNA that is not fully known and little or no expression of the desired transcript.

This information also provides a foundation for primer development in PCR based expression studies, since it will be possible to develop primers in less conserved regions of the gene.

5.12 Sequence analysis

The nucleotide sequence analysis using the BLAST software at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST) revealed the identity with protein kinase family proteins from different organisms. Initial 40 per cent of the sequence showed over 71 per cent homology in different plants like *Ricinus communis, Arabiopsis thaliana,* rice and hypothetical protein of *Vitis vinifera*. Similar results were obtained from Blastx analysis.

The sequence had an ORF with the 264 bp in length as predicted by ORF Finder program. The longest ORF was subjected to Blastp, which showed significant alignment with rice protein kinase domain containing protein with 82 per cent coverage. Functional aspects of the domains present in the sequence were discovered through 'Interproscan'.

EMBOSS Transeq tool was used which can translate specified regions corresponding to the coding regions of sequences. It can translate using the standard ('Universal') genetic code and also with a selection of non-standard codes. Transeq result indicated that coding region translated by +3 frame gave the presence of protein kinase catalytic domain, serine/threonine-protein kinase domain, protein kinase-like domain and serine/threonine-protein kinase-like domain through Interproscan.

The predicted protein was also analysed using the Conserved domain architecture retrieval tool of BLAST at the NCBI server (http://www.ncbi.nlm. nih.gov/BLAST), and one conserved domain was identified as the PKc_like superfamily. Further BLAST analysis of this protein revealed that it has high homology with the hypothetical proteins from *Vitis vinifera* (57%) and protein kinase APK1B homolog of *Ricinus communis* (52%), protein kinase of *Glycine max* (45%) and putative serine/threonine kinase from *Oryza sativa* (59%)- so that it can be defined as multidomain containing homolog of protein kinase superfamily.

Charulata *et al.* (2010) has reported the differential expression of APK1B transcript in foxtail millet (*Setaria italica* L.) during dehydration stress which is a drought tolerant crop and suggests the role in signal transduction during dehydration stress.

Qamar *et al.* (2008) has studied Tomato protein kinase 1b (TPK1b) gene. It encoded a membrane associated functional kinase required for resistance against *Botrytis* and insect herbivory in tomato and it was identified as a homolog of Arabidopsis protein kinase 1b (APK1b) (Hiramaya and Oka, 1992) that has no known function. Hence, the APK1B homologs in various crops shows multiple role in signal transduction.

The TPK1b kinase domain is most closely related to the *Arabidopsis* APK1b, *Brassica* MLPK, and the grapevine predicted protein CAO21648 based on sequence and phylogenetic analysis. *Arabidopsis* APK1a and APK1b are 84

per cent identical and exhibit kinase activities *in vitro* (Hirayama and Oka, 1992). MLPK shares 76 per cent sequence identity with APK1b and is involved in *Brassica* self-incompatibility (Murase *et al.*, 2004). The T-DNA insertion alleles of APK1b show no fertility or self-incompatibility related phenotypes (Kakita *et al.*, 2007).

APK1 was concluded to be a novel type of protein kinase, which could phosphorylate tyrosine, serine, and threonine residues, though tyrosine phosphorylation seemed to occur only on limited substrates. Since the structure of the APK1 N-terminal portion was indicative of N-myristoylation, APK1 might associate with membranes and thereby contribute to signal transduction. The *A. thaliana* genome contained two APK1 genes close to each other (APK1a and APK1b) (Hirayama and Oka, 1992).

As PNK21C showed significant homology with *Arabidopsis thaliana* protein kinase 1b (APK1B), it may have important role in signal transduction pathways during various stress conditions including water stress.

Future line of work on PNK21C includes the study of Cis-regulatory elements by promoter analysis. For getting promoter and 5' end of genes PlantProm database would be useful to design promoter specific primers conserved in TATA box. The function of these protein kinases in signal transduction during water stress is a complex of various pathways, so the large scale study of transcripts by microarray analysis in spice crops like black pepper is needed to exploit novel genes for molecular breeding.

Summary

6. SUMMARY

The present study entitled "Isolation and characterization of water stress activated protein kinase gene from black pepper (*Piper nigrum* L.) var. Kalluvally," was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture (COH), Vellanikkara. The study was intended to enrich the transcript derived sequence information on protein kinase gene from drought tolerant genotype of black pepper by using reverse transcriptase polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE). The results obtained in the study are summarized in this chapter.

1. Plant material (rooted cuttings) for *P. nigrum* var. Kalluvally was maintained under greenhouse condition in mud pots filled with potting mixture. Plants showed vigorous and healthy growth under the uniform conditions.

2. Protein kinase clones named PNK21 and PNK49 differentially expressed in water stress specific cDNA library (Kushwah, 2008) were revived on LB/amp media and presence of insert in each clone confirmed by colony PCR and sent for sequencing.

3. Sequence data obtained was screened for presence of vector and adaptor sequence, analysed using Blastn and Blastx standered tools which showed 935 bp and 697 bp for PNK21 and PNK49 clones respectively.

4. Homology based approach was used to design degenerate primers for RT-PCR to target both 5' and 3' unknown ends of the available cDNA sequence. Primers were designed using Clustalw multi-sequence alignment tool and Primer3 program.

5. Water stress was induced during the period of Jan. to May months under open sunlight with different intervals and frequency. Plants had shown 100 per cent

revival up to the 4th day of drought. The growth and development observed during stress period was comparatively less.

6. The total RNA was isolated from water stressed Kalluvally using TRIzol method as suggested by Chomczynski and Sacchi (1987) with slight modifications. The agarose gel electrophoresis and spectrophotometric analysis revealed that the total RNA isolated was of good quality containing high amount of RNA. This RNA was further used for cDNA synthesis and to carry out reverse transcriptase PCR.

7. Reverse transcription was carried out using RNA dependent DNA polymerase to synthesize cDNA which was further amplified using degenerate primers towards 5' and 3' end.

8. RT-PCR produced ~600 bp sequence for 5' end amplification for PNK21 clone and the amplicon was gel purified and sent for automated sequencing.

9. Forward and reverse sequence data obtained was then overlapped with background sequence. It has shown only 65 bp new sequence at 5' end of PNK21 whereas remaining sequence was completely overlapping.

10. The sequence was merged with PNK21A and it was designated as PNK21B which was further used to design gene specific nested primers for RNA ligase mediated- rapid amplification of cDNA ends (RLM-RACE).

11. Highly pure RNA sample was isolated from stressed plants. The concentration of total RNA used was 10 μg each for 3' and 5' RACE reaction.

12. 3' end of the mRNA was successfully isolated but 5' end of the transcript could not be unraveled

13. 3' RACE-PCR produced ~400 bp and ~350 bp amplicon which were amplified using nested primers for PNK21B. The 400 bp amplicon was eluted and ligated in to pGEMT cloning vector.

14. The cloned product was sequenced and screening for vector and adaptor sequence was done. The overlapping region between PNK21B and 3' RACE sequence was excluded and both sequences were merged using EMBOSS Merger tool. The sequence was designated as PNK21C.

15. A polyadenylation signature sequence TATAAA was found just upstream of the polyA tail which is a consensus of UAUAAA signature sequence from Arabidopsis mRNA.

16. Blastn and Blastx analysis of final enriched sequence showed significant hits with *Vitis* hypothetical protein and with protein kinase genes coding for APK1B, APK1A, protein kinase domain containing protein and several putative kinases.

17. The longest and significant open reading frame predicted using ORF finder program was 264 bp long, coding for *Vitis* hypothetical protein and protein kinase domain containing protein from *Oryza sativa* with 82 per cent coverage.

18. PNK21C nucleotide sequence translated into amino acid sequence by EMBOSS Transeq tool irrespective of presence or absence of stop codon was showing amino acid sequence with +3 frame and was found to have protein kinase domain predicted using CDD analysis. The same sequence had several functional domains like protein kinase-catalytic domain, serine/threonine-protein kinase domain, protein kinase-like domain and serine/threonine-protein kinase-like domain predicted by InterProScan tool.

19. The study could enrich the available information on protein kinase in black pepper and the sequence data could be further reported for obtaining the 5' end of the coding sequence.

References

REFERENCES

Achard, P., Cheng, H., De Grauwe, L., Decat, J., Schoutteten, H. and Moritz, T. 2006. Integration of plant responses to environmentally activated phytohormonal signals. *Sci.* 311: 91–94.

Alamillo, J., Almoguera, C., Bartels, D. and Jordano, J. 1995. Constitutive expression of small heat shock proteins in vegetative tissues of the resurrection plant *Craterostigma plantagineum. Plant Mol. Biol.* 29: 1093–1099.

Almoguera, C., Coca, M.A. and Jordano, J. 1993. Tissue-specific expression of sunflower heat shock proteins in response to water stress. *Plant J.* 4: 947–958.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 1997. 25(17): 3389-4020.

An, X., Lu, J., Huang, J., Zhang, B. and Liu, D., Zhang, X., Chen, J., Zhou, Y. and Tong, Y. 2007. Rapid Assembly of Multiple-Exon cDNA Directly from Genomic DNA. PLoS ONE 2(11): e1179. doi:10.1371/journal.pone.0001179.

Arendse, M.S., Dubery, I.A. And Berger, D.K. 1999. Isolation by PCR-based methods of a plant antifungal polygalacturonase-inhibiting protein gene. *Electron. J. Biotechnol.* 2(3).

Bartels, D. and Sunkars, R. 2005. Drought and salt tolerance in plants. *Crit. Rev. Plant Sci.* 24: 23–58.

Bogeat-Triboulot, M.B., Brosche, M., Renaut, J., Jouve, L., Le, Thiec, D. and Fayyaz, P. 2007. Gradual soil water depletion results in reversible changes of gene expression, protein profiles, ecophysiology and growth performance in *Populus euphratica*, a poplar growing in arid regions. *Plant Physiol.* 143: 876–892.

Bohnert, H.J. and Shen, B. 1999. Transformation and compatible solutes. *Sci. Hort.* 78: 237–260.

Boston, R.S., Viitanen, P.V. and Vierling, E. 1996. Molecular chaperones and protein folding in plants. *Plant Mol. Biol.* 32: 191–222.

Boudsocq, M., Barbier-Brygoo, H. and Lauriere, C. 2004. Identification of nine sucrose nonfermenting 1-related protein kinases 2 activated by hyperosmotic and saline stresses in *Arabidopsis thaliana*. *J. Biol. Chem.* 279: 41758–41766.

Boudsocq, M., Droillard, M.J., Barbier-Brygoo, H., Lauriere, C. 2007. Different phosphorylation mechanisms are involved in the activation of sucrose non-fermenting 1 related protein kinases 2 by osmotic stresses and abscisic acid. *Plant Mol. Biol.* 63: 491–503.

Bowler, C., Van Montagu, M. and Inze′, D. 1992. Superoxide dismutases and stress tolerance. *Annu. Rev. Plant. Physiol. Plant Mol. Biol.* 43:83–116.

Bradham, C.A., Foltz, K.R., Beane, W.S., Arnone, M.I., Rizzo, F., Coffman, J.A., Mushegian, A., Goel, M., Morales, J. and Geneviere, A. M. 2006. The sea urchin kinome: a first look. *Dev. Biol.* 300: 180–193.

Bray, E. 1997. Plant responses to water deficit. *Trends Plant Sci.* 2: 48-54

Bray, E.A., Bailey-Serres, J. and Weretilnyk, E. 2000. Responses to abiotic stresses. In: Gruissem, W., Buchannan, B. and Jones, R. (eds.), *Biochemistry and molecular biology of plants*. American Society of Plant Physiologists, Rockville, MD, pp. 1158–1249.

Caenepeel, S., Charydczak, G., Sudarsanam, S., Hunter, T. and Manning, G. 2004. The mouse kinome: discovery and comparative genomics of all mouse protein kinases. *Proc. Natl Acad. Sci.*, USA. 101: 11707–11712.

Ceccardi, T.L., Meyer, N.C. and Close, T.J. 1994. Purification of a maize dehydrin. *Protein Express. Purif.* 5:266–269.

Chang, C., Kwok, S.F., Bleecker, A.B., and Meyerowitz, E.M. 1993. Arabidopsis ethylene-response gene *ETRJ:* similarity of product to two-component regulators. *Sci.* 262: 539-544.

Chang, S., Pryear, J. and Cairney, J. 1993. A simple and efficient method for isolating RNA from pine trees. *Plant Mol. Biol. Rep.* 11: 113-117.

Chapin, F.S. 1991. Integrated responses of plants to stress. A centralized system of physiological responses. *BioSci.* 40: 29–31.

Charulata, Sahu, P.P. and Prasad, M. 2010. Comparative transcriptome analysis of differentially expressed genes in foxtail millet (*Setaria italica* L.) during dehydration stress. *Biochem. Biophys. Res. Commun.* 393: 720-727.

Chaves, M.M. 1991. Effects of water deficits on carbon assimilation. *J. Exp. Bot.* 42: 1-16.

Chomczynski, P. and Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *An. Biochem.* 162: 156-159.

Close, T.J. 1996. Dehydrins: emergence of a biochemical role of a family of plant dehydration proteins. *Plant Physiol.* 97: 795–803.

Cohen, P. 1988. Protein phosphorylation and hormone action. *Proc. R. Soc. B: Biol. Sci.* 234: 115–144.

Cullen, P.J. and Sprague, G.F.Jr. 2000. Glucose depletion causes haploid invasive growth in yeast. *Proc. Natl. Acad. Sci.*, USA. 97: 13619–13624.

Davies, W.J. and Zhang, J.H. 1991. Root signals and the regulation of growth and development of plants in drying soil. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 42: 55–76.

Davies, W., Kudoyarova, G. and Hartung, W. 2005. Long-distance ABA signaling and its relation to other signaling pathways in the detection of soil drying and the mediation of the plant's response to drought. *J. Plant Growth Reg.* 24: 285–295.

Delfine, S., Alvino, A., Villani, M.C. and Loreto, F. 1999. Restrictions to carbon dioxide conductance and photosynthesis in spinach leaves recovering from salt stress. *Plant Physiol.* 119: 1101–1106.

Diamant, S., Eliahu, N., Rosenthal, D. and Goloubinoff, P. 2001. Chemical chaperones regulate molecular chaperones *in vitro* and in cells under combined salt and heat stresses. *J. Biol. Chem.* 276: 39586–39591.

Diedhiou, C.J., Popova, O.V., Dietz, K.J. and Golldack, D. 2008. The SNF1- type serine–threonine protein kinase SAPK4 regulates stress responsive gene expression in rice. *BMC Plant Biol.* 8: 49.

Dominguez, O. and Lopez-Larrea, C. 1994. Gene walking by unpredictably primed PCR. *Nucl. Acids Res.* 22: 3247-3248.

Dure, L. 1993a. Structural motifs in Lea proteins. In: Close, T.J. and Bray, E.A. (eds.), *Plant response to cellular dehydration during environmental stress.* American Society of Plant Physiologists, Rockville, MD, pp. 91–103.

Dure, L. 1993b. A repeating 11-mer amino acid motif and plant desiccation. *Plant J.* 3: 363–369.

Eisen, J.A., Coyne, R.S., Wu, M., Wu, D., Thiagarajan, M., Wortman, J.R., Badger, J. H., Ren, Q., Amedeo, P. and Jones, K.M. 2006. Macronuclear genome sequence of the ciliate *Tetrahymena thermophila*, a model eukaryote. *PLoS Biol.* 4: 286.

Fan, L. and Neumann, P.M. 2004. The spatially variable inhibition by water deficit of maize root growth correlates with altered profiles of proton flux and cell wall pH. *Plant Physiol.* 135: 2291–2300.

Fan, L., Linker, R., Gepstein, S., Tanimoto, E., Yamamoto, R. and Neumann, P.M. 2006. Progressive inhibition by water deficit of cell wall extensibility and

growth along the elongation zone of maize roots is related to increased lignin metabolism and progressive stelar accumulation of wall phenolics. *Plant Physiol.* 140: 603–612.

Fersht, A.R. 1977. *Enzyme Structure and Mechanism.* W. H. Freeman, San Francisco, pp. 170-175.

Forster, C., Arthur, E., Crespi, S., Hobbs, S.L.A., Mullineaux, P. and Casey, R. 1994. Isolation of a pea (*Pisum sativum*) seed lipoxygenase promoter by inverse polymerase chain reaction and characterization of its expression in transgenic tobacco. *Plant Mol. Biol.* 26: 235-248.

Galle′, A., Haldimann, P. and Feller, U. 2007. Photosynthetic performance and water relations in young pubescent oak (*Quercus pubescens*) trees during drought stress and recovery. *New Phytologist.* 174: 799–810.

Gaxiola, R.A., Li, J., Undurraga, S., Dang, L.M., Allen, G.J., Alper, S.L. and Fink, G.R. 2001. Drought- and salt-tolerant plants result from over-expression of the AVP1 H^+-pump. *Proc. Natl. Acad. Sci.*, USA. 98: 11444–11449.

Goldberg, J.M., Manning, G., Liu, A., Fey, P., Pilcher, K.E., Xu, Y. and Smith, J.L. 2006. The dictyostelium kinome-analysis of the protein kinases from a simple model organism. *PLoS Genet.* 2: 38.

Gomez-Cadenas, A., Verhey, S.D., Holappa, L.D., Shen, Q., Ho, T.H., Walker-Simmons, M.K. 1999. An abscisic acid-induced protein kinase, PKABA1, mediates abscisic acid-suppressed gene expression in barley aleurone layers. *Proc. Natl. Acad. Sci.*, USA. 96: 1767–1772.

Gomez-Cadenas, A., Zentella, R., Walker-Simmons, M.K. and Ho, T.H. 2001. Gibberellin/abscisic acid antagonism in barley aleurone cells: site of action of the protein kinase PKABA1 in relation to gibberellins signaling molecules. *Plant Cell* 13: 667–679.

Gong, D.M., Guo, Y., Jagendorf, A.T. and Zhu, J.K. 2002. Biochemical characterization of the *Arabidopsis* protein kinase SOS2 that functions in salt tolerance. *Plant Physiol.* 130: 256–264.

Grzesiak, M.T., Grzesiak, S. and Skoczowski, A. 2006. Changes of leaf water potential and gas exchange during and after drought in triticale and maize genotypes differing in drought tolerance. *Photosynthetica* 44: 561–568.

Ha¨ rndahl, U., Hall, R.B., Osteryoung, K.W., Vierling, E., Bornman, J.F. and Sundby, C. 1999. The chloroplast small heat shock protein undergoes oxidation-dependent conformational changes and may protect plants from oxidative stress. *Cell Stress Chaperones* 4: 129–138.

Hamada, A.M. 2000. Amelioration of drought stress by ascorbic acid, thiamine or aspirin in wheat plants. *Indian J. Plant Physiol.* 5: 358-364.

Hamilton, E.W. and Heckathorn, S.A. 2001. Mitochondrial adaptations to NaCl. Complex I is protected by anti-oxidants and small heat shock proteins, whereas complex II is protected by proline and betaine. *Plant Physiol.* 126: 1266–1274.

Hanks, S K. 1991. Eukaryotic protein kinases. *Curr. Opin. Stnict. Biol.* 1: 369-3833.

Hanks, S.K., and Quinn, A.M. 1991. Protein kinase catalytic domain sequence database: identification of conserved features of primary structure and classification of family members. *Methods Enzymol.* 200: 38-62.

Hanks, S.K., Quinn, A.M., and Hunter, T. 1988. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Sci.* 241, 42-52.

Hardie, D.G., Carling, D. and Carlson, M. 1998. The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? *Annu. Rev. Biochem.* 67: 821–855.

Hare, P.D., Cress, W.A. and Van, S.J. 1998. Dissecting the roles of osmolyte accumulation during stress. *Plant Cell Environ.* 21:535–553.

Hartl, F.U. 1996. Molecular chaperones in cellular protein folding. *Nat.* 381: 571–579.

Hendrick, J.P. and Hartl, F.U. 1993. Molecular chaperone functions of heat-shock proteins. *Annu. Rev. Biochem.* 62: 349–384.

Hirayama, T. and Oka, A. 1992. Novel protein kinase of *Arabidopsis thaliana* (APK1) that phosphorylates tyrosine, serine and threonine. *Plant Mol. Biol.* 20: 653–662.

Holappa, L. and Walker-Simmons, M.K. 1995. The wheat abscisic acid responsive protein kinase mRNA, PKABA1, is up-regulated by dehydration, cold temperature and osmotic stress. *Plant Physiol.* 108: 1203-1210.

Hong, S.W., Jon, J.H., Kwak, J.M. and Nam, H.G. 1997. Identification of a receptor-like protein kinase gene rapidly induced by abscisic acid, dehydration, high

salt and cold treatments in *Arabidopis thaliana. Plant Physiol.* 113: 1203–1212.

Honigberg, S.M. and Lee, R.H. 1998. Snf1 kinase connects nutritional pathways controlling meiosis in *Saccharomyces cerevisiae. Mol. Cell. Biol.* 18: 4548–4555.

Hopkins, W.G. 1999. The physiology of plants under water stress. In: Hopkins, W.G. (ed.), *Introduction to Plant Physiology.* J. Wiley and Sons, New York. pp. 451-475.

Horwitz, J.P., Chua, J., Curby, R.J., Tomson, A.J. and Fisher, B.E. 1964. Substrates for cytochemical demonstration of enzyme activity. *J. Med. Chem.* 7: 515-516.

Hrabak, E.M., Chan, C.W. and Gribskov, M. 2003. The Arabidopsis CDPK–SnRK superfamily of protein kinases. *Plant Physiol.* 132: 666–680.

Huai, J., Wang, M., He, J., Zheng, J., Dong, Z., Lv, H., Zhao, J. and Wang, G. 2008. Cloning and characterization of the SnRK2 gene family from *Zea mays. Plant Cell Rep.* 27: 1861–1818.

Hunter, T. and Plowman, G.D. 1997. The protein kinases of budding yeast: six score and more. *Trends Biochem. Sci.* 22: 18–22.

Hwang, I.W. and Goodman, H.M. 1995. An *Arabidopsis thaliana* root specific kinase homolog is induced by dehydration, ABA and NaCl. *Plant J.* 8: 3743.

Ingram, J. and Bartels, D. 1996. The molecular basis of dehydration tolerance in plants. *Annu. Rev. Plant Biol.* 47: 377–403.

Ingram, J. and Bartels, D. 1996. The molecular basis of dehydration tolerance in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 47: 377–403.

Iuchi, S., Kobayshi, M., Taji, T., Naramoto, M, Seki, M., Kato, T., Tabata, S., Kakubari, Y., Yamaguchi-Shinozaki, K. and Shinozaki, K. 2001. Regulation of drought tolerance by gene manipulation of 9-cis-epoxycarotenoid, a key enzyme in abscisic acid biosynthesis in *Arabidopsis*. *Plant J.* 27: 325–333.

Jaleel, C.A., Gopi, R., Sankar, B., Manivannan, P., Kishorekumar, A., Sridharan, R. and Panneerselvam, R. 2007. Studies on germination, seedling vigour, lipid peroxidation and proline metabolism in *Catharanthus roseus* seedlings under salt stress. *S. Afr. J. Bot.* 73: 190-195.

Johnson, R., Wagner, R., Verhey, S. and Walker-Simmons, M.K. 2002. The ABA-responsive kinase PKABA1 interacts with a seed-specific ABA response element binding factor, TaABF and phosphorylates TaABF peptide sequences. *Plant Physiol.* 130: 837–846.

Jung, K., Cao, P., Seo, Y., Dardick, C. and Ronald, P. 2010. The rice kinase phylogenomics database: a guide for systematic analysis of the rice kinase super-family. *Trends Plant Sci.* 10: 1-5.

Kakita, M., Murase, K., Iwano, M., Matsumoto, T., Watanabe, M., Shiba, H., Isogai, A. and Takayama, S. 2007. Two distinct forms of M-locus protein kinase localize to the plasma membrane and interact directly with S-locus receptor kinase to transduce self-incompatibility signaling in *Brassica rapa*. *Plant Cell* 19: 3961–3973.

Kavi Kishor, P.B., Hong, Z., Miao, G.H., Hu, C.A.A. and Verma, D.P.S. 1995. Overexpression of pyrroline-5-carboxylate synthatase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiol.* 25: 1387-1394.

Kazuoka, T. and Oeda, K. 1994. Purification and characterization of COR85-oligomeric complex from cold-acclimated spinach. *Plant Cell Physiol.* 35: 601–611.

Kelner, A., Pekala, I., Kaczanowski, S., Muszynska, G., Hardie, D.G. and Dobrowolska, G. 2004. Biochemical characterization of the tobacco 42-kD protein kinase activated by osmotic stress. *Plant Physiol.* 136: 3255–3265.

Kirschbaum, M.U.F. 1987. Water-stress in *Eucalyptus pauciflora* – comparison of effects on stomatal conductance with effects on the mesophyll capacity for photosynthesis and investigation of a possible involvement of photoinhibition. *Planta* 171: 466–473.

Kirschbaum, M.U.F. 1988. Recovery of photosynthesis from water stress in *Eucalyptus pauciflora*–a process in two stages. *Plant Cell Environ.* 11: 685–694.

Kozlowski, T.T. 1976. Water supply and leaf shedding. In: Kozlowski, T. T. (Ed.), *Water Deficits and Plant Growth* Academic Press. New York, pp. 191-223.

Kobayashi, Y., Yamamoto, S., Minami, H., Kagaya, Y., Hattori, T. 2004. Differential activation of the rice sucrose nonfermenting1-related protein kinase2 family by hyperosmotic stress and abscisic acid. *Plant Cell* 16: 1163–1177.

Krupa, A., Anamika, K. and Srinivasan, N. 2006. Genome-wide comparative analyses of domain organisation of repertoires of protein kinases of *Arabidopsis thaliana* and *Oryza sativa*. *Genet.* 380: 1–13.

Kumar, S.G., Mattareddy, A. and Sudhakar, C. 2003. NaCl effects on proline metabolism in two high yielding genotypes of mulberry (*Morus alba* L.) with contrasting salt tolerance. *Plant Sci.* 165: 1245-1251.

Kushiro, T., Okamoto, M., Nakabayashi, K., Yamagishi, K., Kitamura, S., Asami, T., Hirai, N., Koshiba, T., Kamiya, Y. and Nambara, E. 2004. The *Arabidopsis* cytochrome P450 CYP707A encodes ABA 8#- hydroxylases: key enzymes in ABA catabolism. *EMBO J.* 23: 1647–1656.

Kushwah, R. 2008. Development and analysis of ESTs (Expressed Sequence Tags) in black pepper (*Piper nigrum* L.). MSc (Ag) thesis, Kerala Agricultural University, Thrissur.

Kwak, J.M., Moon, J.H., Murata, Y., Kuchitsu, K., Leonhardt, N., DeLong, A. and Schroeder, J.I. 2002. Disruption of a guard cell-expressed protein phosphatase 2A regulatory subunit, RCN1, confers abscisic acid insensitivity in Arabidopsis. *Plant Cell* 14: 2849–2861.

Larcher, W. 1995. Ecophysiology and stress physiology of functional groups. In. *Physiological Plant Ecology*. Springer, Berlin. pp. 286-296.

Lee, G.J., Roseman, A.M., Saibil, H.R. and Vierling, E. 1997. A small heat shock protein stably binds heat-denatured model substrates and can maintain a substrate in a folding-competent state. *EMBO J.* 3: 659–671.

Lei, G., Qiao, D., Bai, L., Xu, H. and Cao, Y. 2008. Isolation and characterization of a mitogen-activated protein kinase gene in the halotolerant alga *Dunaliella salina. J. Appl. Phycol.* 20: 13–17.

Leung, J. and Giraudat, J. 1998. Abscisic acid signal transduction. *Annu. Rev. Plant Biol.* 49: 199–222.

Lexa, M., Horak, J. and Brzobohaty, B. 2001. Virtual PCR. *Bioinf.* 17(2): 192-193.

Li, J., Wang, X.Q., Watson, M.B. and Assmann, S.M. 2000. Regulation of abscisic acid-induced stomatal closure and anion channels by guard cell AAPK kinase. *Sci.* 287: 300–303.

Lichtenthaler, H.K. 1996. Vegetation stress: an introduction to the stress concept in plants. *J. Plant Physiol.* (148) 4–14.

Liu, J., Zeng, H., Li, X., Wang, Y., Tang, W. and Han, L. 2010. Isolation and Characterization of the Betaine Aldehyde Dehydrogenase Gene in *Ophiopogon japonicus. Open Biotechnol. J.* 4: 18-25.

Loomis, W.D. 1974. Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. *Meth. Enzymol.* 31: 528-545.

Ludwig, A.A., Romeis, T. and Jones, J.D. 2004. CDPK-mediated signalling pathways: specificity and cross-talk. *J. Exp. Bot.* 55: 181–188.

Ma, S. and Bohnert, H.J. 2007. Integration of *Arabidopsis thaliana* stress related transcript profiles, promoter structures and cell-specific expression. *Genome Biol.* 8: 49.

Madan, M.S. 2007. Economic analysis of low input technology for increased black pepper (*Piper nigrum* L.) production. *Indian J. Agril. Sci.* 77 (7): 445-7.

Mahajan, S. and Tuteja, N. 2006. Cold, salinity and drought stresses: An overview. *Arch. Biochem. Biophys.* 444: 139-158.

Mali, G.V. 2010. Characterization of subtracted cDNA library for detecting Expressed Sequence Tags (ESTs) specific to drought tolerance in black pepper (*Piper nigrum* L.). MSc (Ag) thesis, Kerala Agricultural University, Thrissur.

Manning, G., Whyte, D.B., Martinez, R., Hunter, T. and Sudarsanam, S. 2002. The protein kinase complement of the human genome. *Sci.* 298: 1912–1934.

Maruyama, K. and Sugano, S. 1994. Oligo-capping: a simple method to replace the cap structure of eukaryotic mRNAs with oligoribonucleotides. *Genet.* 138: 171-174.

Mathai, C.K. 1983. Growth and yield analysis in black pepper varieties (*Piper nigrum* L.) under different light conditions. Ph D Thesis, University of Agricultural Sciences, Bangalore.

Monks, D.E., Aghoram, K., Courtney, P.D., DeWald, D.B. and Dewey, R.E. 2001. Hyperosmotic stress induces the rapid phosphorylation of a soybean phosphatidylinositol transfer protein homolog through activation of the protein kinases SPK1 and SPK2. *Plant Cell* 13: 1205–1219.

Moran, J.F., Becana, M., Iturbe-Ormaetxe, I., Frechilla, S., Klucas, R.V. and Aparicio-Tejo, P. 1994. Drought induces oxidative stress in pea plants. *Planta* 194: 346–352.

Morrison, D.K., Murakami, M.S. and Cleghon, V. 2000. Protein kinases and phosphatases in the Drosophila genome. *J. Cell Biol.* 150: 57–62.

Mullis, K. and Faloona, L. 1987. Specific synthesis of DNA *in vitro* via a polymerase catalyzed chain reaction. *Methods Enzymol.* 155: 335-350.

Mullis, K., Scharf, F.S., Saiki, R., Hprn, G. and Erlich, H. 1986. Specific ezymatic amplification of DNA *in vitro*: The polymerase chain reaction. *Cold Spring Harbor Symp. Quant. Biol.* 51: 263-273.

Mundy, J. and Chua, N.H. 1988. Abscisic acid and water stress induce the expression of a novel rice gene. *EMBO J.* 7: 2279–2286.

Munné-Bosch, S., Jubany-Marí, T. and Alegre, L. 2001. Drought-induced senescence is characterized by a loss of antioxidant defences in chloroplasts. *Plant Cell Environ.* 24: 1319–1327.

Munné-Bosch, S. and Alegre, L. 2004. Die and let live: leaf senescence contributes to plant survival under drought stress. *Func. Plant Biol.* 31: 203-216.

McCue, K.F. and Hanson, A.D. 1990. Drought and salt tolerance: toward understanding and application. *Trends Biotechnol.* 8: 358–362.

McNeil, S.D., Nuccio, M.L. and Hanson, A.D. 1999. Betaines and related osmoprotectants: targets for metabolic engineering of stress resistance. *Plant Physiol.* 120: 945–949.

Mellor, H. and Parker, P.J. 1998. The extended protein kinse C superfamily. *Biochem. J.* 332: 281–292.

Miranda-Saavedra, D. and Barton, G.J. 2007. Classification and functional annotation of eukaryotic protein kinases. *Proteins* 68: 893–914.

Mittler, R. 2002. Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci.* 7: 405–410.

Mittler, R. 2006. Abiotic stress, the field environment and stress combination. *Trends Plant Sci.* 11: 15–19.

Miyashita, K., Tanakamaru, S., Maitani, T. and Kimura, K. 2005. Recovery responses of photosynthesis, transpiration and stomatal conductance in kidney bean following drought stress. *Environ. Exp. Bot.* 53: 205–214

Mizoguchi, T., Irie, K. Hirayama, T., Hayashida, N., Yamaguchi-Shinozaki, K., Matsumoto, K. and Shinozaki, K. 1996. A gene encoding a mitogen-activated protein kinase kinase kinase is induced simultaneously with genes for a mitogen-activated protein kinase and an S6 ribosomal protein kinase by touch, cold and water stress in *Arabidopsis thaliana. Proc. Natl. Acad. Sci.*, USA. 93: 765-769.

Monks, D.E., Aghoram, K., Courtney, P.D., DeWald, D.B. and Dewey, R.E. 2001. Hyperosmotic stress induces the rapid phosphorylation of a soybean phosphatidylinositol transfer protein homolog through activation of the protein kinases SPK1 and SPK2. *Plant Cell* 13: 1205–1219.

Moran, J.F., Becana, M., Iturbe-Ormaetxe, I., Frechilla, S., Klucas, R.V. and Aparicio-Tejo, P. 1994. Drought induces oxidative stress in pea plants. *Planta* 194: 346–352.

Morrison, D.K., Murakami, M.S. and Cleghon, V. 2000. Protein kinases and phosphatases in the Drosophila genome. *J. Cell Biol.* 150: 57–62.

Mullis, K. and Faloona, L. 1987. Specific synthesis of DNA *in vitro* via a polymerase catalyzed chain reaction. *Methods Enzymol.* 155: 335-350.

Mullis, K., Scharf, F.S., Saiki, R., Hprn, G. and Erlich, H. 1986. Specific ezymatic amplification of DNA *in vitro*: The polymerase chain reaction. *Cold Spring Harbor Symp. Quant. Biol.* 51: 263-273.

Mundy, J. and Chua, N.H. 1988. Abscisic acid and water stress induce the expression of a novel rice gene. *EMBO J.* 7: 2279–2286.

Munné-Bosch, S., Jubany-Marí, T. and Alegre, L. 2001. Drought-induced senescence is characterized by a loss of antioxidant defences in chloroplasts. *Plant Cell Environ.* 24: 1319–1327.

Munné-Bosch, S. and Alegre, L. 2004. Die and let live: leaf senescence contributes to plant survival under drought stress. *Func. Plant Biol.* 31: 203-216.

Muoma, J., Ombori, O. and Jesse, M. 2010. Signal transduction one of the current molecular approaches in the management of drought stress in the Sub-Saharan region. *Biotechnol.* 9(4): 469-476.

Murase, K., Shiba, H., Iwano, M., Che, F.S., Watanabe, M., Isogai, A. and Takayama, S. 2004. A membrane-anchored protein kinase involved in *Brassica* self-incompatibility signaling. *Sci.* 303: 1516–1519.

Mustilli, A.C., Merlot, S., Vavasseur, A., Fenzi, F. and Giraudat, J. 2002. *Arabidopsis* OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. *Plant Cell* 14: 3089–3099.

Nambara, E. and Marion-Poll, M. 2005. Abscisic acid biosynthesis and catabolism. *Annu. Rev. Plant Biol.* 56: 165–185.

Nath, A.K., Kumari, S. and Sharma, D.R. 2005. *In vitro* selection and characterization of water stress tolerant cultures of bell pepper. *Indian J. Plant Physiol.* 10: 14-19.

Neumann, P.M. 1995. The role of cell wall adjustment in plant resistance to water deficits. *Crop Sci.* 35: 1258–1266.

Noctor, G. and Foyer, C. 1998. Ascorbate and glutathione: keeping active oxygen under control. *Annu. Rev. Plant. Physio. Plant Mol. Biol.* 49: 249–279.

Ochman, H., Gerber, A.S. and Hartl, D.L. 1988. Genetic applications of an inverse polymerase chain reaction. *Genet.* 120: 621-623.

Ohara, O., Dorit, R.L. and Gilbert, W. 1989. One sided polymerase chain reaction: The amplification of cDNA. *Proc. Natl. Acad. Sci., U.S.A.* 86: 5673-5677.

Okuma, E., Soeda, K., Tada, M. and Murata, Y. 2003. Exogenous proline mitigates the inhibition of growth of *Nicotiana tabacum* cultured cells under saline conditions. *Soil Sci. Plant Nutr.* 46:257-263.

Osakabe, Y., Mizuno, S., Tanaka, H., Maruyama, K. 2010. Overproduction of the membrane-bound receptor-like protein kinase1, RPK1, enhances abiotic stress tolerance in *Arabidopsis*. *J. Biol. Chem.* 285(12): 9190–9201.

Padilla, F.M. and Pugnaire, F.I. 2007. Rooting depth and soil moisture control Mediterranean woody seedling survival during drought. *Funct. Ecol.* 21: 489–495.

Park, S., Li, J., Pittman, J.K., Berkowitz, G.A., Yang, H., Undurraga, S., Morris, J., Hirschi, K.D. and Gaxiola, R.A. 2005. Up-regulation of an H+-pyrophosphatase (H^+-PPase) as a strategy to engineer drought-resistant crop plants. *Proc. Nat. Acad. Sci.*, USA. 102: 18830–18835.

Parker, J.D., Rabinovitch, P.S. and Burmer, G.C. 1991. Targeted gene walking polymerase chain reaction. *Nucl. Acids Res.* 19: 3055-3060.

Pillay, V.S., Sasikumaran, S. and Ibraqhim, K.K. 1988. Effect of rainfall on yield of black pepper. *In*: *Agrometerology of Plantation Crops.* Kerala Agricultural University, Trichur. pp. 152 –159.

Plowman, G.D., Sudarsanam, S., Bingham, J., Whyte, D. and Hunter, T. 1999. The protein kinases of *Caenorhabditis elegans*: a model for signal transduction in multicellular organisms. *Proc. Natl. Acad. Sci.*, USA. 96: 13603–13610.

Polidoros, A.N., Pasentsis, K. and Tsaftaris, A.S. 2006. Rolling circle amplification-RACE: a method for simultaneous isolation of 5′ and 3′ cDNA ends from amplified cDNA templates. *BioTechniques* 41: 35-42.

Portillo, M., Fenoll, C. and Escobar, C. 2006. Evaluation of different RNA extraction methods for small quantities of plant tissue: Combined effects of reagent type and homogenization procedure on RNA quality-integrity and yield. *Physiologia Plantarum* 128: 1–7.

Price, A.M., Atherton, N.M. and Hendry, G.A.F. 1989. Plants under drought-stress generate activated oxygen. *Free Rad. Res. Commun.* 8: 61–66.

Proebsting, E.L.Jr. and Middleton, J.E. 1980. The behavior of peach and pear trees under extreme drought stress. *J. Amer. Soc. Hort. Sci.* 105: 380–385.

Qamar, A.S., Chai, M., Luo, H., Song, F. and Mengiste, T. 2008. Tomato Protein Kinase 1b Mediates Signaling of Plant Responses to Necrotrophic Fungi and Insect Herbivory. *Plant Cell* 20: 1964-1983.

Rabbani, M.A., Maruyama, K., Abe, H., Khan, M.A., Katsura, K., Ito, Y., Yoshiwara, K., Seki, M., Shinozaki, K. and Yamaguchi-Shinozaki, K. 2003. Monitoring expression profiles of rice (*Oryza sativa* L.) genes under cold, drought and high-salinity stresses and ABA application using both cDNA microarray and RNA gel blot analyses. *Plant Physiol.* 133: 1755–1767.

Richard, S., Morency, M.J., Drevet, C., Jouanin, L. and S´eguin, A. 2000. Isolation and characterization of a dehydrin gene from white spruce induced upon wounding, drought and cold stresses. *Plant Mol. Biol.* 43: 1–10.

Ronde, J.A. Mescht, A.V. and Steyn, H.S.F. 1999. Proline accumulation in response to drought and heat stress in cotton. *Afr. Crop Sci. Soc.* 1-11.

Rood, S.B., Patino, S., Coombs, K. and Tyree, M.T. 2000. Branch sacrifice: cavitation-associated drought adaptation of riparian cottonwoods. *Trees* 14: 248–257.

Russell, B.L., Rathinasabapathi, B. and Hanson, A.D. 1998. Osmotic stress induces expression of choline monooxygenase in sugar beet and amaranth. *Plant Physiol.* 116: 859–865.

Sabehat, A., Lurie, S. and Weiss, D. 1998. Expression of small heat-shock proteins at low temperatures. *Plant Physiol.* 117: 651–658.

Saito, S., Hirai, N., Matsumoto, C., Ohigashi, H., Ohta, D., Sakata, K. and Mizutani, M. 2004. *Arabidopsis* CYP707As encode (+)-abscisic acid 8#-hydroxylase, a key enzyme in the oxidative catabolism of abscisic acid. *Plant Physiol.* 134: 1439–1449.

Samaras, Y., Bressan, R.A., Csonka, L.N. Gracia-Rios, M.G., Paino D'Urzo, M. and Rhoes, D. 1995. Proline accumulation during drought and salinity. In: Smirnoff, N. (Ed.). *Environment and plant metabolism: flexibility and accumulation.* Oxford, UK: BIOS Scientific Publishers, pp. 161-187.

Schenk, P.W. and Snaar-Jagalska, B.E. 1999. Signal perception and transduction: the role of protein kinase. *Biochem. Biophys. Acta* 1449: 1–24.

Seki, M., Narusaka, M., Ishida, J., Nanjo, T., Fujita, M., Oono, Y., Kamiya, A., Nakajima, M., Enju, A. and Sakurai, T. 2002. Monitoring the expression profiles of 7,000 Arabidopsis genes under drought, cold and high salinity stresses using a full-length cDNA microarray. *Plant J.* 31: 279–292.

Serrano, R. and Gaxiola, R. 1994. Microbial models and stress tolerance in plants. *Crit. Rev. Plant Sci.* 13: 121-138.

Serrano, R., Mulet, J.M., Rios, G., Marquez, J.A., de Larrinoa, I.F., Leube, M.P., Mendizabal, I., Pascual-Ahuir, A., Proft, M., Ros, R. and Montesinos, C. 1999. A glimpse of the mechanisms of ion homeostasis during salt stress. *J. Exp. Bot.* 50: 1023–1036.

Shaefer, B. 1995. Revolution in rapid amplification of cDNA ends: new strategies for polymerase chain reaction cloning of full-length cDNA ends. *Analyt. Biochem.* 227: 255-273.

Sheen, J. 1998. Mutational analysis of protein phosphatase 2C involved in abscisic acid signal transduction in higher plants. *Proc. Natl. Acad. Sci.,* USA. 95: 975–980.

Shen, Q., Gomez-Cadenas, A., Zhang, P., Walker-Simmons, M.K., Sheen, J., Ho, T.H. 2001. Dissection of abscisic acid signal transduction pathways in barley aleurone layers. *Plant Mol. Biol.* 47: 437–448.

Sheveleva, E., Chmara, W., Bohnert, H.J. and Jensen, R.G. 1997. Increased salt and drought tolerance by D-ononitol production in transgenic *Nicotiana tabacum* L. *Plant Physiol.* 115: 1211– 1219.

Shinozaki, K. and Yamaguchi-Shinozaki, K. 1996. Molecular responses to drought and cold stress. *Curr. Opinion Biotechnol.* 7: 161-167.

Shinozaki, K. and Yamaguchi-Shinozaki, K. 1997. Gene expression and signal transduction in water stress response. *Plant Physiol.* 115: 327–334.

Shinozaki, K., Yamaguchi-Shinozaki, K. and Seki, M. 2003. Regulatory network of gene expression in the drought and cold stress responses. *Curr. Opinion Plant Biol.* 6: 410–417.

Shinozaki, K. and Yamaguchi-Shinozaki, K. 2007. Gene networks involved in drought stress response and tolerance. *J. Exp. Bot.* 58: 221-227.

Silver, J. and Keerikatte, V. 1989. Novel use of polymerase chain reaction to amplify cellular DNA adjacent to an integrated provirus. *J. Virol.* 63: 1924-1928.

Sinclair, T.R. and Muchow, R.C. 2001. System analysis of plant traits to increase grain yields on limited water supplies. *Agron. J.* 93: 263–270.

Sivamani, E., Bahieldin, A., Wraithc, J.M., Al-Niemia, T., Dyera, W.E., Hod, T.H.D. and Qu, R. 2000. Improved biomass productivity and water use efficiency under water deficit conditions in transgenic wheat constitutively expressing the barley HVA1 gene. *Plant Sci.* 155: 1–9.

Skriver, K. and Mundy, J. 1990. Gene expression in response to abscisic acid and osmotic stress. *Plant Cell* 2: 503–512.

Smirnoff N. 1993. The role of active oxygen in the response of plants to water deficit and desiccation. *New Phytologist* 125: 27–58.

Sofo, A., Dichio, B., Xiloyannis, C. and Masia, A. 2004. Effects of different irradiance levels on some antioxidant enzymes and on malondialdehyde content during rewatering in olive tree. *Plant Sci.* 166: 293–302.

Spices Board. 2008. *Spice Wise Area and Production* [online]. Available: http://www.indianspices.com/pdf/spicewisearprd.xls. [29 July 2010].

Stokes, S.B., Rugh, C.L. and Kamalay, J.C. 1990. RNA and DNA isolation from recalcitrant plant tissues. *Biotechniques* 9: 268-272.

Stone, J.M. and Walker, J.C. 1995. Plant protein kinase families and signal transduction. *Plant Physiol.* 108: 451-457.

Suprunova, T., Krugman, T., Distelfeld, A., Fahima, T., Nevo, E. and Korol, A. 2007. Identification of a novel gene (Hsdr4) involved in water-stress tolerance in wild barley. *Plant Mol. Biol.* 64: 17-34.

Sze, H., Lia, X. and Palmgrenb, M.G. 1999. Energization of plant cell membranes by H-pumping ATPases: regulation and biosynthesis. *Plant Cell* 11: 677–690.

Taji, T., Ohsumi, C., Iuchi, S., Seki, M., Kasuga, M., Kobayashi, M., Yamaguchi-Shinozaki, K. and Shinozaki, K. 2002. Important roles of drought and cold-inducible genes for galactinol synthase gene in stress tolerance in *Arabidopsis thaliana*. *Plant J.* 29: 417–426.

Thankamani, C.K., Chempakam, B. and Ashokan, P.K. 2003. Water stress induced changes in enzyme activities and lipid per oxidation in black pepper (*Piper nigrum* L). *J. Med. Arom. Plant sci.* 25 (3): 646-650.

Thomashow, M.F. 1998. Role of cold-responsive genes in plant freezing tolerance. *Plant Physiol.* 118: 1–7.

Thomashow, M.F. 1999. Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annu. Rev. Plant Biol.* 50: 571– 599.

To¨ ro¨ k, Z., Goloubinoff, P., Horva´ th, I., Tsvetkova, N.M., Glatz, A., Balogh, G., Varvasovszki, V., Los, D.A., Vierling, E., Crowe, J.H. and Vı´gh, L. 2001. Synechocystis HSP17 is an amphitropic protein that stabilizes heat-stressed membranes and binds denatured proteins for subsequent chaperone-mediated refolding. *Proc. Natl. Acad. Sci.,* USA. 98: 3098–3103.

Triglia, T., Peterson, M.G. and Kemp, D.J. 1988. A procedure for *in vitro* amplification of DNA segments that lie outside the boundaries of known sequences. *Nucl. Acids Res.* 16: 8186.

Triglia, T., Peterson, M.G. and Kemp, D.J. 1988. A procedure for *in vitro* amplification of DNA segments that lie outside the boundaries of known sequences. *Nucl. Acids Res.* 16: 8186.

Ullmann, A., Jacob, F. and Monod, J. 1967. Characterization by *in vitro* complementation of a peptide corresponding to an operator-proximal segment of the β-galactosidase structural gene of *Escherichia coli. J. Mol. Biol.* 24: 339-341.

Umezawa T, Fujita M, Fujita Y, Yamaguchi-Shinozaki K, Shinozaki K. 2006a. Engineering drought tolerance in plants: discovering and tailoring genes unlock the future. *Curr. Opinion Biotechnol.* 17: 113–122.

Umezawa, T., Okamoto, M., Kushiro, T., Nambara, E., Oono, Y., Seki, M., Kobayashi, M., Koshiba, T., Kamiya, Y. and Shinozaki, K. 2006 (b). CYP707A3, a major ABA 8#-hydroxylase involved in dehydration and rehydration response in *Arabidopsis thaliana. Plant J.* 46: 171–182.

Umezawa, T., Yoshida, R., Maruyama, K., Yamaguchi-Shinozaki, K. and Shinozaki, K. 2004. SRK2C, a SNF1-related protein kinase 2, improves drought tolerance by controlling stress-responsive gene expression in *Arabidopsis thaliana. Proc. Natl. Acad. Sci.,* USA. 101: 17306–17311.

Unyayar, S., Kele, Y. and Unatl, E. 2004. Proline and ABA levels in two sunflower genotypes subjected to water stress. *Bulg. J. Plant Physiol.* 30 (3-4): 34-47.

Urao, T., Katagiri, T., Mizoguchi, T., Yamaguchi-Shinozaki, K., Hayashida, N. and Shinozaki, K. 1994. Two genes that encode Ca2+- dependent protein kinases are induced by drought and high-salt stresses in *Arabidopsis thaliana. Mol. Gen. Genet.* 244: 331-340.

Vasantha, S., Thomas, T.V., Ramadasan, A. and Zachariah, T.J. 1989. Plastid pigments of black pepper cultivars, an evaluation of physiological parameters. *Indian J. Plant Physiol.* 33: 363-366.

Vierling, E. 1991. The roles of heat-shock proteins in plants. *Annu. Rev. Plant Biol.* 42: 579–620.

Vinocur, B. and Altman, A. 2005. Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. *Curr. Opin. Biotechnol.* 16(2): 123-132.

Wahid, P and Sitepu, D. 1987. *Current Status and Future Prospect of Pepper Development in Indonesia.* FAO, Regional Office for Asia and Pacific, Bangkok.

Wang, A., Doyle, M. and Mark, D. 1989. Quantitation of mRNA by the polymerase chain reaction. *Proc. Natl. Acad. Sci.* 86: 9717-9721.

Ward, P., Equinet, L., Packer, J. and Doerig, C. 2004. Protein kinases of the human malaria parasite *Plasmodium falciparum*: the kinome of a divergent eukaryote. *BMC Genomics* 5: 79.

Waters, E.R., Lee, G.J. and Vierling, E. 1996. Evolution, structure and function of the small heat shock proteins in plants. *J. Exp. Bot.* 47: 325–338.

Wrzaczek, M. and Hirt, H. 2001. Plant MAP kinase pathways: how many and what for? *Biol. Cell* 93: 81–87.

Wu, Y., Sharp, R.E., Durachko, D.M. and Cosgrove, D.J. 1996. Growth maintenance of the maize primary root at low water potentials involves increases in cell wall extension properties, expansin activity and wall susceptibility to expansins. *Plant Physiol.* 111: 765–772.

Xiong, L.M., Schumaker, K.S. and Zhu, J.K. 2002. Cell signal during cold, drought, and salt stress. *Plant Cell suppl.* 165– 183.

Xu, D., Duan, X., Wang, B., Hong, B., Ho, T.H.D. and Wu, R. 1996. Expression of a late embryogenesis abundant protein gene HVA1, from barley confers tolerance to water deficit and salt stress in transgenic rice. *Plant Physiol.* 110: 249–257.

Yadav, S.K., Jyothi, L.N., Maheswari, M., Vanaja, M. and Venkateswarlu, B. 2005. Influence of water deficit at vegetative, anthesis and grain filling stages on water relation and grain yield in sorghum. *Indian J. Plant Physiol.* 10: 20-24.

Yamaguchi-Shinozaki, K. and Shinozaki, K. 2005. Organization of cis-acting regulatory elements in osmotic- and cold-stress-responsive promoters. *Trends Plant Sci.* 10: 88–94.

Yang, J., Zhang, J., Ye, Y., Wang, Z., Zhu, Q. and Liu, L. 2004. Involvement of abscisic acid and ethylene in the responses of rice grains to water stress during filling. *Plant Cell Environ.* 27: 1055–1064.

Yoon, H.W., Kim, M.C. and Shin, P.G. 1997. Differential expression of two functional serine/threonine protein kinases from soybean that have an unusual acidic domain at the carboxy terminus. *Mol. Gen. Genet.* 255: 359–371.

Yoshida, R., Hobo, T., Ichimura, K., Mizoguchi, T., Takahashi, F., Alonso, J., Ecker, J.R. and Shinozaki, K. 2002. ABA-activated SnRK2 protein kinase is required for dehydration stress signaling in *Arabidopsis*. *Plant Cell Physiol.* 43: 1473–1483.

Zdobnov, E.M. and Apweiler, R. 2001. Inter- ProScan-an integration platform for the signature- recognition methods in InterPro. *Bioinf.* 17:847-848.

Zhang, J.Z., Creelman, R.A. and Zhu, J.K. 2004. From laboratory to field: Using information from Arabidopsis to engineer salt, cold and drought tolerance in crops. *Plant Physiol.* 135: 615–621.

Zhu, B.C., Su, J., Chan, M.C., Verma, D.P.S., Fan, Y.L. and Wu, R. 1998. Over-expression of [delta]-pyrroline-5-carboxylate synthatase gene and analysis of tolerance to water-stress and salt-stress in transgenic rice. *Plant Sci.* 25: 41-48.

Zhu, J.K. 2002. Salt and drought stress signal transduction in plants. *Annu. Rev. Plant Biol.* 53: 247–273.

Zhu, J.K., Hasegawa, P.M. and Bressan, R.A. 1997. Molecular aspects of osmotic stress in plants. *Crit. Rev. Plant Sci.* 16: 253–277.

Appendices

Appendix I

Details of available sequence data available on protein kinase genes

Sl. No	Designation of clone	Size (bp)	Protein coded (Blastn, Blastx)
1.	PNK21	721	Protein kinase
3.	PNK49	697	Receptor protein kinase

PNK-21 clone (*Piper nigrum* Kalluvally-21)

CGTGGTCGCGGCCGAGGTGTCAGACTTTGGATTGGCTCGCCAAGGGCCTGGTGAAGGACTTA
GTCATGTCTCAACTGCGGTTGTTGGGACTATAGGGTATGCAGCACCAGAATATATTCAAACT
GGACGTTTGACGGCTAAAAGTGATGTTTGGAGCTATGGAGTTGTCCTCTATGAACTGATCAC
TGGTAGAAAGCCAATAGACCGTAATCGTCCTAAGGGTGAACATAAACTACTTGAGTGGGTGA
AGCCTTATTTATCAGAGAAGCGTTTTTCCATGATTCTTGATCCAAGAATACAAGAATAGATT
TCCCTGAAGTCGGCCCAAAAGCTTGCCGCTGTTGCAAACAAATGCCTTGTACGACAGCAGAA
GGCACGTCCAAAGATGAGTGAGATTTTAGCGATTGTGAAGCAAATTGTGGAGTCATCAACAA
CTGGGTCTCCTCAACCTGCCATCAAAATCCTAGATTCAAGTGAAGTCTCCAATGAAGAACAC
AAAAACAGAAGCCGATTGAAACGAGCCATTGCTTATCTTAATATGGGTGGAGATGGCAAGAG
GTTTTACTGGTGGAAGTTCAAATCCAAGCTCAGTAAAGTTAAGTTGTGGAAGACGAAGATAG
GACTAATGGTTTCTAGGCTAGCGTCATGATAAGAAACCAGGCATAGGAGAAGTTGGGAAGTT
TTTAGAGATGGCCTAAAGAATCCACCATAAAGTGTCAGT

PNK49 clone (*Piper nigrum* Kalluvally-49)

TCGAGCGGCCGCCCGGGCAGGTACTTCTCTGAGGTGAATTTGTTGGGGAAGAGCAATCATGC
AGCAACTTATAAAGGAATGTTGAGAGATGGTTCTTTTGTTGTTGTGAAAAGATTTAGCAAAA
CCAGTTGCAAATCAGAGGAGGTGGAATTCCTGAAAGGGCTCAAGATCCTTATGTCGTTGCAG
CATGAAAACCTTGTTAGGCTTAAGGGTTTTTGCTGCTCTAAATCTAGAGGTGAATGTTTTCT
TGTATATGATTTTGTTGTTAATGGAAATTTGCTGCAATATCTTGATTCTAGAGATGGAAGTG
GGAGAATTCTTAATTGGTCTACCAGAGTATCAATCATTCACGGCATCGCCAAAGCTATTCGC
TACCTACATGCCAGCAACCCTAGCAGGCCTTCACTTGTTCATCAGAACATCTCAGCTCAGAA
GATCCTCTTGGATAAGCATTTCAAACCATTGCTTTCAGACTCTGGCTTCCACAAGCTCCTGG
CCAACGATGCTATCTTTTCTACTATTAAATCTAGTGCTGCCATGGGGGTACCTCGGCCGCGA
CCACGCCCTCCCCCTGACTCGTGGGCTCGGTCTTTCGCTGCGGCGACGTACCGCTCTCTCAA
GGGGTAACGTTACCCAAAATCGGGTAAGCAGAAAAAATGGGACAAAGGCCACAAAGCCGGAA
CTTAAGGCCCGTCTG

Appendix II

Composition of different media used in the study

1. Luria Bertani (LB) broth

Tryptone	-	10 g
Yeast Extract	-	5 g
NaCl	-	5 g
pH adjusted to	-	7± 0.2
Distilled water	-	to make up to 1000ml

2. Luria Bertani agar medium

Tryptone	-	10 g
Yeast Extract	-	5 g
NaCl	-	5 g
Agar	-	20g
pH adjusted to	-	7± 0.2
Distilled water	-	make up to 1000ml

Appendix III

Composition of the reaction mixture for colony PCR

➢	Template DNA	- 2.0µl
➢	10X Taq assay buffer	- 2.5µl
➢	dNTP mix (10mM)	- 1.0µl
➢	Forward primer	- 1.0µl
➢	Reverse primer	- 1.0µl
➢	Taq DNA polymerase (0.3 U)	- 2.0µl
➢	Autoclaved distilled water	- 15.5µl
		25 µl

PCR cycle set for colony PCR

➢	Initial denaturation	- 94°C for 2 min	
➢	Denaturation	- 94°C for 45sec	} 30 cycles
➢	Annealing	- 55°C for 1 min	
➢	Extension	- 72°C for 2 min	
➢	Final extension	- 72°C for 10 min	
➢	Cooling of samples	- 4°C for 10 min	

Details of primers used for colony PCR

T7 (Forward) -TAATACGACTCACTATAGGG

SP6 (Reverse) -ATTTAGGTGACACTATAGAA

Appendix IV

Buffers and dyes used in gel electrophoresis

1. 6x Loading/ tracking dye

Bromophenol blue	-	0.25%
Xylene cyanol	-	0.25%
Glycerol	-	30%

The dye was prepared and kept in fridge at 4^0C

2. Ethidium bromide (intercalating dye)

The dye was prepared as a stock solution of 10 mg/ ml in water and was stored at room temperature in a dark bottle.

3. 50x TAE buffer (pH 8.0)

Tris base	-	242.0 g
Glacial acetic acid	-	57.1 ml
0.5M EDTA (pH 8.0)	-	100 ml
Distilled water	-	1000 ml

The solution was prepared and stored at room temperature

4. 10X MOPS buffer (PH 7.0)

200mM MOPS (PH-7.0)	-	41.85 gm
80mM sodium acetate	-	6.56 gm
10mM EDTA	-	3.725 gm
DEPC water	-	1000 ml

The solution was prepared in DEPC autoclaved water and stored at room temperature

Appendix V

Reagents and primers used for RLM-RACE PCR

Amount	Component	Storage
12 µl	Calf Intestine Alkaline Phosphatase	-20^{0}C
50 µl	10X CIP Buffer	-20^{0}C
12 µl	Tobacco Acid Pyrophosphatase	-20^{0}C
50 µl	10X TAP Buffer	-20^{0}C
12 µl	T4 RNA Ligase	-20^{0}C
50 µl	10X T4 RNA Ligase Buffer	-20^{0}C
10 µl	Ammonium Acetate Stop Solution	-20^{0}C
10 µl	M-MLV Reverse Transcriptase	-20^{0}C
200 µl	DNTP Mix (2.5 mM each dNTP)	-20^{0}C
200 µl	10X RT Buffer	-20^{0}C
25 µl	10X PCR Buffer	-20^{0}C
25 µl	Random Decamers (50µM)	-20^{0}C
25 µl	RNase inhibitor (10 U/µL)	-20^{0}C
25 µl	Mouse Thymus RNA (1 mg/mL)	-20^{0}C
1.75 ml	Nuclease-free Water	Any temp.

RACE primers and adapter used for RLM-RACE PCR

Component	Concn.	Sequence (5' to 3') (bp)
5' RACE Adapter	0.3 µg/µL	GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGC UUUGAUGAAA
3' RACE Adapter	0.3 µg/µL	GCGAGCACAGAATTAATACGACTCACTATAGGT12VN
5' RACE Outer Primer	10 µM	GCTGATGGCGATGAATGAACACTG
5' RACE Inner Primer	10 µM	CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG
3' RACE Outer Primer	10 µM	GCGAGCACAGAATTAATACGACT
3' RACE Inner Primer	10 µM	CGCGGATCCGAATTAATACGACTCACTATAGG

Abstract

ISOLATION AND CHARACTERIZATION OF WATER STRESS ACTIVATED PROTEIN KINASE GENE FROM BLACK PEPPER *Piper nigrum* L. VAR. KALLUVALLY

By

BANKAR ASHOK DNYANDEO
(2009-11-101)

ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture

Kerala Agricultural University, Thrissur

CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY

COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR - 680 656

KERALA, INDIA

2011

ABSTRACT

Water stress is identified as one of the main constraint for enhancing the productivity in black pepper. To survive stress, plants employ a complex set of distinct signaling pathways that trigger stress-specific tolerance or avoidance in the organism as a whole. An important biochemical mechanism for regulating such pathways is reversible protein phosphorylation which is mediated by protein kinases. Gaining an understanding of the mechanisms that regulate the expression of these genes and functional annotation of their transcripts will be necessary for the genetic improvement of plants cultivated in extreme environments.

Genotypic variations for drought tolerance have been reported in black pepper and the variety Kalluvally is one of the drought tolerant genotype of black pepper (Thankamani, 2003). In the previous studies at the Centre, water stress specific cDNA library of Kalluvally was constructed by suppression subtractive hybridization (SSH) (Kushwah, 2008). Several protein kinase genes were found to be up-regulated during the study.

The present investigation was undertaken to obtain full length coding sequence information on the partial clones of protein kinase genes available in the cDNA library by reverse transcriptase polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE). The clones named PNK21 and PNK49 were selected from the library. The clones were revived on LB/ampicillin media and subjected to full length sequencing. Degenerate primers were designed based on protein kinase gene sequences of various crops, obtained from NCBI database to amplify the unknown ends of the transcripts by RT- PCR.

Plants raised under greenhouse were screened for revival after water stress with different interval and frequency under open condition. The total RNA was isolated using TRI® reagent from stressed plants and subsequently cDNA was synthesized. The PCR amplification was carried out using degenerate primers designed for all three clones. Amplicons of size of ~600 bp for PNK21 clone and

~600 bp for PNK49 clone were obtained. Direct sequencing of PCR product of PNK21 clone was done.

The sequence data obtained was merged and analysed using bioinformatics tools. Blastn analysis revealed ~50 per cent coverage with the cDNA sequences for protein kinase from database. So, further primers were designed to amplify the full length cDNA sequence by RNA ligase mediated-RACE.

The 3' end of the PNK21 cDNA was successfully amplified using RLM-RACE PCR with amplicon size of ~400 bp. The purified PCR product was ligated in pGEMT plasmid vector and cloned. The recombinant *E. coli* cells were selected based on blue white screening on LB agar containing ampicillin with X-gal and IPTG. After confirmation of the insert by colony PCR, the clones were sequenced.

The finally enriched sequence was analysed using bioinformatics tools. Blastn and Blastx revealed maximum similarity with *Ricinus communis* APK1B protein kinase. The sequence indicated open reading frame and conserved domains for protein kinase and polyadenylation signal site TATAAA was found just upstream of the polyA tail at 3' end when analysed with different Bioinformatics tools.