DEVELOPMENT AND ANALYSIS OF ESTs (EXPRESSED SEQUENCE TAGS) IN BLACK PEPPER (*Piper nigrum* L.)

By

RENU KUSHWAH

THESIS

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Faculty of Agriculture

Kerala Agricultural University, Thrissur

Centre for Plant Biotechnology and Molecular Biology

COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR - 680 656

KERALA, INDIA

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DECLARATION

I, hereby declare that this thesis entitled **"Development and analysis of ESTs (Expressed Sequence Tags) in black pepper (*Piper nigrum* L.)"** 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 **Renu Kushwah**

08.09.2008

CERTIFICATE

Certified that this thesis entitled **"Development and analysis of ESTs (Expressed Sequence Tags) in black pepper (*Piper nigrum* L.)"** is a bonafide record of research work done independently by **Ms. Renu Kushwah** under my guidance and supervision and that it has not formed the basis for the award of any degree, diploma, fellowship or associateship to her.

Vellanikkara
08.09.2008

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

CERTIFICATE

We, the undersigned members of the advisory committee of **Ms. Renu Kushwah** (2006-11-104), a candidate for the degree of **Master of Science in Agriculture**, with major field in **Plant Biotechnology**, agree that the thesis entitled **"Development and analysis of ESTs (Expressed Sequence Tags) in black pepper (*Piper nigrum* L.)"** may be submitted by **Ms. Renu Kushwah** in partial fulfillment of the requirement for the degree.

Dr. P.A. Nazeem
(Major advisor)
Professor and Head
Centre for Plant Biotechnology and Molecular Biology
College of Horticulture
Vellanikkara

Dr. D. Girija
(Member, Advisory committee)
Professor
Centre for Plant Biotechnology and
Molecular Biology (CPBMB)
College of Horticulture
Vellanikkara

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

Dr. S. Manjula
(Member, Advisory committee)
Scienitist B
Department of Plant Molecular Biology
Rajiv Gandhi Centre for Biotechnology
Thiruvananthapuram

External Examiner
.

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Dedicated to
My
BELOVED
MASTER

ABBREVIATIONS

ABA	**Abscisic Acid**
ABF	**ABA Responsive Element Binding Factor**
ABRE	**ABA Responsive Element**
NH_4OAc	**Ammonium acetate**
β	**Beta**
bp	**Base pair**
BLAST	**Basic Local Alignment Search Tool**
CBF	**C- repeats Binding Factor**
CPBMB	**Centre for Plant Biotechnology and Molecular Biology**
cDNA	**Complementary DNA**
CDD	**Conserved Domain Database**
^oC	**Degree Celsius**
DREB	**Dehydration Responsive Element Binding**
DNA	**Deoxyribonucleic acid**
dNTPs	**Deoxyribo Nucleoside Triphosphate**
DEPC	**Diethyl pyrocarbonate**
DIC	**Distributed Information Centre**
ds	**Double stranded**
EDTA	**Ethylene diamine tetra acetic acid**
ESTs	**Expressed Sequence Tags**
GSS	**Genomic Survey Sequence**
HSP	**Heat Shock Protein**
pH	**Hydrogen ion concentration**
IPTG	**Isopropyl thio galactoside**
Kb	**Kilo base**
LB	**Luria Bertani**
L	**Litre**
LEA	**Late Embryogenesis Abundant**

mRNA	**messenger RNA**
MOPS	**3-(N-Morpholino)-propanesulfonic acid**
µg	**Micro gram**
µl	**Micro Litre**
ml	**Milli litre**
mM	**Milli Molar**
M	**Molar**
NCBI	**National Centre for Biotechnology information**
ng	**Nano gram**
nm	**Nano meter**
NCED	**9-cis-Epoxycarotenoid Dioxygenase**
OD	**Optical density**
ORF	**Open Reading Frame**
%	**Percentage**
PCR	**Polymerase Chain Reaction**
P5CS	**Δ^1-pyrroline-5-carboxylate synthetase**
QTL	**Quantitative Trait Loci**
RT- PCR	**Reverse Transcriptase Polymerase Chain Reaction**
rpm	**revolutions per minute**
RNA	**Ribonucleic acid**
RNase	**Ribonuclease**
SCAMP	**Secretory Carrier Membrane Protein**
SA- PMPs	**Streptavidin- Paramagnetic Particles**
SSH	**Suppression subtractive Hybridization**
TAE	**Tris Acetate EDTA**
TIP	**Tonoplast Intrinsic Protein**
UV	**Ultra violet**
UTR	**Untranslated Region**
v/v	**volume/volume**
X-gal	**5bromo-4-chloro-3-indoyl-β-D-galactosidase**

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Introduction

1. INTRODUCTION

Black pepper is the most important spice of the world and is commonly known as the 'black gold'. It therefore is rightly called '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 patterns and the occurrence of drought in such areas may devastate the plantations of black pepper completely.

It has been estimated that approximately US$ 10 billion of primary food production is lost annually because of insufficient rainfall or lack of rain together. In view of this looming water crisis, breeders in both the public and private sectors are devoting increasing attention and resources to developing new high yielding cultivars endowed with higher yield potential and stability across a broad range of water availability (Blum, 1996; Richards *et al*., 2002). Global warming, no longer a conjecture but an alarming reality and the ensuing increased unpredictability of the intensity and frequency of rainfall patterns further underline the urgency and need for a more effective improvement of crop yield under drought conditions.

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. Unravelling the mechanisms involved in drought tolerance in the resistant genotypes, could help the molecular breeding of economical crops in a long way.

Environmental abiotic stress such as drought severely impairs plant growth and development and limits crop production. In order to survive and adapt to this stress, plants must modulate various physiological and metabolic responses

based on the stress signals (Bohnert *et al.*, 1995). Thus identifying and understanding these mechanisms of drought is crucial for the development of tolerant commercial cultivars. Tolerance to drought involves complex mechanisms working in combination in order to avoid or tolerate water deficits. Many genes are reported to be involved in drought conditions which function not only in directly protecting the cells but also in the regulation of gene expression and signal transduction (Shinozaki and Yamaguchi, 1997). The molecular basis for plant tolerance to water stress remains unclear since several regulatory mechanisms are involved in the stress signal pathways thus making drought stress a multigenic character. Therefore, for producing drought tolerant plants it is important to understand this complex networks for which it becomes necessary to identify and characterize the genes that respond to water stress.

One of the powerful techniques of molecular biology to identify these differentially expressed genes is Suppression Subtractive Hybridization (SSH). It is a method of detecting and isolating gene sequences that are differentially expressed. Way *et al*. (2005) have successfully used this technique to identify genes expressed in wheat undergoing gradual water deficit stress. Gazendam and Oelofse (2007) have used this technique to isolate cowpea genes conferring drought tolerance.

The basic tool which is now becoming popular because of its various advantages for analysis of the isolated genes is Expressed Sequence Tags (EST), generated via partial sequencing of cDNA clones. This resource contains a vast amount of information about the genes that are expressed in higher plants at different developmental stages and under a variety of environmental conditions. Thus, EST development is a systematic means to catalog the transcriptome and profiling of the transcripts with genes and allow studies of complex cellular processes including mechanisms of plant adaptation to a variety of biotic and abiotic factors (Chen *et al*., 2002). Once identified, these genes can be used in developing transgenics.

With this background the present study was undertaken to develop and analyze ESTs with special reference to drought tolerance in black pepper variety Kalluvally using suppression subtractive hybridization.

Review of Literature

2. REVIEW OF LITERATURE

Back pepper, christened as 'King of spices' and 'black gold' is the most widely used spice in the world occupying a position that is supreme and unique and is the most important export oriented spice crop of India. Black pepper occupies an area of 253130 ha with production of 90856 tonnes (2005-2006). Kerala is in the foremost position contributing about 95 percent of area (24055/ha) and production (74980 tonnes) of the crop in the country. However, in productivity India occupies the last position among the leading black pepper producing countries in the world (Alagappan and Manoharan, 2001).

More than 80 percent of the cultivable land in the world is under rainfed condition. In such areas, drought is the major constraint for the crop productivity and so in plantation crops since they are widely grown in different soil types such as sandy, sandy loam, laterite and forest soils in the state of Kerala, Karnataka, Tamilnadu and Andhra Pradesh (Rajagopal and Nareshkumar, 2003).

Even though Kerala receives good rains, rainfall is not distributed uniformly and the crop suffers due to moisture stress from December to May (Ravindran *et al.,* 2002). Increasing the crop area under irrigation has several limitations, water resources being the major one and water requirement of black pepper being fairly high. Since, the availability of water has become a limiting factor; further increase in crop area under irrigation is very difficult. Then, the approach to mitigate such problems has to be to use the available water source with high productivity efficiency. Thus, it becomes necessary to identify the varieties, which are tolerant to water stress conditions in the field and to evolve management strategies for conserving available water resources to over come adverse effects of drought. In the breeding strategies, exploitation of the identified varieties which are not only drought tolerant, but also have the potential for high yield under limited water conditions, would be an important step for overall improvement of crop productivity in drought prone areas. Taking into the above

considerations black pepper variety Kalluvally was selected for the present study as this variety was reported to be one of the hardy genotypes among six other cultivars (Thankamani *et al.,* 2003). In the present study, an attempt has been made to identify the genes that are differentially expressed during water stress conditions and thus develop and analyze Expressed Sequence Tags (ESTs) through subtracted cDNA library construction using suppression subtractive hybridization (SSH). A concise review of the previous research studies in relation to the present study has been done. The contents of this chapter are placed under the following heads:

2.1 Introduction to drought
2.2 Crop losses due to drought
2.3 Responses of plants to drought conditions
2.4 Molecular basis of drought tolerance
2.5 Isolation of differentially expressed genes using SSH.
2.6 Development of ESTs
2.7 Future perspectives

2.1 Introduction to Drought

Abiotic stress is the primary cause of crop loss world wide, reducing average yields for most of the major crop plants by more than 50 percent. Among the abiotic factors that have shaped and continue shaping plant evolution, water availability is the most important (Bray *et al.*, 1997).

In meteorological term, drought may be defined as lack of precipitation over a prolonged time period. In agronomical term, drought refers to a situation where any area receives annual rainfall less than its average rainfall. In physiological terms, drought is a situation where transpiration rate exceeds absorption rate, so plant experiences a stress.

Drought is a major abiotic stress that severely affects agricultural systems and food production. It is generally accepted to be the widest spread abiotic stress experienced by crop plants and the major limiting factor to crop production in most areas of the world, especially in the tropics. Intermittent drought at critical stages of many crops reduces yield.

Despite many decades of research, drought continues to be a challenge to agricultural scientists in general and to plant breeders in particular. This is due to the unpredictability of its occurrences, severity, timing and duration; and the interaction of drought with other abiotic stresses (Ceccarelli and Grando, 1996).

2.2 Crop losses due to Drought

Drought can significantly reduce crop yields and restrict the latitudes and soils on which commercially important species can be cultivated (Blum, 1985). In black pepper a rainless period extending up to three months together with a frequent day temperatures of 35^{o}C are deleterious to the crop. During the six months from December to May, even the total rainfall received is scanty. The sudden dying of vines in hot summer was found to be due to the intense heat along with low soil temperature, which affected the base of vine (Ramadasan and Vasantha, 1994). In any such yellowing and subsequent death of plants, there is also the involvement of root damaging agents such as the burrowing nematode and *Phytophthora capsici*. Extensive damage to the feeder root system by these organisms' leads to an induced stress, subsequent yellowing of leaves, leaf shedding and vine death during the severe drought period. Vijayakumar *et al.*, (1984) observed leaf yellowing leading to development of necrotic patches and eventual loss of vines in post monsoon and summer season was considered to be the major cause for the development of the disorder. It was found that thousands of pepper plants dried up in Kannur district during the drought period of 1973, 1977, 1983, 1987 and 1989.

During 2004 drought in India, 62 million hectares (30%) of total crop area affected and food grains production fell to 24 million tonnes from normal (12%). A survey was conducted during the 6th Asia regional maize workshop in India in November 1995 to determine the effects of drought and low nitrogen on Asia maize yields. The results revealed that half of the total maize area in Asia has been affected by both stresses and yield loss due to drought was estimated to be 10 to 75 percent (Logroiio and Lothrop, 2006).

An annual estimated loss in peanut production equivalent to over (US $ 208 million) could be recovered through genetic enhancement for drought resistance with a benefit: cost ratio of 5:2 (Johansen and Nigam, 1994). The estimate of maize production losses due to drought in Indonesia was about 15 percent or about 1.2 million tonnes (Dahlan *et al.*, 2006).

According to a survey, conducted by Indian ministry of agriculture covering the 1987-1988 drought, the most severe since the mid 1960s, the gross domestic product of India decreased 7 percent on account of the loss of crops. Total loss of crop product accounted for 36million tonnes, mainly due to losses in wheat, rice and coarse cereals (Chaturvedi, 1994).

In Georgia, losses due to 2006 drought was about $ 819.4 million for all agricultural products including $ 239.5 million for cotton, $ 101.4 million for other field crops including peanuts, $ 40.1 million for corn, $ 25.3 million for tobacco, $ 107.6 million for hay and forage and $ 224.3 million for pastures (Annon, 2006).

2.3 Response of plants to drought conditions

Whole plants respond to drought through morphological, physiological and metabolic modifications occurring in all plant organs. At the cellular level plant responses to water deficit result from cell damage, whereas other responses

may correspond to adaptive processes. Although a large number of drought-induced genes have been identified in a wide range of plant species, a molecular basis for plant tolerance to water stress remain far from being completely understood (Ingram and Bartels, 1996).

2.3.1. Physiological, morphological and biochemical responses

Plants that are subjected to water stress lose water from the leaf tissues, stem and respond with a set of biochemical and physiological adaptations (Stankovic, 1996). Initial phenotypic responses involve stomata closure followed by changes in stomatal distribution. (Mansfield and Atkinson, 1990) and alteration in the root and shoot ratio. These processes occur in order to reduce water loss through both reduced transpiration and increased water uptake. These phenotypic manifestations result from a new set of organ and tissue specific process, which involves accumulation of increased amount of solutes in roots (Sharp and Davis, 1979), changes in hydrolytic conductivity coefficient, changes in cell elasticity (Fiscus, 1975; Stevdle and Zimmermann, 1977) and cell enlargement (Hsiao, 1973).

Drought decreases cuticular transpiration in seedlings (Beugtson *et al.*, 1978) and increase plasma membrane permeability (Quartacci and Navarilzzo, 1992). It reduces leaf area and protoplast volume (Saradadevi *et al.,* 1996) and decreases photosynthesis reorganizing the photosystem II (PSII), through increased phosphorylation of the PSII core coupled with increased D1 protein synthesis (Giardi *et al.,* 1996). Some plants turn from C_3 to CAM photosynthetic pathway so as to increase water use efficiency.

2.3.1.1 Black pepper

Thomas *et al.* (1990) studied the proline accumulation potential of ten selected cultivars of back pepper under PEG induced stress. Accumulation of

proline was higher and earlier in cvs. Kottandan and Neelamundi than control. Maximum decline in the accumulated proline was observed during recovery from water stress. Vasantha *et al.* (1991) reported that absolute proline content during water stress in black pepper ranged from 1.96 to 3.48 µmoles.

During moisture stress, expansion of leaves in black pepper decreased before the soil moisture reached a critical levels (Ramadasan and Vasantha, 1994). Root to shoot ratio was higher in cv. Kalluvally as compared to other cultivars. The content of epicuticular ranged from 1.46 to 2.08 mg/cm^3. The activity of nitrate was alone found to decline in all cultivars under water stress. Various physiological and biochemical parameters such as membrane leakage, relative water content (Rahman *et al*., 1999) enzyme activities viz. catalase, superoxide dismutase, peroxidase (Pederson and Aust, 1973; Kellog and Fridovich, 1975; Chempakam *et al*., 1993) and polyphenol oxidase and lipid peroxidation (Chempakam *et al.*, 1993) have been worked out to assess the effect of water stress on crop and its relative tolerance. Krishnamurthy *et al.* (2000) reported that in different black pepper genotypes, relative water content decreased and membrane leakage increased due to water stress. The activity of catalase and superoxide dismutase decreased while that of peroxidase and polyphenol oxidase increased with the intensity of the stress in all the genotypes.

Thankamani *et al.* (2003) found that the drought resistant cultivars showed a higher percentage increase in enzyme activities of peroxidase and superoxide dismutase on exposure to water stress than the drought susceptible cultivars. When severely water stressed, high levels of lipid peroxidation were observed in the drought susceptible cultivars compared to the tolerant cultivars. Chlorophyll and leaf epicuticular wax content of four black pepper varieties at mild, moderate, severe and zero water stress was studied by Thankamani and Ashokan (2002). They observed that under moderate water stress, chlorophyll a content decreased in all the varieties except Poonjarmunda and Uthirankotta -2. Severe stress decreased chlorophyll a, b and total content in all the varieties. Total chlorophyll

content was not affected, however chlorophyll a/b content varied with water stress. Maximum wax content was observed under severe water stress, which was relatively high in varieties Kalluvally – 4, Padarpan, Poonjarmunda, Kumbakodi, Panniyur-5 and Uthirankotta.

2.3.1.2 Other plants

Liu and Li (2005) studied the effects of soil water regime in two wheat cultivars, differing in drought tolerance with respect to root respiration, photosynthesis and grain yield. They found that the drought tolerance cultivar (Dingxi 24), had a higher biomass and metabolic activity under severe drought stress compared to the sensitive cultivar (Longchun 139-2), which resulted in further limitation of grain yield. Results showed that root respiration, carbohydrates allocation and grain yield were closely related to soil water status in wheat cultivars.

Pinheiro *et al.* (2004) studied drought tolerance in four clones of *Coffea canephora* (Robusta coffee) in relation to protection against oxidative stress when subjected to long-term drought. They found that drought tolerant clones were better able to maintain their leaf status than drought sensitive clones after withholding irrigation. Regardless of the clones irrigated, the net carbon assimilation rate decreased under drought stress. There was an increase in superoxide dismutase (Clones 109A and 120), ascorbate peroxidase (Clones 14, 46 and 109A), catalase, and guaiacol peroxidase (Clones 46 and 109A) and also in glutathione reductase (Clone 46) and dehydroascorbate reductase (Clone 109A).

Dulai *et al*. (2005) studied the responses of thermal stability of photosystem II to drought in bread wheat and in *Aegilops* species. The results indicated that the three-day drought treatment did not affect a considerable water loss in leaves and parallel with this, a significant heat tolerance increase of PS II

was not observable. But, it was observed that some *Aegilops* genotypes originating from arid habitats had better heat tolerance than wheat during drought.

Yin *et al.* (2004) found that exogenous ABA application in two Poplar species significantly affected morphological and physiological properties such as decreasing dry matter accumulation, specific leaf area and gas exchange and increasing at least relatively dry matter allocation into the root fraction, endogenous ABA content and δ^{13} C, under both well watered and water stressed conditions.

Susihoto and Berninger (2007) studied the interactions between morphological and physiological drought responses in *Eucalyptus microtheca* and found that the growth of the seedlings decreased and allocation patterns changed so that allocation to the roots increased. However, changes in photosynthesis and stomatal conductance under drought were modest.

Zayed *et al.* (1998) described that drought stressed mung bean (*Vigna radiata*) seedlings have increased concentration of hydrolytic enzymes such as α-amylase that resulted in an increased content of soluble polysaccharides. The accumulation of such organic solutes resulted improvement in cytoplasmic osmoregulation and thus increased plant tolerance to drought.

Cui *et al.* (2004) studied the effect of drought on physiological aspects of Crassulacean acid metabolism in Doritaenopsis, an orchid. They found that under light and dark conditions photosynthesis, stomatal conductance and transpiration decreased with increasing the duration of drought. Also, there was an increase in reduced sugar content while total sugar and starch content decreased. On imposition of drought there was an increase in phosphoenol pyruvate carboxylase and peroxidase activity while, superoxide dismutase activity decreased.

Bastide *et al.* (1993) showed that the tissue of *Xerosicyos*, an orchid response to water stress by accumulation of ABA that resulted in closing of stomata and limiting the CO_2 uptake, as compared to well watered control.

2.4 Molecular basis of drought tolerance

The complex plant response to abiotic stress involves many genes and biochemical- molecular mechanisms. A complex of signal transduction events that have not been clearly delineated induces changes in gene expression. Various genes respond to drought stress in several species, and functions of their gene products have been predicted from sequence homology with known proteins. Such genes that are induced during drought stress conditions provides tolerance to plant by functioning directly in protecting cells from water deficit by the production of important metabolic proteins and indirectly by regulating other genes for signal transduction (Shinozaki *et al.,* 2003).

These gene products can be classified into three major groups:

1. Those that encode products and directly protect plant cells against stresses.
   E.g. Heat stress proteins or chaperones, LEA proteins, osmoprotectants, antifreeze proteins, detoxification enzymes and free radicals scavengers (Bray *et al*., 2000).
2. Those that are involved in signaling cascades and in transcriptional control.
   E.g. MAPKs, CDPKs (Ludwig *et al*., 2004) and kinase (Zhu, 2001), phosopholipases (Frank et al., 2000) and transcriptional factors (Shinozaki and Yamaguchi-Shinozaki, 2000).
3. Those that are involved in water and ion uptake and transport such as aquaporins and ion transporters (Blumwald, 2000).

2.4.1 Drought associated genes and proteins

2.4.1.1 Heat shock proteins and chaperones

Heat shock proteins (Hsps) and molecular chaperons, as well as late embryogenesis abundant (LEA) protein families, are reported to be involved in plant drought stress tolerance (Wang *et al*., 2003; 2004). High temperature and drought stress can cause denaturation and dysfunction of many proteins.

Hsps and LEA proteins help to protect against stresses by controlling the proper folding and conformation of both structural (*i.e.* cell membrane) and functional (*i.e.* enzymes) proteins (Almoguera and Jordano, 1992; Vierling, 1991) Small hsps are also found to be associated with plant desiccation tolerance. These act as molecular chaperones during seed dehydration and first few days of rehydration (Hoekstra *et al.*, 2001). It has been shown that two of hsps, hsp 70 in maize and hsp 27 in soybean can also be induced by water stress (Sachs and David, 1986).

Over expression of LEA proteins was correlated in several cases with desiccation tolerance, although the actual function of these proteins is still unknown (Villalobos *et al.,* 2004). Recently, over expression of HVA1, a group 3 LEA protein isolated from barley (*Hordeum vulgare*) conferring dehydration tolerance to transgenic plants was reported (Chandra Babu *et al.*, 2004).

2.4.1.2 Reactive oxygen species

Stress induced production of reactive oxygen species (ROS) is another aspect of environmental stress in plants (Mittler, 2002). Alleviation of oxidative damage by the use of different antioxidants and ROS scavengers can enhance plant resistance to drought. Over expression of aldehyde dehydrogenase AtALDH3 gene in Arabidopsis conferred tolerance to drought (Sunkar *et al.*,

2003). The transgenic plants showed improved tolerance to dehydration; as well as to other type of stress suggesting that aldehyde dehydrogenase can maintain membrane integrity under osmotic stress. Aldehyde dehydrogenase catalyzes the oxidation of toxic aldehydes, which accumulates as a result of side reactions of ROS with lipids and proteins (Kirch *et al.,* 2004).

2.4.1.3 ABA responsive genes

Several different stresses may trigger the same or similar transduction pathways. The plant hormone ABA also accumulates in response to the physical phenomena of loss of water due to drought or other stresses and alleviation in endogenous ABA content is known to induce certain water deficit induced genes. Therefore, ABA accumulation is a step in one of the signal transduction pathways that induces genes during water deficit. Various protein kinases have been reported in plants and are thought to function in phosphorylation processes in various signal transduction pathways, including water stress and ABA responses.

Recently, 9-cis-epoxycarotenoid dioxygenase gene (NCED), involved in ABA synthesis has been found to be strongly induced under water deficit in the eight days old cowpea plants (Iuchi *et al.,* 2000).A cDNA, pKABA1, corresponding to a protein kinase, which is induced by ABA, has been isolated (Anderberg and Walker-Simmons, 1992).

Available evidences suggests that stress induced responses may be ABA mediated or independent of ABA (Shinozaki and Yamaguch-Shinozaki, 1997). ABA mediated gene response may require or may not require protein synthesis to take place. The induction of mRNA of rd22 gene by ABA, which showed homology to an unidentified seed protein of *Vicia faba*, required protein synthesis to take place since cycloheximide inhibited induction of the gene (Yamaguch-Shinozaki and Shinozaki, 1999).

2.4.1.4 Signaling cascades and transcriptional control

Genes involved in signaling cascades and in transcriptional control in drought tolerance such as kinases, phospholipases (Thiery *et al*., 2004) and transcription factors (e.g. heat shock factor) and the C- repeat binding factor /dehydration responsive element binding protein (CBF/DREB) and ABA responsive element binding factor/ ABA responsive element (ABF/ABRE) families have been extensively studied (Zhang *et al*., 2004).

There are some transcriptional factors such as MYB and MYC that are involved in drought tolerance. These factors function as transcription activators in the dehydration (Abe *et al*., 1997).

Upon over expression of DREB1A (a dehydration responsive element binding protein) under the control of rd29a promoter in *A. thaliana*, a number of stress tolerance genes were expressed and resulted in an improved tolerance under drought and several stresses (Kasuga *et al.*, 1999).

Analysis of another gene of DRE- binding protein DREB 2 showed that its promoter was induced under water stress in transgenic Arabidopsis (Nakasimha *et al.,* 2000). These genes do not require ABA for their expression but do respond to exogenous ABA.

2.4.1.5 Aquaporins

Apart from the above proteins, assisting in maintaining the dehydration status, drought or osmotically stressed plants, synthesize several genes which produce water channel proteins and water transport proteins such as membrane proteins of family aquaporins that can alter the cellular water potential and thus, protect against water deficit (Chrispeds and Agre, 1994).

Neale *et al*. (2000) reported the involvement of tonoplast intrinsic protein (TIP) a plant aquaporins during severe drought stress in resurrection grass (*Sporobolus stapfianus*) along with other proteins.

2.4.2 Drought associated genes and metabolites

Severe drought stress causes detrimental changes in cellular components. Wide ranges of metabolites that can prevent these detrimental changes have been identified. Theses are referred to as 'compatible solutes' and are also known as 'osmoprotectants' because their accumulation is assumed to have a putative protective function against water deficit. These include amino acid (e.g. proline), quaternary and other amines (e.g. glycine betaine and polyamines) and a variety of sugar alcohols (e.g. mannitol and trehalose) [Yancey *et al*., 1982].

2.4.2.1 Amino acids

Proline accumulation was correlated with improved plant performance under water stress. Transgenic tobacco over expressing P5CS (Δ^1-pyrroline-5-carboxylate synthetase; the enzyme involved in the proline biosynthesis from L-glutamate via. Δ^1-pyrroline-5-carboxylate) from moth bean (*Vigna acontifolia*) lead to 10 to 18 fold increase in proline content and showed better growth under water stress compared to the wild type (Kavi *et al.,* 1995).

To examine the possibility that plant growth reduction in response to osmotic stress might actually result from osmolyte accumulation, proline levels were manipulated by expressing mutated derivatives of P5CS from tomato in *Sacchromyces cerevisiae* (Maggio *et al*., 2002). The levels of proline accumulation and the cell growth were inversely correlated in cells grown under normal osmotic conditions.

2.4.2.2 Amines

Glycine betaine is a widely studied osmoprotectants, the accumulation of which has been studied with respect to modifications of several metabolic steps. Transgenic tobacco over expressing bet B gene (synthesizing betaine aldehyde dehydrogenase) from *E. coli* showed better performance under osmotic stress conditions (Holmstrom *et al*., 1994). Weretilynk and Hauson (1990) reported the expression of betaine aldehyde dehydrogenase gene in response to drought as well as salinity.

Plant polyamines are also involved in plant response to drought stress. Transgenic rice plants expressing *Datura stramonium* ADC under the control of the monocot Ubi-1 promoter produced much higher levels of putrescine under drought stress, promoting spermidine and spermine synthesis and ultimately protecting the plants from drought (Capell *et al.*, 2004).

2.4.2.3 Sugars and sugar alcohols

Overall carbon metabolism and the level of specific sugars are severely affected by abiotic stress such as drought. In *Setaria sphacelata*, a naturally adopted C4 grass, photosynthetic carbohydrate content was studied under conditions of both rapid and slow water deficit. In short term stress experiments, a decrease in sucrose and starch content was observed and in long-term experiment, a higher amount of soluble sugars and a lower amount of starch were found. The shift of metabolism might occur because starch synthesis and dehydration are more affected than sucrose synthesis (Silva and Arrabaca, 2004).

Trehalose, a rare non-reducing sugar, is present in many bacteria and fungi and in some desiccation tolerant higher plants. Rice tolerance to multiple abiotic stresses through engineering trehalose over expression was reported (Garg *et al*., 2002).

Mannitol is another sugar alcohol that accumulates upon water stress and other stresses. Transgenic wheat expressing the mannitol-1-phosphatase dehydrogenase gene (mtlD) of *E.coli* was significantly more tolerant to water and salt stress (Abebe *et al*., 2003)

2.5 Isolation of Differentially Expressed Genes through SSH

In higher eukaryotes, programs of differential gene expression mediate biological processes such as cellular growth and organogenesis. To understand the molecular regulation of these processes, the relevant differentially expressed genes of interest must be identified, cloned and studied in detail. Subtractive cDNA hybridization has been a powerful approach to identify and isolate cDNAs of differentially expressed genes (Duguid and Dinauer, 1990; Hara *et al*., 1991; Hendrick *et al*., 1984). Numerous cDNA subtraction methods have been reported, although these methods have been successful in some cases, they require several rounds of hybridization and are not well suited for the identification of rare messages (Sargent and David, 1983; Davis *et al*., 1984). A new PCR based cDNA subtraction method, termed as suppression subtractive hybridization (SSH) overcomes the technical limitations of traditional subtraction methods (Diatchenko *et al*., 1996; Gurskaya *et al*., 1996).

SSH is a powerful procedure that offers the advantage of being able to compare two mRNA populations and isolate the cDNAs of genes that are either over expressed or exclusively expressed in one population and not in the other (Kamakura *et al*., 1999; Ablett *et al*., 2000).

Way *et al*. (2005) identified and characterized genes involved in wheat adaptation to water deficit. Differentially expressed genes in wheat plants subjected to a progressive drought stress were isolated through suppression subtractive hybridization.

Gazendam and Oelofse (2007) used SSH technique to identify and isolate the genes conferring drought tolerance in cowpea and constructed a subtracted cDNA drought expression library.

de los Reyes *et al.* (2003) have developed ESTs in rice seedlings that represents an important subset of cold stress regulated genes by constructing subtracted cDNA library through SSH.

Dicto and Manjula (2005) have successfully utilized SSH in identification of genes from *Piper colubrinum* that were differentially expressed in response to the signaling molecule, Salicylic acid.

Park *et al.* (2004) have constructed subtractive cDNA library and generated EST database for the genes involved in secondary metabolism in *Camellia sinensis* (tea), using suppression subtractive hybridization.

Ok *et al*. (2003) have successfully utilized SSH technique to identify differentially expressed genes during flower development in Carnation. Chang *et al.* (2006) identified specially transcribed genes in sterile anther of wheat through suppression subtractive hybridization.

2.6 Development of Expressed Sequence Tags (ESTS)

Defining the transcriptome of a complete, multicellular eukaryote is a daunting challenge. The two most widely used and comprehensive approaches are whole genome sequencing coupled with application of gene prediction algorithms (Mathe *et al*., 2002) and single pass sequencing of cDNAs to obtain expressed sequence tags (ESTs; Adams *et al.*, 1991). Among newer approaches that have not been used as widely are targeted sequencing of gene rich regions, identified either as being hypomethylated (Rabinowicz *et al*., 1999; Bedell *et al*., 2005) or enriched in single copy sequences (Peterson *et al*., 2002), and several analysis of

gene expression (Velculescu *et al.*, 1995). No one methodological approach however is sufficient while others are in a practical sense incapable of identifying every potentially expressed gene.

Among available approaches development of ESTs provides a number of substantial advantages (Pratt *et al*., 2005):

1. It is much less expensive route to gene discovery than whole genome sequencing.
2. It offers unambiguous identification of transcribed genomic sequences.
3. It results in a cDNA resource that can serve broad scientific community.
4. It provides at no additional cost the templates suitable for cDNA based microarray applications.
5. It also provides information about gene expression as a function of developmental stage, organ and / or environmental parameters at the time plant material is harvested for RNA isolation.
6. It can reveal information about several transcript properties, including untranslated region (UTR) structures, polyadenylation signals and alternate splicing.

Because of these and other advantages, several EST projects in commercially important crop species have been initiated.

2.6.1 ESTs for abiotic stress

Diab *et al*. (2004) have developed ESTs to identify drought inducible genes and differentially expressed genes by QTL mapping in barley. Sharma and Kumar (2005) identified three drought modulated expressed sequence tags in tea using differential display mRNA technique.

Zheng *et al*. (2006) have developed expressed sequence tags specifically induced by water stress to analyze the expression profiles of maize seedlings under abscisic acid (ABA) treatment, high salinity and cold stress conditions.

Jin *et al.* (2006) generated ESTs by large scale sequencing of cDNA clones to identify the genes and characterize the complexity of adaptation of *Leymus chinensis* to high pH sodic soil.

Iturriaga *et al*. (2006) generated EST database to understand the molecular basis of dehydration tolerance by constructing a cDNA library from *Selaginella lepidophylla*, a resurrection plant.

A collection of ESTs from cDNA libraries from leaves and roots of Burma mangrove treated with NaCl, mannitol, phytohormones and dehydration was developed and analyzed (Miyama *et al*., 2006).

Wang *et al*. (2006) generated and analyzed ESTs to investigate the expression profile of *Tamarix androssowii*, a salt tolerant woody plant species, in response to $NaHCO_3$ stress.

Mishra *et al.* (2007) developed ESTs to identify differentially regulated transcripts in response to abiotic (salinity, drought and cold) stresses from subtracted cDNA libraries of Pearl millet.

Quaggiotti *et al.* (2007) generated ESTs to identify candidate genes involved in the regulation of the response to chromium in *Salix* species, chosen on the basis of their suitability in phytoremediation techniques.

Diab *et al* (2008) have developed ESTs for drought tolerance in durum wheat. They investigated the genetic and physiological bases of drought tolerance

in durum wheat by QTL mapping and mapping candidate genes derived from differentially expressed genes.

2.6.2 Other applications of ESTs

Besides use in identification of genes involved in abiotic stress, ESTs has also been developed for use in other applications.

Suiba *et al.* (2005) have developed EST database for *Lathyrus sativus* to identify the gene sequences similar to other plant genes functionally associated with disease response.

ESTs provide a valuable tool that can be used to identify genes in secondary metababolite synthesis. Jung *et al.* (2003) have developed Ginseng (*Panax ginseng*) ESTs to identify genes involved in biosynthesis of ginsenosides.

For refined annotation of the Arabidopsis genome Zhu *et al*. (2003) developed ESTs by complete EST mapping.

Peng *et al*. (2007) generated ESTs in *Vitis vinifera*, wine grape and table grape to identify new candidate genes that are involved in the development of berry.

Schlueter *et al*. (2004) have successfully utilized EST database to resolve evolutionary events like finding out gene duplications in major crop species such as *Zea mays*, *Oryza sativa*, *Sorghum bicolor*, *Hordeum vulgare, Solanum tuberosum*, *Lycopersicon esculentum*, *Medicago truncatula* and *Glycine max.*

Keilin *et al.* (2007) studied the expression pattern of grape bud during different developmental stages of bud by developing grape bud EST.

ESTs have also been developed to study the expression patterns in ornamental plants like Petunia (Shimamura *et al*., 2007) and *Phalaenopsis equestris*, an orchid (Tsai *et al*., 2006).

ESTs have proved to be powerful tool for gene discovery, gene mapping and for the analysis of quantitative traits. Pang *et al.* (2005) have developed EST libraries for gene discovery and marker development in cultivated peanut (*Arachis hypogaea*). Genetic relationships of D genome species between *G. arboreum* and *G. ramimondii* in *Gossypium* were studied by developing EST-SSR markers from EST library (Guo *et al*., 2007).

The growth pattern of plants can be easily studied using ESTs. Lee *et al.* (2005) generated ESTs to study the changes in gene expression during the growth phase of Poplar in suspension culture.

MicroRNAs (miRNAs) are a class of non coding RNAs that regulate gene post- transcriptional expression in animals and plants. Comparatively genomic computational methods have been developed to predict new miRNAs in worms, humans and Arabidopsis. An EST-GSS (Genomic survey sequence) based combine approach for the detection of novel miRNAs in *Gossypium hirsutum* was carried out by Qiu *et al*. (2007).

2.7 Future perspectives

Complementary DNA microarray analysis can be carried out to investigate the expression levels of high number of genes or clones simultaneously. Further, expression patterns of interesting genes that emanate from the microarray study, can be investigated by employing real time RT-PCR on RNA samples extracted from differentially drought treated plants.

The sequences obtained from the drought specific subtracted cDNA library can be used to generate probes to isolate genomic DNA containing the corresponding genes and to provide markers for physical maps.

Differential screening, *In situ* mRNA hybridization and use of reporter genes (GUS, GFP) under the control of water stress inducible promoters can be used to confirm the localization of the expressed genes.

ESTs are versatile and thus can be utilized further for multiple applications. Recently, applying DNA bar coding in EST project, Qui *et al*. (2003), categorized maize cDNA libraries using distinct 6-bp DNA sequences to track the origin of ESTs from specific mRNA pools.

ESTs have also become invaluable resources in the area of proteomics for peptide identification and proteome characterization, particularly in the absence of complete genome sequence information (Lisacek *et al*., 2001; Kim *et al.*, 2003).

The drought inducible genes identified can be used independently or in combination for producing drought tolerant crop plants by molecular breeding.

Materials and Methods

3. MATERIALS AND METHODS

The study on 'Development and analysis of ESTs (Expressed Sequence Tags) in black pepper (*Piper nigrum*) was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), IT – BT Complex, College of Horticulture, Vellanikkara during the period from August, 2006 to July, 2008. Materials used and methodologies adopted for the study are described in this chapter.

3.1 Materials

3.1.1 Plant materials

Black pepper (*Piper nigrum*) variety Kalluvally was used in the study and it was obtained from the 'Pepper Research Station' of Kerala Agricultural University located at Panniyur, Kannur. The cuttings were raised in polybags and maintained in green house for six months and later transferred to earthen pots of 12' diameter holding 8 kg potting mixture, which were then maintained in open conditions.

3.1.2 Chemicals, glassware and plastic ware

All the chemicals used in the study were of good quality (AR/GR grade) obtained from various firms such as Merck, Sisco Research Laboratories, Himedia and Sigma. Molecular grade enzymes, buffers, and DEPC treated water were supplied by Bangalore GeNei Ltd. RNase ZAP was obtained from Ambion, Inc, USA. Various kits such as PCR – Select ^{TM} cDNA Subtraction kit and Advantage 2 Polymerase Mix were procured from CLONTECH, USA. PolyATtract mRNA isolation kit and pGEMT vector system were obtained from Promega Corporation, USA. Competent cells preparation kit was obtained from GeNei, Bangalore. All the plastic ware used was procured from Axygen and Tarsons India Ltd.

3.1.3 Laboratory equipments

Instruments available at CPBMB were utilized for the work while Bioinformatics softwares were accessed from the Distributed Information Centre (DIC).

3.2 Methods

3.2.1 Estimation of soil moisture

For estimation of soil moisture during water stress, 6 months old fifteen potted plants (five potted plants with three replications each) of uniform size were selected and numbered as BP1 to BP15. All the pots were watered on first day and soil was collected from three different pots. From the second day onwards irrigation was withheld and soil sample (25 g) was collected from the three respective pots on 2^{nd}, 3^{rd}, 4^{th} and 5^{th} day. Soil moisture of the above collected soil samples was estimated using Gravimetric – oven drying method. The procedure followed is described below

1. About 25 g of soil was taken from each of the three different pots and transferred to pre-weighed aluminum boxes.
2. The weight of the aluminium box containing moist soil was recorded.
3. The moist soil was kept in aluminum boxes and dried at 105^{o}C for about 24 hrs in the hot air oven.
4. Weight of the sample after cooling was recorded.
5. Weight was recorded at regular intervals till a constant weight was obtained.
6. The following observations were taken
    a. Weight of empty aluminium box – W1 (g)
    b. Weight of aluminium box + moist soil – W2 (g)
    c. Weight of aluminium box + oven dried soil – W3 (g)

Moisture content was determined using the following formula

$$\% \text{ moisture on weight basis (W)} = \frac{(W2-W1) - (W3-W1)}{(W3-W1)} \times 100$$

Where,

(W2-W1) = Weight of moist soil

(W3-W1) = Weight of oven dried soil

(W3-W1) = Weight of oven dried soil

3.2.2 Estimation of tolerance of plants to water stress

For knowing the correct stage for leaf sample collection from water stressed plants required for total RNA isolation, an experiment was conducted in March, 2008 to estimate the plant's tolerance to different levels of water stress. For this 6 months old thirty potted plants (six potted plants each in the five lots) were selected and labeled as BP1 to BP30. On the first day all the plants were irrigated and then gradually subjected to water stress by withholding water from second day onwards. After observing wilting symptoms on the shoot tip, five plants were watered immediately after wilting with equal amount of water (750 ml), which was sufficient to bring the moisture level to field capacity. Next five plants were watered after 24 h of wilting. Similarly, the next lot was watered at 36 and 48 h after wilting. Observations were taken based on the days taken by the plants to wilt and the ability of the plants to revive. Therefore, finally watering was restricted when the next set of plants were unable to recover.

3.2.3 Total RNA isolation and handling

A. General precautions

B. Sample collection

C. RNA isolation

A. General precautions

Before starting RNA isolation, certain precautions were strictly followed to avoid contamination and degradation; so as to obtain intact and high quality RNA. All the glassware, mortar and pestle, microtips and microcentrifuge tubes were treated with autoclaved DEPC treated water and then autoclaved. Solutions like 80 percent ethanol and MOPS buffer were prepared with autoclaved DEPC treated water. Electrophoresis unit was first wiped with RNAse ZAP and then washed with DEPC treated water. Gloves were worn throughout the experiment.

B. Sample collection

Young, tender leaves from healthy plants watered daily and water stressed (water withheld for two days) plants were collected early in the morning for RNA isolation. Leaves were then washed quickly with DEPC treated water.

C. RNA isolation

Isolation of good quality and quantity of RNA is a pre requisite for employing Suppression Subtractive Hybridization (SSH). The procedure described by Chomczynski and Sacchi (1987) was followed with modifications for isolation of RNA. The modifications include second time addition of chloroform and avoiding centrifugation after adding Trizol.

Reagents

Trizol reagent (Invitrogen)
Chloroform
Ice cold Isopropanol
80 percent ethanol (v/v)
Autoclaved DEPC treated water

Procedure

1. 0.1 g of leaf tissue was weighed and ground in liquid nitrogen using mortar and pestle.
2. After it became fine powder, more of liquid nitrogen was added and the leaf powder was brought to the bottom of the mortar.
3. One ml of Trizol per 100 mg of leaf tissue was added and the homogenate incubated till the solution became brown.
4. The homogenate was transferred to a 2 ml microcentrifuge tube.
5. Two hundred microlitres of chloroform was added to the homogenate and the contents mixed by shaking. It was then kept at room temperature for 2 min.
6. The contents were then centrifuged at 13,000 g at 4^{o}C for 15 min.
7. The supernatant was transferred to a fresh microcentrifuge tube.
8. Steps 5 & 6 were repeated .The supernatant was transferred to a fresh tube.
9. Five hundred microlitres of ice cold isopropanol was added to the supernatant. The contents were mixed by gentle inversion and centrifuged at 13,000 g at 4^{o}C for 15 min.
10. The supernatant was discarded and the pellet was washed with one ml of 80 percent ethanol by centrifugation at 7500 g at 4^{o}C for 10 min.
11. The supernatant was discarded and the pellet was air dried under laminar air flow for 10 min.
12. The pellet was dissolved in 20µl of autoclaved DEPC treated water.

3.2.4. Quality and quantity analysis of total RNA

3.2.4.1 Quality analysis by agarose gel electrophoresis

Agarose gel electrophoresis was performed to check the quality of RNA and mRNA, following the method as described by Sambrook *et.al.* (1989).

Materials for agarose gel electrophoresis

1) Agarose (GeNei, Low EEO)

2) 10X MOPS buffer (pH 7.0)

3) 6X Loading/ Tracking dye

4) Electrophoresis unit, power pack (GeNei), casting tray, comb

5) Ethidium bromide solution (0.5µg/ml)

6) UV transilluminator (Herolab ^{R})

7) Gel documentation and analysis system (UVP, GelDoc It™ imaging system; UK)

Chemical composition of buffers and dye are given in Annexure I.

Procedure

1. Six hundred ml of 1X MOPS buffer was prepared by diluting 10X stock solution with autoclaved DEPC treated water. This buffer was used to fill the electrophoresis tank and to prepare the gel.
2. The open ends of gel casting tray were sealed with cello tape and placed on a horizontal platform. The comb was placed at one end properly.
3. 0.7 percent agarose was dissolved properly in 1X MOPS buffer by boiling in a microwave oven.
4. The solution was cooled to lukewarm temperature and ethidium bromide was added (0.5 µg /ml) as an intercalating dye of RNA, to help its visualization under UV rays.
5. The solution was poured into the gel casting tray and allowed to solidify.
6. After the gel was completely set (30 to 45 min. at room temperature), the comb and cello tape were carefully removed.
7. The gel was placed in the electrophoresis tank containing 1X MOPS buffer (with the wells near the cathode) such that it is submerged to a depth of 1 cm.

8. One microlitre of 6X gel loading dye was mixed with 2µl of RNA and then loaded in the wells with the help of a micropipette. λ DNA /*Eco*RI + *Hae*III double digest (Bangalore GeNei) marker was also added in the first well.
9. The cathode and anode were connected to the power pack and the gel was run at a constant voltage of 100 volts.
10. The power was turned off when the loading dye moved 2cm from the wells.
11. The gel was placed in a gel documentation system (UVP, GelDoc It™ Imaging system, UK), bands were visualized under UV light and the gel image was documented.

3.2.4.2 Spectrophotometric analysis of RNA

The quantity and quality of RNA was estimated using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). Before taking sample readings, the instrument was set to zero by taking 1µl autoclaved distilled water as blank. One microlitre from each sample was quantified and was measured in ng/µl. The absorbance of nucleic acid samples were measured at a wavelength of 260 nm and 280 nm and OD_{260}/OD_{280} and OD_{260}/OD_{230} ratios were recorded to assess the purity of RNA. A ratio of 1.8 to 2.0 for OD_{260}/OD_{280} and above 1.0 for OD_{260}/OD_{230} indicated good quality RNA.

Good quality total RNA from normal plants and water stressed plants were pooled separately.

3.2.5 mRNA isolation

PolyA mRNA was isolated from pooled total RNA of normal plants and water stressed plants using PolyATtract mRNA isolation kit. Precautions as described in section 3.2.3.A. were followed to avoid degradation of mRNA. The

Schematic diagram of the PolyATtract mRNA isolation procedure is given in fig. 1.

Details of reagents provided in the kit are given in Annexure II.

Procedure

I. Annealing of Probe

1. The total RNA (normal and water stressed separately) obtained in step 3.2.3.C. was brought to a final volume of 500µl using RNase free water in a sterile, RNase free 1.5 ml microcentrifuge tube.
2. The tubes were placed in water bath at 65^{o}C for 10 min.
3. Three microlitres of the Biotinylated Oligo (dT) probe and 13µl of 20X SSC were added to the RNA samples and mixed gently.
4. Incubated at room temperature until cooled.

II. Preparation of Stock solution of 0.5X SSC and 0.1X SSC

1. 1.2 ml of sterile 0.5X SSC was prepared by adding 1.170ml of RNase free water to 30µl 20X SSC.
2. 1.4 ml of sterile 0.1X SSC was prepared by adding 1.393ml RNase free water to 7µl 20X SSC.

III. Washing of Streptavidin – Paramagnetic particles (SA-PMPs)

1. One micro centrifuge tube (0.6ml vol.) each of SA-PMPs per isolation supplied in the kit, were resuspended by gently flicking at the bottom of the tube until completely dispersed, then captured by placing the tubes in magnetic stand until the SA-PMPs were collected at the side of the tube.
2. The supernatant was carefully removed. The particles were not centrifuged.

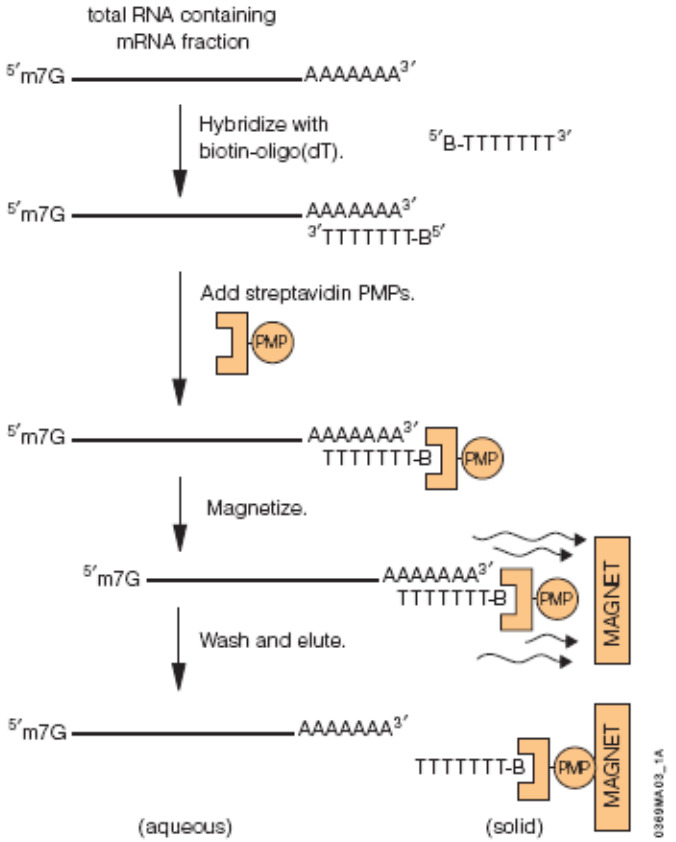


Fig. 1 Schematic diagram of the PolyATtract mRNA isolation procedure

3. SA-PMPs were washed three times with 0.5X SSC (300µl/wash), each time being captured using the magnetic stand and carefully removed the supernatant.
4. The washed SA-PMPs were resuspended in 100µl 0.5X SSC.

IV. Capturing and washing of annealed Oligo (dT) – mRNA hybrids

1. The entire contents of the annealing reaction (Step I.3) were added to the tube containing the washed SA-PMPs.
2. The contents were then incubated at room temperature for 10 min and gently mixed by inverting once in every 2 min.
3. SA-PMPs were captured using magnetic stand and supernatant was carefully removed without disturbing SA-PMP pellet.
   (The supernatant was saved in this step, until it was certain that satisfactory binding and elution of mRNA has occurred)
4. The particles were washed four times with 0.1X SSC (300µl/wash) by gently flicking the bottom of the tube until all the particles were resuspended. After the final wash, the supernatant was removed as much as possible without disturbing SA-PMP particles.

V. Elution of mRNA

1. The SA-PMP pellet obtained in step IV.4 was resuspended in 100µl of RNase free water.
2. The SA-PMPs were magnetically captured and eluted mRNA was transferred to a sterile RNase free tube. The particles were not discarded.
3. The elution step was repeated by resuspending SA-PMP pellet in 150µl of RNase free water. The capture step was repeated and elute was pooled with the mRNA eluted in Step V.2.

A. Concentrating mRNA

Normal mRNA and stressed mRNA obtained from the above procedure were concentrated for further use in SSH using the following steps,

1. 0.1 volume of 3M sodium acetate (pH5.2) and one volume of isopropanol were added to the eluted mRNA samples obtained in step 3.2.5.A.V.3 and then incubated at -20^{o}C overnight.
2. Next morning the concentrated samples were centrifuged at 12,000g for 10 min. The mRNA pellet was resuspended in one ml of 80 percent ethanol and centrifuged again.
3. The pellet was air dried and dissolved in 4µl of RNase free water.

B. Quality and quantity analysis of mRNA

The quality and quantity of mRNA (normal and stressed) was analyzed by agarose gel electrophoresis and using NanoDrop® ND-1000 spectrophotometer following the procedure as described in the section 3.2.4.2 A ratio greater than 1.9 for OD_{260}/OD_{280} and greater than 1.0 for OD_{260}/OD_{230} indicated good quality mRNA.

3.2.6 Suppression Subtractive Hybridization (SSH)

For isolating drought specific genes that were expressed in water stressed conditions in black pepper variety Kalluvally, a powerful technique Suppression Subtractive Hybridization (Diatchenko *et.al.*, 1996) was employed using PCR Select ^{TM} cDNA Subtraction Kit. This technique enables researchers to compare two populations of mRNA and obtain genes that are expressed in one population but not in the other. The procedure prescribed in the kit was followed for the Control subtraction first, after obtaining positive results a forward experimental subtraction was performed with normal and water stressed mRNA.

Details of reagents provided in the kit for each procedure is given in Annexure III.

A. Control subtraction

A complete 'Control subtraction' was first performed to estimate both the yield and size distribution of synthesized ds cDNA with Control PolyA RNA (from human skeletal muscle) provided in the kit and also to validate reagents provided in the kit. The control ds cDNA that contained specific transcripts (differentially expressed) were denoted as 'tester' while the reference cDNA as 'driver'.

I. First strand cDNA synthesis

This procedure was performed with the control PolyA RNA provided in the kit. The skeletal muscle cDNA made in this section, served as control driver cDNA in later steps.

1. The following components were combined over ice in a sterile 0.5 ml microcentrifuge tube

Components	**Vol. per reaction (µl)**
Control Poly A RNA (2µg)	2.0
cDNA Synthesis Primer (10µM)	1.0
Sterile H_2O	2.0
Total volume	**5.0**

2. The contents were mixed and briefly spun.
3. The contents were then incubated at 90^{o}C for 2 min in a thermal cycler (Eppendorf Master cycler, personal; Eppendorf Germany)
4. Immediately cooled on ice for 2 min and centrifuged briefly.
5. The following reagents were further added for the reaction

Components	Vol. per reaction (µl)
5X First strand Buffer	2.0
dNTP Mix (10mM each)	1.0
Sterile H_2O	1.0
AMV Reverse Transcriptase (20 units/µl)	1.0
Total volume (including 5µl)	**10.0**

6. The tubes were gently vortexed and briefly centrifuged.
7. The tubes were then incubated at 42^{o}C for 1.5 h in a thermal cycler.
8. First strand cDNA synthesis was terminated by keeping the tubes on ice and immediately proceeded to section II.

II. Second strand cDNA synthesis

1. The following components were added over ice to the first strand synthesis reaction tubes (10µl)

Components	Vol. per reaction (µl)
Sterile H_2O	48.4
5X Second Strand Buffer	16.0
dNTP Mix (10 mM)	1.6
20X Second Strand Enzyme Cocktail	4.0
Total volume (including 10µl from first strand reaction mix)	**80.0**

2. The contents were mixed and spun briefly.
3. The contents were incubated at 16^{o}C for 2 hrs in a thermal cycler.
4. Two microlitres (6 units) of T_4 DNA Polymerase was added and mixed well.
5. The contents were again incubated at 16^{o}C for 30 min in a thermal cycler.

6. Four microlitres of 20X EDTA/Glycogen mix was added to terminate Second Strand synthesis.
7. The contents were then transferred to a new 0.6ml digestion tubes.
8. Hundred microlitres of phenol: chloroform: isoamyl alcohol (25:24:1) was added.
9. The contents were vortexed thoroughly and centrifuged at 14,000 rpm for 10 min at room temperature to separate phases.
10. The top aqueous layer was carefully collected and placed in a fresh 0.6 ml digestion tube. Inter and lower phases were discarded.
11. Hundred microlitres of chloroform: isoamyl alcohol (24:1) was added.
12. Steps 9 and 10 were repeated.
13. Forty microlitres of 4M NH4OAc and 300µl of 95 percent ethanol were added.
14. The contents were vortexed thoroughly and centrifuged at 14,000 rpm for 20 min at room temperature.
15. The supernatant was collected carefully.
16. The pellet was overlaid with 500µl of 80 percent ethanol.
17. The contents were centrifuged at 14,000 rpm for 10 min.
18. The supernatant was removed and the pellet was air dried for 10 min under laminar air flow.
19. The precipitate was dissolved in 50µl of sterile H_2O.
20. Six microlitres of ds cDNA obtained in step 19 was separated for analysis of ds cDNA synthesis.

III. A. *Rsa*I Digestion

This step generated shorter, blunt ended ds cDNA fragments that were optimal for subtraction and required for adaptor ligation in Section IV.

1. The following reagents were added over ice

Components	**Vol. per reaction (µl)**
ds cDNA	43.5
10X *Rsa*I Restriction Buffer	5.0
RsaI (10units/µl)	1.5

2. The contents were mixed by vortexing and briefly centrifuged.
3. The tubes were incubated at 37^{o}C for 1.5 hrs.
4. 5µl of the digest mixture was set aside to analyze the efficiency of RsaI digestion.
5. 2.5µl of 20X EDTA/Glycogen mix was added to terminate the reaction.
6. Fifty microlitres of phenol: chloroform: isoamyl alcohol (25:24:1) was added.
7. The contents were vortexed thoroughly and centrifuged at 14,000 rpm for 10 min at room temperature to separate phases.
8. The top aqueous layer was carefully collected and placed in a fresh 0.6 ml digestion tube.
9. Fifty microlitres of chloroform: isoamyl alcohol (24:1) was added.
10. Steps 7 & 8 were repeated.
11. Twenty five microlitres of 4M NH_4OAc and 187.5µl of 95 percent ethanol were added.
12. Step 7 was repeated.
13. Supernatant was removed carefully.
14. The pellets were overlaid with 200µl of 80 percent ethanol.
15. The contents were centrifuged at 14,000rpm for 15 min.
16. The supernatant was removed carefully and the pellets were air dried for 5 to10 min under laminar air flow.
17. The pellet was dissolved in 5.5µl of H_2O and stored at -20^{o}C.

B. Analysis of *Rsa*I Digestion

To analyze the *Rsa*I Digestion efficiency of ds cDNA, 2.5µl of undigested ds cDNA (from section II) and 5µl of RsaI digested cDNA (from section III.A) were electrophoresed on 1 percent agarose gel in 1X TAE buffer and observed in a UV transilluminator.

IV.A. Adaptor Ligation

The cDNA obtained in step III.A.17.was taken in two separate tubes: one aliquot was ligated with Adaptor 1 (Tester 3-1) and the second was ligated with Adaptor 2R (Tester 3-2) as prescribed in the kit. After the ligation reactions were set up, portions of each tester tube were combined so that the cDNA was ligated with both the adaptors (unsubtracted Tester 3-c). Each unsubtracted tester control cDNA served as a positive control for ligation and later served as a negative control for subtraction. Adaptors were not ligated to the driver cDNA.

1. Preparation of control skeletal muscle tester cDNA.

    a. ϕX 174/*Hae*III control DNA (provided in the kit) was diluted with sterile H_2O to a final concentration of 150ng/ml.
    b. One microlitre of control skeletal muscle cDNA (Step III.A.17) was mixed with 5µl of the diluted ϕX 174/*Hae*III control DNA (150ng/ml).
    c. This served as the skeletal muscle tester cDNA.

2. Preparation of adaptor ligated tester cDNA

    A ligation Master Mix was prepared by combining the following reagents in a 0.5 ml microcentrifuge tube

Components	Vol. per reaction(µl)
Sterile H_2O	3.0
5X Ligation buffer	2.0
T_4 DNA ligase (400 units/µl)	1.0

It was ensured that we have sufficient Master Mix for all ligations plus one additional reaction.

3. The reagents were added in the sequential order as given in the table 1. Mixture was pipetted up and down to mix thoroughly.

Table 1 Setting up the ligation reactions

Components	Tester 3-1 (1)	Tester 3-2 (2)
Diluted tester cDNA	2µl	2µl
Adaptor 1 (10µM)	2µl	-
Adaptor 2R (10µM)	-	2µl
Master Mix	6µl	6µl
Final volume	**10µl**	**10µl**

4. Preparation of unsubtracted tester control 3-c

   In a fresh 0.5 ml microcentrifuge tube, 2µl of Tester 3-1 and 2µl of Tester 3-2 was added.

5. All the tubes: Tester 3-1, Tester 3-2 and Tester 3-c were spun and incubated at 16^{o}C overnight
6. One µl of EDTA/Glycogen Mix was added to stop ligation reaction.
7. All the samples were heated at 72^{o}C for 5 min to inactivate the activity of ligase.
8. The tubes were briefly centrifuged.

9. One µl of unsubtracted tester control 3-c was removed and diluted into one ml with H_2O.
10. Samples were stored at -20^{o}C.

B. Analysis of ligation

The following PCR experiment was performed to verify that at least 25 percent of the cDNAs have adaptors on both ends.

1. One µl of ligated cDNA from Section IV.A was diluted into 200µl of H_2O.
2. The reagents in Table 2 was combined in four separate tubes:

Table 2 Setting up the ligation analysis reaction for Control Subtraction

Components	**Tube 1 (µl)**	**Tube 2 (µl)**	**Tube 3 (µl)**	**Tube 4 (µl)**
Tester 3-1(Ligated to Adaptor 1)	1	1	-	-
Tester 3-2 (ligated to Adaptor 2R)	-	-	1	1
G3PDH3' Primer (10µM)	1	1	1	1
G3PDH5'Primer (10µM)	-	1	-	1
PCR Primer 1 (10µM)	1	-	1	-
Total volume	**3**	**3**	**3**	**3**

3. A Master Mix for all the reaction tubes plus one additional tube was prepared. The reagents were added in the sequential order as given in the table 3. Contents were mixed well by vortexing and briefly centrifuged.
4. Twenty two microlitres of Master Mix was aliquotted into each of the reactions from Step 2.
5. Mixed well by vortexing and briefly centrifuged.

Table 3 Preparation of the ligation analysis PCR Master Mix

Components	Vol Per reaction (µl)	Reaction mix for 5 Nos. (µl)
Sterile H_2O	18.5	92.5
10X PCR Reaction Buffer	2.5	12.5
dNTP Mix (10mM)	0.5	2.5
50X Advantage cDNA polymerase Mix	0.5	2.5
Total volume	**22.0**	**110.0**

6. The reaction mix was incubated at 75^{o}C for 5 min in a thermal cycler.
7. Immediately the following PCR programme was commenced:

Step1: 94^{o}C for 30 sec - Initial denaturation
Step2: 94^{o}C for 30 sec - Denaturation
Step3: 65^{o}C for 30 sec - Annealing } 25 cycles
Step 4: 68 ^{o}C for 2.5 min - Extension
Step 5: 72 ^{o}C for 2 min - Final extension
Step 6: 4 ^{o}C for 10 min - Cooling of samples

8. Five microlitres from each reaction was analyzed on 2 percent agarose gel.

V. First Hybridization

In the following procedure, an excess of driver cDNA was added to each tester cDNA, samples were heat denatured and allowed to anneal.

Procedure

1. 4X Hybridization Buffer was warmed to room temperature for 15 to 20 min.
2. The reagents given in Table 5 were combined over ice in the order shown

Table 4 Setting up the First Hybridization

Components	**Hybridization Sample 1 (Tester 3-1)**	**Hybridization sample 2 (Tester 3-2)**
RsaI digested cDNA (Step III.A.17)	1.5µl	1.5µl
Adaptor 1 ligated Tester 1-1 (Step IV.A.9)	1.5µl	-
Adaptor 2R ligated Tester 1-2 (Step IV.A.9)	-	1.5µl
4X Hybridization Buffer	1.0µl	1.0µl
Total volume	**4.0µl**	**4.0µl**

3. The samples were overlaid with one drop of mineral oil and briefly centrifuged.
4. Incubated at 98^{o}C for 1.5 min in a thermal cycler.
5. Incubated at 68^{o}C for 8 h and then immediately proceeded to Second hybridization.

VI. Second Hybridization

The two samples from the first hybridization were mixed together and freshly denatured cDNA was added to further enrich for differentially expressed sequences.

1. The following reagents were added into a sterile 0.2 ml microcentrifuge tube over ice

Components	**Vol. per reaction (µl)**
Driver cDNA (Step III.A.17)	1.0
4X Hybridization Buffer	1.0
Sterile H_2O	2.0

2. One micro litre of this mixture was placed in a 0.2 ml microcentrifuge tube and one drop of mineral oil was overlaid.
3. Incubated at 98^{o}C for 1.5 min in a thermal cycler.
4. The tube of freshly denatured driver was removed from the thermal cycler and the following procedure was used to mix the driver simultaneously with hybridization samples 1 and 2 (prepared in section V. Table 5).

    a. Mineral oil was carefully removed from the tubes.
    b. The micropipette was set to 10µl and the hybridization sample 2 was carefully drawn along with small amount of airspace.
    c. Freshly denatured solution (Step VI.4) was drawn carefully along with airspace.
    d. The entire mixture was transferred to the tube containing hybridization sample 1 (stepV.5).
    e. The contents were mixed by pipetting up and down.

5. Briefly centrifuged the tube.
6. Incubated at 68^{o}C overnight.
7. Two hundred microlitres of dilution buffer was added and mixed by pipetting.
8. The reaction was heated at 68^{o}C for 7 min in a thermal cycler.
9. Stored at -20^{o}C

VII. PCR Amplification

In this section differentially expressed cDNAs were selectively amplified.

A. First PCR

1. The following PCR templates were prepared

    a. One micro litre of each diluted cDNA (*i.e.* each subtracted sample from step VI.9 and the corresponding diluted unsubtracted tester control from step IV.A.10) was added into an appropriately labeled 0.2 ml microcentrifuge tube.
    b. One microlitre of the PCR control subtracted cDNA (provided in the kit) was added into an appropriately labeled 0.2 ml microcentrifuge tube.
    c. A master mix was prepared for all the primary PCR tubes plus one additional tube. For each reaction planned, the reagents given in Table 6 was combined in the order shown:

Table 5 Preparation of the primary PCR Master mix

Components	Vol per reaction (µl)	Master mix for 4 samples (µl)
Sterile H_2O	19.5	780.0
10X PCR Reaction Buffer	2.5	10.0
dNTP Mix (10mM)	0.5	2.0
PCR Primer 1 (10mM)	1.0	4.0
50X Advantage cDNA Polymerase Mix	0.5	2.0
Total volume	**24.0**	**96.0**

2. The contents were mixed well by vortexing and the tubes briefly centrifuged.
3. Twenty four microlitres of Master Mix was added into each of the reaction tubes prepared in step 1.
4. The reaction mix was incubated at 75^{o}C for 5 min in a thermal cycler to extend the adaptors and immediately performed the PCR.
5. The following PCR programme was adopted:

Step1: 94^{o}C for 30 sec - Initial denaturation
Step2: 94^{o}C for 30 sec - Denaturation
Step3: 66^{o}C for 30 sec - Annealing } 27 cycles
Step 4: 72^{o}C for 1.5 min - Extension
Step 5 4^{o}C for 10 min - Cooling of samples

6. Eight micro litres aliquots were set aside and run on the same gel which was used to analyze the secondary PCR products.

B. Second PCR

1. Three microlitres of each primary PCR product was diluted in 27µl of H_2O.
2. One microlitre each of the diluted sample was taken in a labeled 0.2 ml microcentrifuge tube.
3. Master Mix for all the secondary PCR reactions plus one additional reaction was prepared by adding the reagents in a sequential order as given in the table 7.
4. The contents were mixed well by vortexing and centrifuged briefly.
5. Twenty four micro litres of Master Mix was added into the sample tubes of step 2.

Table 6 Preparation of the secondary PCR master mix

Components	Vol. per reaction (µl)	Master mix for 4 Nos. (µl)
Sterile H_2O	18.5	74.0
10X PCR Reaction Buffer	2.5	10.0
Nested PCR Primer1 (10µM)	1.0	4.0
Nested PCR Primer 2R (10µM)	1.0	4.0
dNTP Mix (10mM)	0.5	2.0
50X Advantage cDNA Polymerase Mix	0.5	2.0
Total volume	**24.0**	**96.0**

6. The following PCR programme was performed immediately.

Step1: 94^{o}C for 30 sec - Denaturation
Step2: 68^{o}C for 30 sec - Annealing
Step3: 72^{o}C for 1.5 min - Extension
} 12 cycles

7. Samples (8µl) from each reaction were analyzed on a 2 percent agarose gel and run in 1X TAE buffer.
8. The left over reaction products (17µl) were stored at -20^{o}C.

Forward experimental subtraction

A forward experimental subtraction was performed with normal and water stressed mRNA obtained in step 3.2.5. The ds cDNA obtained from normal mRNA population was denoted as the 'driver' and water stressed cDNA as the 'tester'. Forward experimental subtraction was carried out as described for Control subtraction in section 3.2.6.1 except the following differences:-

1. In First strand cDNA synthesis, two reactions were carried out with 4µl each of normal and water stressed Poly A mRNA.

2. In adaptor ligation step, each tester cDNA was taken in two separate tubes. The tester which was ligated to Adaptor 1 was labeled as Tester 1-1 and the other which was ligated with Adaptor 2R as Tester 1-2. Step 1 was avoided as described in section IV.A. One microlitre of *Rsa*I digested experimental cDNA (step III.A.17.) was diluted with 5µl of sterile H_2O and then followed the same procedure as described in section IV.A. After the ligation reactions were set up, portions of each tester tubes were combined and labeled as unsubtracted Tester 3-c.

3. Analysis of ligation
   a. One micro litre of each ligated cDNA tester 1-1 and 1-2 was diluted with 199µl of sterile H_2O.
   b. The reagents in Table 8 was combined in two tubes:
   c. A master mix for all the tubes plus one additional tube was prepared. The reagents were added in the sequential order as given in table 9.
   d. The contents were mixed well by vortexing and briefly centrifuged.
   e. Twenty microliters of master mix was aliquotted into each of the reactions from step 2.
   f. Mixed well and briefly centrifuged.
   g. The reaction mix was incubated at 75^{o}C for 5 min in a thermal cycler.
   h. Immediately the following PCR programme was commenced.

Step 1: 94^{o}C for 30 sec - Initial denaturation
Step 2: 94^{o}C for 30 sec - Denaturation
Step 3: 46.5^{o}C for 1 min - Annealing
Step 4: 72^{o}C for 2 min - Extension
} 30 cycles

Step 5: 72^{o}C for 10 min - Final extension

Step 6: 4^{o}C for 10 min - Cooling of samples

Table 7 Setting up the ligation analysis reaction for experimental tester

Components	**Tube 1 (µl)**	**Tube 2 (µl)**
Tester 1-1 (Ligated to Adaptor 1)	2	-
Tester 1-2 (Ligated to Adaptor 2R)	-	2
Actin 5' (Forward Primer)	1	1
Actin 3' (Reverse Primer)	1	1
Total volume	**4**	**4**

Table 8 Preparation of the ligation analysis PCR Master Mix for experimental tester

Components	**Per reaction (µl)**	**Reactions mix for 3 in nos. (µl)**
Sterile H_2O	16	48
10X PCR Buffer	2.5	7.5
dNTP mix	2	6
50X Advantage Polymerase mix	0.5	1.5
Total volume	**21**	**21**

4. The primary PCR products (8µl) were analyzed separately on 2 percent agarose gel and run in 1X TAE buffer.

5. After analyzing the second PCR products on agarose gel, the gel was saved for eluting the amplicons.

3.2.7 Gel elution of PCR amplified fragments

Smear of subtracted sample that was obtained after Second PCR on gel was eluted using AxyPrep DNA Gel Extraction Kit (Axygen, Biosciences). Procedure was followed as per the manufacturer's guidelines.

1. cDNA smear of subtracted sample was excised from the gel using a sterile, sharp scalpel avoiding much exposure to UV on a transilluminator.
2. Gel slice was weighed in a 1.5 ml microcentrifuge tube.
3. Added 3X gel volume of gel solubilization buffer (DEA buffer).
4. The gel was resuspended in gel solubilization buffer by vortexing. The mix was heated at 75^{o}C until the gel was completely dissolved. Intermittent vortexing was given every 2 to 3 min to enhance gel solubilization.
5. Added 0.5X gel solubilization volume of binding buffer (DEB buffer) and mixed properly.
6. A spin column was placed in a 2 ml collection tube. The solubilized gel slice was transferred into the spin column that was assembled in the 2 ml collection tube and centrifuged at 12,000g for 1 min.
7. The filtrate was discarded. Added 500µl of wash buffer (W1) to the spin column and centrifuged at 12,000g for 30 sec.
8. The filtrate was discarded and 700µl of desalting buffer (W2) was added and centrifuged at 12,000g for 30 sec.
9. A second wash was given by adding 700µl of desalting buffer (W2), followed by centrifugation at 12,000g for 30 sec to ensure the complete removal of salt. The filtrate was discarded and spin column was again placed in collection tube.
10. Column was again centrifuged for 1 min at 12,000g to remove any residual buffer.
11. Spin column was transferred to a fresh 1.5ml microcentrifuge tube. The eluent was prewarmed at 65^{o}C to improve the elution efficiency.

12. To elute the DNA, 7µl of eluent was added to the centre of the spin column. It was allowed to stand for 1 min at room temperature. Then centrifuged at 12,000g for 1 min.
13. Eluted cDNA fragments were checked on 0.7 percent agarose gel and stored at – 20^{o}C for further cloning works.

3.2.8 Transformation

3.2.8.1 Preparation of competent cells

Competent cells required for plasmid transformation were prepared using the competent cell preparation kit of GeNei, Bangalore in SOB agar media. (Chemical compositions of media are given in Annexure IV)

Procedure

Day I

The desired strain JM 109 was streaked on SOB plate from master plate. The plates were incubated at 37^{o}C for 16 to18 hrs.

Day II

1. Inoculated 5 to 6 moderately sized colonies from the SOB plate in 50 ml SOB broth in a 500ml flask.
2. Incubated the broth on a rotary shaker at 37^{o}C; 160 rpm. When the OD reached 0.3 at A600, the growth of the bacteria was arrested quickly by chilling for 2 min on ice.
3. The entire culture was transferred into a 50ml sterile polypropylene tube and centrifuged at 3500 rpm for 15 min at 4^{o}C.
4. The supernatant was discarded. Keeping the bottle on ice, the bacterial cell pellet was resuspended very gently in 16.6 ml ice cold Solution A (provided in the kit).

5. Kept on ice for 20 min and then centrifuged at 3500 rpm for 15 min at 4^{o}C.
6. The supernatant was discarded and the pellet was chilled on ice.
7. The pellet was resuspended in 3 ml of ice cold Solution A.
8. The suspension was left on ice for 10 min and aliquots of 100µl were added in chilled 1.5 ml microcentrifuge tubes.
9. The competent cells were covered with aluminium foil and were stored at -70^{o}C.

3.2.8.2 Screening of competent cells

The competent cells prepared were screened to check their transformation efficiency, by transforming them using a plasmid (pUC18) containing ampicillin resistance marker. The procedure is as follows:

1. Prepared 50 ml LB media and 50 ml LB broth.
2. The competent cells kept at -70^{o}C were thawed on ice.
3. pUC18 was diluted to 1:10 dilution. Added 1µl of diluted pUC18 to thawed competent cells.
4. The contents were mixed gently and kept on ice for 40 min.
5. Meanwhile, the water bath was set to 42^{o}C.
6. The tube was rapidly taken from ice and a heat shock at 42^{o}C was given exactly for 90 sec. Without shaking, the tube was placed back on ice for 5 min.
7. Added 250µl of LB broth to vial under sterile conditions and was inverted twice to mix the contents.
8. The tube was incubated at 37^{o}C for 1 hour with shaking.
9. The transformed cells (100, 150, 200µl) were plated on LB agar/ampicillin (50mg/l) overlaid with IPTG (6µl) and X gal (60µl).
10. The plates were incubated overnight at 37^{o}C.

3.2.9 cDNA cloning

The eluted product was cloned in pGEMT vector system supplied by Promega.

3.2.9.1 Ligation

The appropriate amount of PCR product (insert) required for ligation was calculated by estimating the quantity of eluted cDNA using NanoDrop Spectrophotometer. For this, 0.25µl of eluted cDNA was diluted to 5µl with sterile H_2O and then taken reading. The amount of PCR product required was calculated by the following relationship

$$\text{ng of insert} = \frac{\text{ng of vector x Kb size of insert}}{\text{Kb size of vector}} \text{ X Insert: vector molar ratio}$$

Ligation procedure was followed as per the manufacturer's guidelines.

Procedure

1. The pGEMT vector was briefly centrifuged to collect contents at the bottom of the tubes.
2. The following ligation reaction was set up as described below:

Components	**Per reaction**
2X Ligation buffer	5.0µl
pGEMT vector (50ng)	1.0µl
Eluted product	3.0µl
T_4 DNA Ligase (3units/µl)	1.0µl
Total volume	**10.0µl**

3. The reaction was mixed by pipetting and incubated for 1 hr at room temperature. Then it was kept at 4^{o}C overnight.

3.2.9.2 Transformation of ligated product

Reagents

1. Ampicillin – 5mg/ml in water
2. IPTG – 200mg/ml in water
3. X-gal – 10mg/ml in DMSO

The procedure followed for plasmid DNA transformation is as follows:

The ligated PCR product was added to 100µl of thawed competent cells and kept on ice for 40 minutes. Heat shock was given at 42^{o}C for 90 seconds in a water bath and immediately placed back in ice for 5 minutes. LB medium (250µl) was added to the cells and incubated at 37^{o}C for 1 hr on a shaker at 160 rpm. The aliquots of transformed cells (100, 150 and 100µl) were plated on LB agar/Ampicillin (5 mg/ml) / IPTG (6µl) / X-gal (60µl) plates and incubated overnight at 37^{o}C.

3.2.9.3 Selection and Maintenance of Recombinants

The recombinant clones were selected based on blue white screening. Percentage of recombinants was calculated by the following formula:

$$\textbf{\% of recombinants} = \frac{\text{No. of white colonies}}{\text{Total No. of colonies}} \text{ X } 100$$

All the white colonies were carefully transferred to the prepared LB/ampicillin grid plates and the colonies were numbered in each plate. The plates were incubated at 37^{o}C overnight and further stored at 4^{o}C. Each colony was given an identity number to indicate the plant source, the plate number and

colony number. E.g. PNK 1(1) indicating *Piper nigrum* var; Kalluvally colony 1 in grid plate 1.

3.2.9.4 Isolation of Recombinant Plasmid DNA

Plasmid DNA was isolated from 30 white colonies selected randomly using alkaline mini prep procedure as described by Birnboim and Doly (1979).

Reagents

Solution I - Resuspension buffer

Solution II - Lysis Buffer

Solution III- Neutralization buffer

LB medium

Ampicillin (50mg/L)

Chemical compositions of the reagents are given in Annexure IV.

Procedure

1. **Harvesting**
   a. A single colony of bacteria was transferred into 3ml LB broth/ampicillin in autoclaved test tubes
   b. The cultures were incubated overnight at 37^{o}C; 160 rpm.
   c. Poured 1.5 ml of culture into a microcentrifuge tube (1.5 ml) and centrifuged at 12,000rpm for 1 min at 4^{o}C.
   d. The supernatant was discarded leaving the bacterial pellet as dry as possible.

2. **Lysis by alkali**
   a. The bacterial pellet was resuspended in 100µl of ice cold Solution I by vigorous vortexing.

b. To the above, 200µl of freshly prepared lysis buffer was added, mixed well by inverting the tube rapidly five times.
c. Added 150µl of ice cold Solution III to the above, vortexed gently in an inverted position for 10 seconds and kept on ice for 5 min
d. The contents were centrifuged at 12,000 rpm for 5 min at 4^{o}C and the supernatant was transferred to a fresh microcentrifuge tube.
e. To the supernatant, 0.6 volume of ice cold isopropanol was added and kept at room temperature for 2 min.
f. The contents were centrifuged at 12,000 rpm for 5 min at 4^{o}C and the supernatant was discarded.
g. The pellet was rinsed with one ml of 70 percent (v/v) ethanol.
h. The tube was centrifuged at 12,000 rpm for 10 min at 4^{o}C.the supernatant was discarded and the pellet was air dried for 10 min
i. The pellet was finally dissolved in 30µl autoclaved distilled water.
j. Five microlitres from each sample was analyzed on 0.8 percent agarose gel.

3.2.10. Confirmation of presence of insert

Confirmation of insert presence was carried out by performing PCR amplification of recombinant plasmid DNA using T7 and SP6 Primers. The following PCR template was prepared

1. Diluted 1µl of plasmid DNA (≈25 ng) to 10µl with sterile H_2O.
2. One microlitre of diluted plasmid DNA was added in a new PCR tube.
3. A Master Mix was prepared and the reagents were in the sequential order as shown in the table 10.
4. The contents were mixed well and briefly centrifuged.
5. Twenty four microlitres of Master Mix was aliquotted into reaction from step b.
6. The following PCR programme was run immediately:

Step1: 94^{o}C for 2 min - Initial denaturation
Step2: 94^{o}C for 45sec - Denaturation
Step3: 41^{o}C for 1 min - Annealing } 30 cycles
Step 4: 72^{o}C for 2 min - Extension
Step 5: 72^{o}C for 10 min - Final extension
Step 6: 4^{o}C for 10 min - Cooling of samples

7. The PCR products were analyzed on 1 percent agarose gel.

Table 9 Preparation of PCR Master mix

Components	**Vol per reaction (µl)**
10X Taq buffer	2.5
dNTP Mix (10mM)	1.0
T7 Primer (27.5nM)	1.0
SP6 Primer(31.3nM)	1.0
Taq DNA polymerase (0.3 units)	1.0
Sterile H_2O	16.5
Total volume	**24.0**

3.2.10. Preparation of stabs

LB agar medium was melted, cooled to 42^{o}C, added ampicillin and poured into storage vials aseptically in a laminar air flow. After solidification, single colony was inoculated into the medium using sterile bacterial loop. The vials were incubated at 37^{o}C overnight and further stored at 4^{o}C.

3.2.11. Sequencing of cDNA clones

According to the technical programme only fifteen clones were to be sequenced, but five more were sent and in total twenty clones were sequenced. Out of twenty, fifteen stabs of recombinant clones were sent for sequencing to DNA sequencing facility of Bangalore GeNei. The Primers used for sequencing were T7, SP6 and M13. Rest five sequences were sent to Bioserve DNA sequencing facility (Bioserve, Hyderabad). This firm used the T7 Primer for sequencing.

3.2.12. *In silico* analysis of sequences

The cDNA sequences obtained were analyzed with various online bioinformatics tools.

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.

2. Homology search

The nucleotide sequence of all the twenty 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. The best sequence alignment results were noted and saved.

3. Detection of Open Reading Frame (ORF)

To find the open reading frame of the insert nucleotide sequence, the programme '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.

4. Amino acid analysis

The nucleotide sequences were translated into amino acid sequences using the tool Translate (http://www.expasy.ch/tools/dna.html) provided by ExPAsy. The amino acid sequences obtained were analyzed to locate functional domains using 'InterProScan' (www.ebi.ac.uk/InterProScan/; Zdobnov *et al.*, 2001).The conserved regions were detected using Conserved domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml CDD) provided by NCBI.

3.2.13. Submission of sequences in the database

All the twenty sequences were submitted in dbEST (www.ncbi.nlm. nih.gov/dbEST), by giving the details in the format prescribed by databank. dbEST is an EST databank provided by NCBI.

3.2.14. Assigning the serial number to clones

After analyzing the sequence data using various bioinformatics tools, the clones were serially numbered from 1 to 20 based on their significance in relation to abiotic stress response already reported.

Results

4. RESULTS

The results of the study entitled 'Development and analysis of ESTs (Expressed Sequence Tags) in black pepper (*Piper nigrum*) var, Kalluvally' undertaken during the period from 2006 to 2008 at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University, Vellanikkara are presented in this chapter.

4.1. Soil moisture levels during water stress

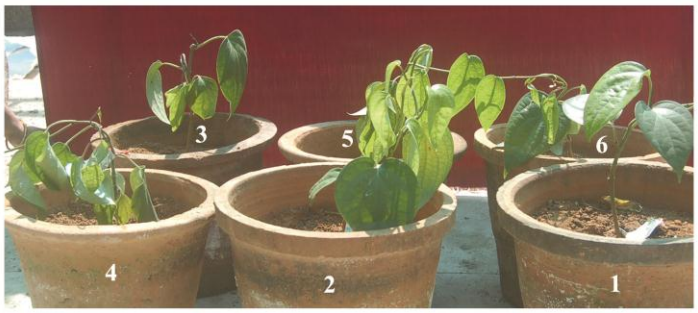
Soil moisture was estimated from fifteen potted plants (five potted plants each in three lots) using the Gravimetric – oven drying method. The percentage soil moisture on dry weight basis was 30.15 in soil samples that were watered upto field capacity, on the same day. The soil moisture content recorded was only 2.77 percent, 96 h after watering (Table 10).

4.2. Tolerance of var. Kalluvally to water stress

The potted plants were subjected to water stress by withholding water after giving a uniform irrigation. It was observed that from the third day onwards, the shoot tips along with young, tender leaves started to wilt (Plate 1A). The plants which were watered on the same day after wilting were able to revive quickly. Those which were irrigated within 24, 36 and 48 h after wilting also revived within varying periods of time. However, none of the plants which were watered 60 h after wilting could revive back to normal condition (Table 11).

4.3. Isolation and analysis of total RNA

Total RNA was isolated from normal and water stressed plants (water withheld for two days, Plate 1B) using trizol reagent. The samples were run on 0.7 percent agarose gel in 1X MOPS buffer. Three intact bands corresponding to 28S,



A. Response of potted plants at different periods after withholding irrigation

B. Stages of the plant selected for total RNA isolation

0 – Normal (Irrigated daily)
1 – 1 day after irrigation
2 – 2 days after irrigation
3 – 3 days after irrigation
4 – 4 days after irrigation
5 – 5 days after irrigation
6 – 6 days after irrigation

Plate 1 Tolerance of variety Kalluvally to different levels of water stress

Table 10 Soil moisture content (dry weight basis) at different intervals after irrigation

S.No.	Period after irrigation (h)	Moisture content (%)
1	4	30.15
2	24	23.86
3	48	21.20
4	72	9.03
5	96	2.77

Table 11 Recovery of var: Kalluvally upon irrigation after water stress

Irrigation after period of wilting (h)	% recovery after irrigation	Time taken for recovery (days)
Within 6h	100	Same day
24	100	1
36	100	2
48	80	3
60	Nil	Nil

18S and 5S rRNA + tRNA were obtained (Plate 2A), indicating the quality of RNA. Total RNA from both the samples quantified spectrophotometrically is presented in Table 12. The quantity of RNA isolated from normal and stressed plants were analyzed to be 0.253 and 0.252 µg μl^{-1} respectively. The ratio for OD_{260}/OD_{280} for the samples was greater than 1.8 indicating pure RNA free from DNA and protein contamination. The ratio for OD_{260}/OD_{230} was greater than 1.0 indicating that the samples were free from polysaccharides and polyphenols.

4.4. mRNA isolation

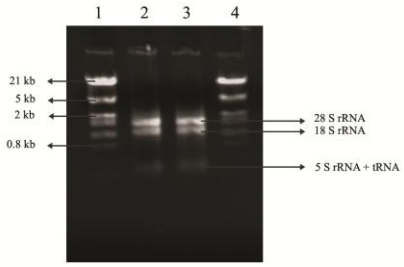
PolyA mRNA was isolated from normal and water stressed total RNA using PolyATtract mRNA isolation kit. The samples were run on 0.7 percent agarose gel in 1X MOPS buffer. PolyA mRNA appeared as a smear of size greater than 0.5 kb in both the samples (Plate 2B). The mRNA samples quantified spectrophotometrically (Table 13), for both the samples gave a ratio greater than 1.9 at OD_{260}/OD_{280} and it was greater than 1.0 at OD_{260}/OD_{230} indicating good quality, pure mRNA.

4.5. Suppression Subtractive Hybridization (SSH)

SSH was performed to isolate the genes that were differentially expressed in water stressed conditions in black pepper variety Kalluvally using PCR Select ^{TM} cDNA Subtraction Kit. A complete Control subtraction was performed first and after obtaining valid results, a forward experimental subtraction was carried out.

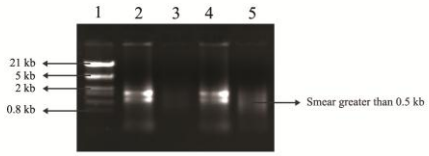
4.5.1. Control Subtraction

A complete Control subtraction was performed as prescribed in the kit using the PolyA RNA (from human skeletal muscle) provided in the kit as control. The First and Second strand cDNA was synthesized successfully and the minute,



Lane 1 & 4 : λ DNA/ *Eco*R I *Hae* III digest
Lane 2 : Total RNA from normal plants
Lane 3 : Total RNA from stressed plants

A. Total RNA isolated from normal and water stressed Kalluvally plants



Lane 1 : λ DNA/ *Eco*R I *Hae* III digest
Lane 2 : Total RNA from normal plants
Lane 3 : mRNA from normal plants
Lane 4 : Total RNA from stressed plants
Lane 5 : mRNA from stressed plants

B. mRNA isolated from normal and water stressed Total RNA

Plate 2 Nature of total RNA and mRNA isolated from leaves of var. Kalluvally

Table 12 Quality and Quantity of total RNA isolated from leaves collected at different conditions

Sample	Absorbance at 260nm	Absorbance at 280nm	OD_{260}/OD_{280}	OD_{260}/OD_{230}	Quantity ($\mu g\ \mu l^{-1}$)
Normal	6.3	3.0	2.07	1.4	0.253
Stressed	6.3	3.1	2.04	1.57	0.252

Table 13 Quality and Quantity of mRNA isolated from leaves collected at different conditions

Sample	Absorbance at 260nm	Absorbance at 280nm	OD_{260}/OD_{280}	OD_{260}/OD_{230}	Quantity ($\mu g\ \mu l^{-1}$)
Normal	0.44	0.19	2.26	1.76	0.017
Stressed	0.54	0.25	2.11	1.75	0.021

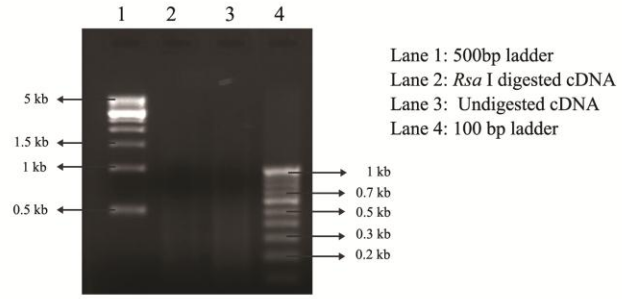
creamy, white pellet obtained after ethanol precipitation was finally dissolved in 50µl of sterile water. The cDNA sample (44µl) obtained was digested with *Rsa*I enzyme and compared with the undigested product (6µl) by agarose (1%) gel electrophoresis.

The undigested cDNA appeared as smear ranging from 0.5 to 3kb, while the digested cDNA was of smaller size ranging from 0.1 to 2 kb (Plate 3A) indicating proper digestion of the cDNA. The sample after *Rsa*I digestion was further used for preparation of tester and driver cDNA.

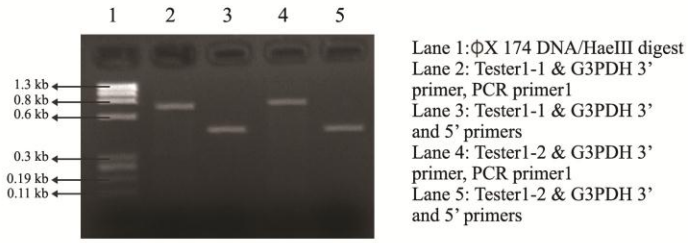
The tester populations prepared out of *Rsa*I digest were successfully ligated with the two different adaptors and was confirmed through PCR amplification. The ligated products after PCR amplification when analyzed on 2 percent agarose gel showed a typical result as specified in the user's manual (Plate 3B). Different banding patterns were observed in all the four samples as described in the users manual provided thus confirming the ligation efficiency. This was due to the differential addition of primers.

Subtractive hybridization was performed in two steps using adaptor ligated testers and driver cDNA. The first hybridization was performed with excess of driver cDNA which was added to the hybridization sample 1 and 2 having the tester cDNA (Adaptor ligated products). This was followed by second hybridization in which the two samples from the first hybridization were mixed together and freshly denatured driver cDNA was added and incubated overnight at 68^{o}C for further enrichment of differentially expressed sequences.

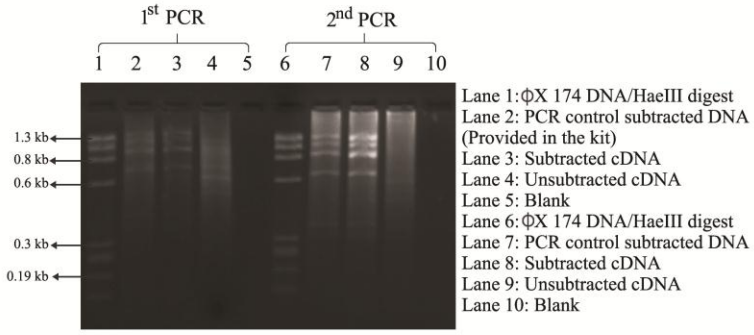
Differentially expressed cDNAs were selectively amplified during the first and second PCR. Both the PCR products were analyzed on 2 percent agarose gel along with PCR control subtracted cDNA (provided in the kit). The results obtained are presented in plate 3C. The ϕX 174/*Hae*III digest used as the marker, the control subtracted cDNA provided in the kit and the subtracted sample showed



A. Analysis of *Rsa*I digestion



B. Analysis of Adaptor ligation



C. PCR products after Control subtraction

Plate 3 Analysis of the reaction products in the Control subtraction

similar banding patterns and were different from the unsubtracted sample. The results confirmed the efficiency of control subtraction.

4.5.2 Forward experimental subtraction

A forward experimental subtraction was carried out using normal ‘driver’ and water stressed ‘tester’ mRNA.

4.5.2. 1. First and second strand cDNA synthesis

First strand cDNA synthesis was carried out with each 4µl of driver and tester Poly A mRNA. The clear solution obtained after first strand cDNA synthesis in both the samples, was carried over for the synthesis of second cDNA strand. Minute, creamy white pellet was obtained after ethanol precipitation. It was finally dissolved in 50µl of sterile water.

4.5.2. 2. *Rsa*I digestion

Tester and driver cDNA (44µl) obtained were digested with *Rsa*I enzyme and compared with the undigested products (6µl). The digested and undigested products when analyzed on 1 percent agarose gel, indicated a smear of size 0.2 to 3kb for undigested cDNA and a smear of size 0.1 to 2kb for digested cDNA (Plate 4A), confirming the efficiency of *Rsa*I digestion

4.5.2. 3. Adaptor ligation

Two tester populations were created from *Rsa*I digested tester (stressed) sample .One population was ligated with Adaptor 1 (Tester 1-1) and the other population with Adaptor 2R (Tester 1-2). An unsubtracted Tester 1-c was created by combining the two tester populations which contained both the adaptors.

Ligation efficiency was checked by using actin primers. The two tester populations when amplified with forward and reverse actin primers and analyzed on 2 percent agarose gel gave two distinct bands indicating the efficiency of ligation and correctness of the procedure (Plate 4B).

4.5.2. 4. First and Second Hybridization

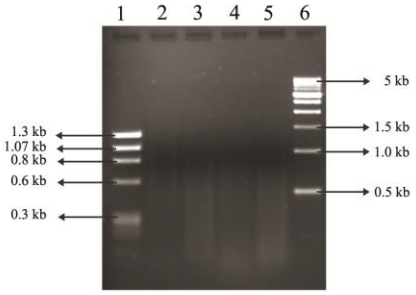
Subtractive hybridization was performed in two steps using RsaI digest of normal cDNA as the driver. The first hybridization was performed with excess of driver cDNA which was added to the hybridization sample 1 and 2 having the tester cDNA (Adaptor ligated products). This was followed by second hybridization in which the two samples from the first hybridization were mixed together and freshly denatured driver cDNA was added and incubated overnight at 68^{o}C for further enrichment of differentially expressed sequences.

4.5.2. 5. PCR amplification

Differentially expressed cDNAs were selectively amplified during the first and second PCR. Analysis of both PCR amplifications was carried out on 2 percent agarose gel separately. In subtracted sample, the primary PCR product appeared as a smear ranging from 0.2 to 1.5kb without any distinct bands, while the smear size of unsubtracted sample (1-c) was greater than 2kb without any distinct bands (Plate 5A). Second PCR amplification further enriched the primary PCR products and thus the smear for subtracted sample became brighter and clearly distinguished from the smear observed in case of unsubtracted sample (Plate 5B).

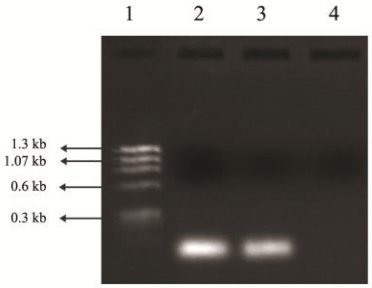
4.6. Gel elution of PCR product

The smear obtained in subtracted sample after second PCR, was eluted and checked on 0.8 percent agarose gel. A smear with good concentration was



Lane 1: ΦX 174 DNA/*Hae*III digest
Lane 2: *Rsa* I digested driver cDNA
Lane 3: Undigested driver cDNA
Lane 4: *Rsa* I digested tester cDNA
Lane 5: Undigested tester cDNA
Lane 6: 500 bp ladder

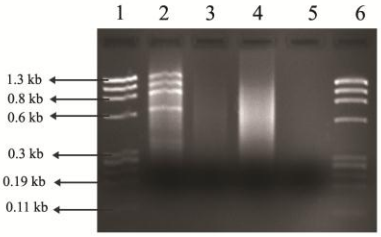
A. RsaI digestion in Forward Experimental subtraction



Lane 1: ΦX 174 DNA/*Hae*III digest
Lane 2: Tester1-1, Actin 3' & 5' primers
Lane 3: Tester1-2, Actin 3' & 5' primers
Lane 4: Blank

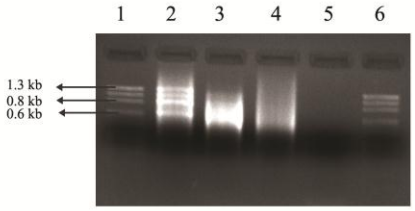
B. Amplification of tester populations with actin primers

Plate 4 Analysis of *Rsa*I digestion and adaptor ligation for forward experimental subtraction



Lane 1:ΦX 174 DNA/*Hae*III digest
Lane 2: Control subtracted DNA
Lane 3: Subtracted sample
Lane 4: Unsubtracted sample
Lane 5: Blank
Lane 6:ΦX 174 DNA/*Hae*III digest

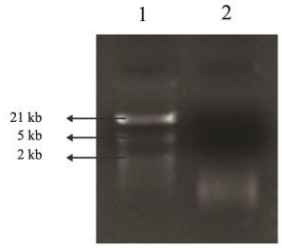
A. Amplification after first PCR



Lane 1:ΦX 174 DNA/*Hae*III digest
Lane 2: Control subtracted DNA
Lane 3: Subtracted sample
Lane 4: Unsubtracted sample
Lane 5: Blank
Lane 6:ΦX 174 DNA/*Hae*III digest

B. Amplification after second PCR

Plate 5 PCR analysis of forward experimental subtraction



Lane 1: λ DNA/ EcoR I Hae III digest
Lane 2: Eluted smear of subtracted sample

Plate 6 Eluted smear obtained from subtracted sample

observed (Plate 6) similar to that obtained during PCR amplification, thus indicating good recovery of fragments from the gel and its suitability for cloning in a vector. The quantity estimated was 0.6ng cDNA μl^{-1} based on absorbance at 260 nm.

4.7. Transformation

4.7.1. Preparation and screening of competent cells

The competent cells prepared in section 3.2.8.A were checked for competence by transforming the plasmid (pUC18) having ampicillin resistance. A large number of blue colonies were obtained (Plate 7A) which indicated a high degree of transformation efficiency. Thus the competent cells prepared were found ideal for cloning amplicons.

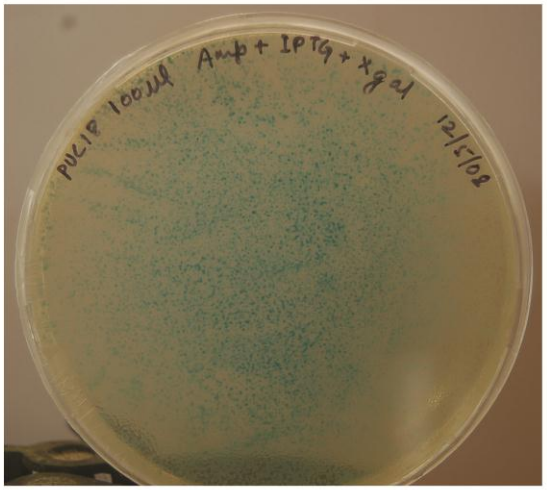
4.1.1 Ligation

The amount of PCR product (insert) required for ligation was calculated using the equation described in section 3.2.9.I. Thus, 30ng of insert was used per 50ng of vector for ligation reaction using pGEMT vector, considering the fact that the eluted sample had 0.6ng cDNA μl^{-1}

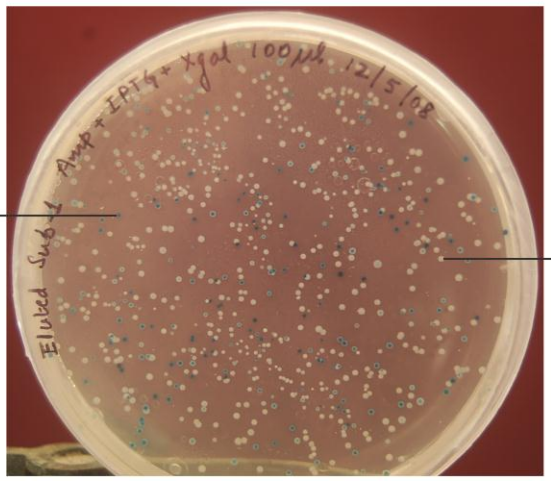
4.1.2 Transformation of ligated product

The ligated product was used to transform the prepared competent cells using the heat shock method and was incubated at 37^{o}C.

Large number of blue and white colonies was obtained after overnight incubation when the transformed cells were cultured in LB/ampicillin media, in the three plates; overlaid with X gal and IPTG (Plate 7B). The details of recombinants obtained in the three plates are given below,



A. Checking the competence of *E.coli* cells



B. Blue white screening of transformed *E. coli.* Cells

White colonies are transformed and blue colonies are non recombinant

Plate 7 Efficiency of the competent cells and screening of the transformants

Details	Plate 1	Plate 2	Plate 3
No. of white colonies	215	235	350
No. of blue colonies	200	212	325
Total no. of colonies	415	447	675
Recombination efficiency	51 %	52%	51%

The mean percentage of recombinants was calculated to be 51.3 percent. The white colonies were transferred to new LB/ampicillin plates which constituted the subtracted cDNA library (Plate 8).

4.8. Screening of the transformed colonies

The plates containing the transformed colonies were screened for recombinant plasmid. The plasmid DNA isolated from white and blue colonies when viewed after electrophoresis in 0.7 percent agarose gel indicated difference in molecular weight. Most of the white colonies had greater plasmid size (Plate 9) than the blue ones, due to the presence of insert. This confirmed the presence of the insert in the plasmid.

4.9. Confirmation of recombination by PCR

The plasmid was further checked for the presence of insert by PCR amplification using T7 and SP6 primers. The PCR products when checked on 1 percent agarose gel showed amplicons of different sizes. Higher molecular weight bands were obtained in PCR products of plasmid DNA isolated from white colonies than the PCR products of those isolated from blue colony (Plate 10). This confirmed the presence of insert in the plasmid.

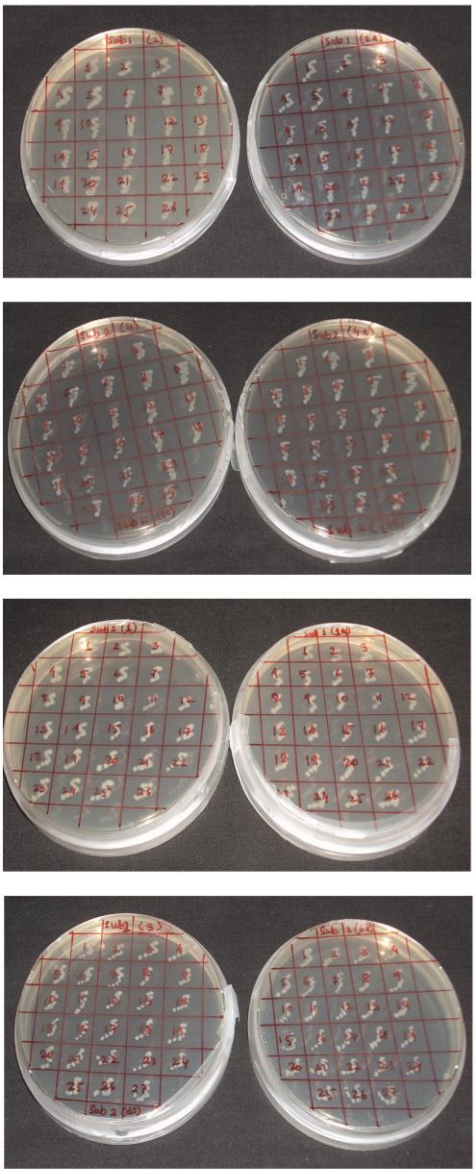
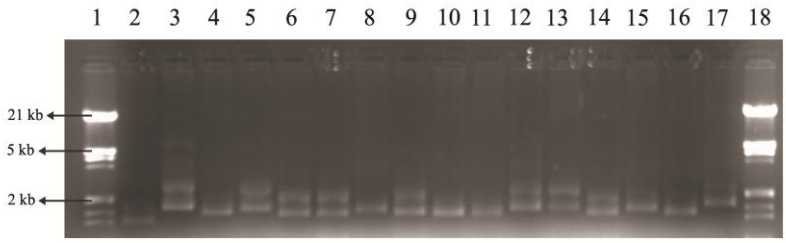


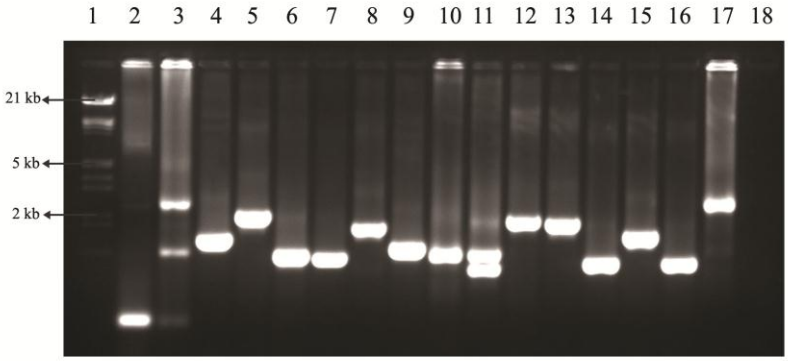
Plate 8 Maintenance of subtracted cDNA library



Lane 1 & 18: λ DNA/ *Eco*R I *Hae* III digest
Lane 2: Plasmid isolated from blue colony
Lane 3 to 17: Plasmid isolated from white colonies (1 to 15)

Plasmids isolated from blue colony and white colonies

Plate 9 Confirmation of inserts in the plasmid DNA



Lane 1: λ DNA/ *Eco*R I *Hae* III digest
Lane 2: PCR product of plasmid isolated from blue colony
Lane 3 to 17: PCR product of plasmid isolated from white colonies (1to15
Lane 18: Blank

PCR amplification of plasmid isolated from blue and white colonies with T7 and SP6 primers

Plate 10 Confirmation of recombinants through PCR analysis

4.10. Sequencing of cDNA clones

Twenty random clones in which the presence of insert was confirmed were screened out and send for automated sequencing. The outsourcing facility available at Genie, Bangalore and Bioserve, Hyderabad was utilized. The sequence data were obtained from the firm within 15 days in the form of electropherogram and nucleotide sequence. The sequences thus obtained were referred to as ESTs.

4.11. Sequence data analysis

The sequence data obtained from the sequencing agency for the twenty Clones 1 to 20 are given in Annexure V and their interpretations are given here under,

Clone 1 [PNK 11(2)]

The sequence data obtained for the clone 1 was of 696bp in size and vector screening indicated vector sequence from 405 to 579bp. The details of results obtained are presented in Table 14 and Fig. 2. After vector and adaptor screening the total sequence obtained for further analysis was 521bp as detailed below,

Vector/Adaptor edited Sequence

5'TTTCGAGCGGCGCCCGGGGCGGTAAGTGGCCCGGGTGGAGAGGAGC
AGCGGCAGGTTCTTGAGGAGGTTTAGGTTGCCGGAGAATGCTAAGGTG
GAGGAGGTGAAGGCGGGGGTGGAGAACGGGGTGCTCACTGTGACTGT
GCCCAAGGTGGAGGTGAAGAAGTCTGAGGTTAAGGCCATTGAAATCT
CTGGAAACTAAGAGTTTCGAAAAAGATAATATTGTGTCAAGGCCATGT

Table14 Results of sequence data analysis for the Clone 1 [PNK 11(2)]

1. Initial Sequence length **:** 696
2. Vector sequence **:** 405-579
3. Sequence length after Vector/Adaptor Screening : 521
4. Blastn Results:

Accession No.	Description	Q.Coverage	Evalue	Max. Id.
EU636239.1	*Malus x domestica* cultivar fuji Heat shock protein 17.5 mRNA	33%	5e-45	84%
AJ582679.1	*Castanea sativa* mRNA for cytosolic classI heat shock protein	32%	4e-39	82%
AM709758.1	*Triticum monococcum* mRNA for 16.9kDa heat shock protein	32%	2e-38	82%

5. Blastx Results:

Accession No.	Description	Evalue	Score (Bits)
AAC01560.1	Heat shock protein 16.5 (*Agrotis stolonifera*)	0.12	39.3
ACD03605.1	Small heat shock protein 16.9kDa (*Triticum aestivum*)	0.44	37.4
CAA45862.1	18kDa heat shock protein (*Hordeum vulgare*)	0.44	37.4

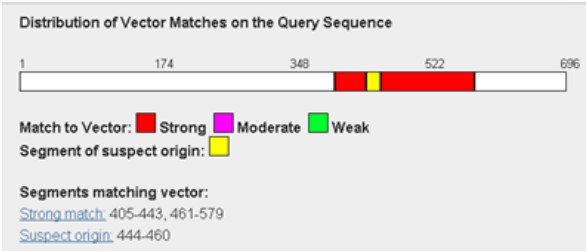
6. ORFs Available:

ORF Location	ORF length	Frame
1-180	180	-3

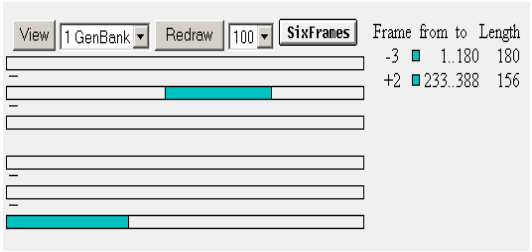
7. CDD available : No CDD

8. Interproscan scan results:

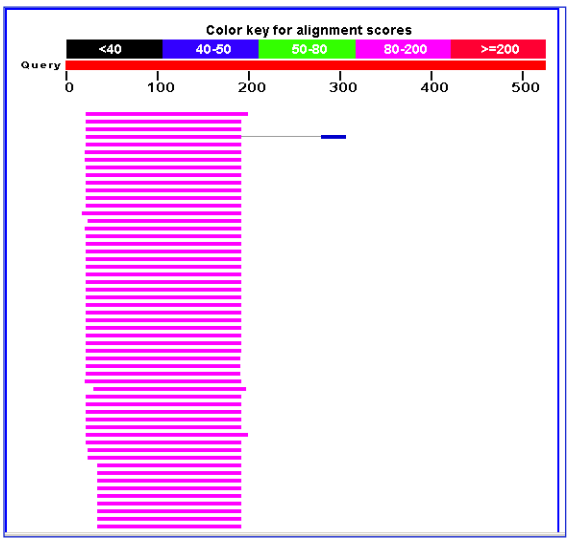
Database entry	Description
PF00011	Heat shock protein HSP20
PTHR11527	Small heat shock protein family
PTHR11527-SF14	HSP-17
tmhmm	Transmembrane helices (1)



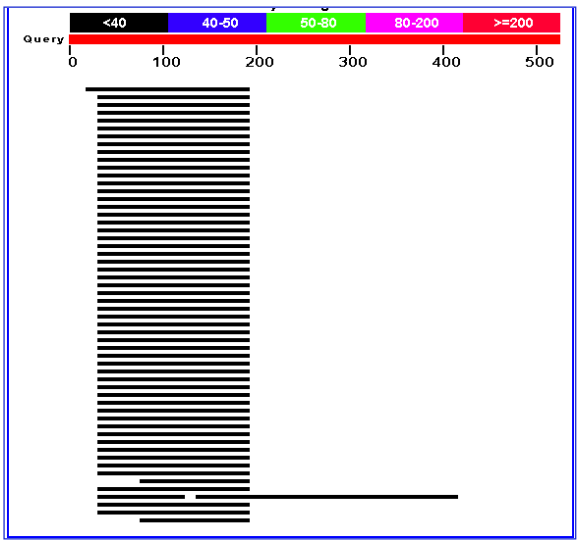
VecScreen output



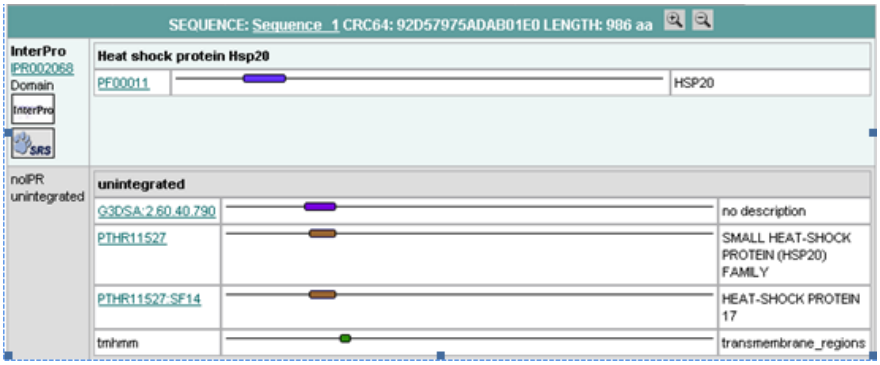
Open reading frames in the cloned sequence



Blastn output



Blastx output



Interproscan result

Fig. 2 Results of sequence data analysis for the Clone 1 [PNK 11(2)]

GTGTCACCACGCTTTTCGTGAATGTGTTTGGTCTCGGTTGGAGCTTGTT
TTCTTTATCTTCGTCTTGTAATAAGGTGATGAATGGGTCTATGCGAGGA
TCGGTGTTGATGTCAGTTAGTATGAATGGGACATTCAGTTTAAAACTG
TTATAATTTGATGTCTGATTTTTAATCACTAGTGCGGCCGCCTGCAGAA
CCGCTCAACAAATTCCAACACAACCATAACGAAGCCGGAAAACCATA
AAGGGTTAAAGCCCTGGGGTGCCCTAATGAATGAACAAACCCA**3'**

Blastn and Blastx results indicated homology with several Heat shock proteins from different organisms. In Blastn results, 30 percent of the sequence also showed over 80 percent homology to the mRNA encoding heat shock proteins in different plants like *Malus*, *Castanea*, *Triticum* etc. The sequence also showed similarity (score- 37 to 39 bits) with heat shock proteins of *Agrotis*, *Triticum*, *Hordeum* etc.

The sequence was found to have two ORFs with the longest one being 180 bp in length. The Interproscan results indicated the presence of heat shock protein domains for HSP-20 and HSP-17 and a small heat shock protein family. The sequence also had one transmembrane helix but did not have any conserved domain.

Clone 2 [PNK 1(1)]

The sequence data obtained for the clone 2 was of 526bp in size and vector screening indicated vector sequence from 1 to 71bp. The details of results obtained are presented in Table 15 and Fig. 3. After vector and adaptor screening the total sequence obtained for further analysis was 453bp as detailed below:-

Vector/Adaptor edited Sequence

5'GTGGTCGCGGCCGAGGTACTTCAGATGCAGGGGCCTGCTCAGAAGT
CCGCTTCTTCAAGGAGGCTCCAACTGCTTCTGTATCATCAGTAGCAAA

Table 15 Results of sequence data analysis for the Clone 2 [PNK1(1)]

1. Initial Sequence length **:** 526
2. Vector sequence **:** 1-71
3. Sequence length after Vector/Adaptor Screening **:** 453
4. Blastn Results:

Accession No.	Description	Q.Coverage	Evalue	Max. Id.
DQ822940.1	*Glycine max* MYB transcription factor MYB 155 mRNA	15%	4e-07	80%
AB210849.1	*Lemna qibba* LqLHY H1 mRNA	13%	2e-05	81%
AY611029.1	*Castanea sativa* late elongated hypocotyl (LHY) mRNA	15%	6e-05	78%

5. Blastx Results:

Accession No.	Description	Evalue	Score (Bits)
CAN81352.1	Hypothetical protein (*Vitis vinifera*)	2e-05	51.6
AAU20773.1	Late elongated hypocotyl(*Castanea sativa*)	7e-05	49.5
ABH02882.1	MYB transcription factor MYB 156 (*Glycine max*)	2e-04	48.5

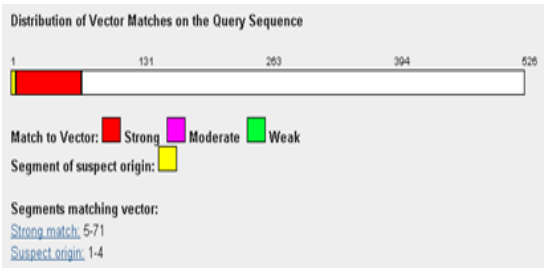
6. ORFs Available:

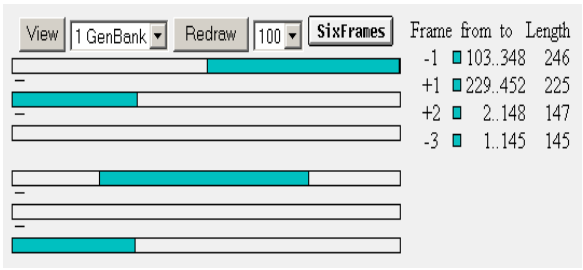
ORF Location	ORF length	Frame
103-348	246	-1

7. CDD available : No CDD

8. Interproscan scan results:

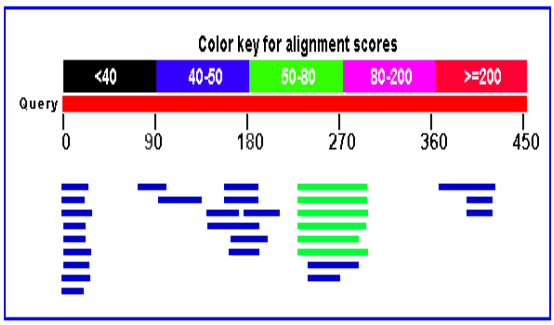
Database entry	Description
tmhmm	Transmembrane helices (4)

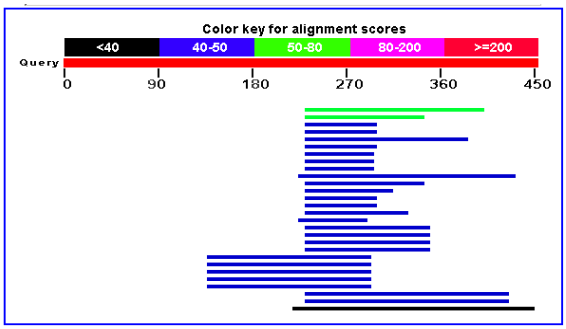




VecScreen output

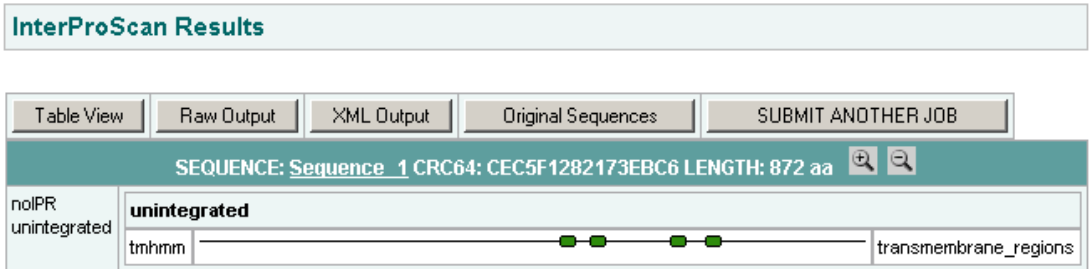
Open reading frames in the cloned sequence





Blastn output

Blastx output



Interproscan result

Fig. 3 Results of sequence data analysis for the Clone 2 [PNK1 (1)]

ATCAAGCCTCATAAGTGCATATTCATACAAAGAACTTGTTGCTCCCGC
CAATGATAAAAATGGGCAGTCAAAAGTTGAAGCAGGAACAAATGAAC
AGAAGATAGGTTTACTGAATCAACCGCGGTCAACAGAAGCTGGGGCG
ACTGGCCTTTCAAGCGCTTTTTACCAGAGAAGTTCTGCCACAAAGCTTC
TCTCCACCTCATGACGATGCAAGAAGCAAGGTGCCCACCAAGAGCATA
GCAGAGGACGAGCATACGAGGCAAGCTTCAAGGCAAGTTGAAGGAGT
GAGCAAGCTCAAGCTGGATTTGGAGCTTGGCTGCAGGATGCAACCATC
TCCCTCTCATGCGGTTCCTGCCCGA**3**’

Blastn result indicated that 15 percent of the sequence showed 80 percent homology with *Glycine max* MYB transcription factor. Blastx result indicated that the sequence had homology (score- 50 bits) with a hypothetical protein of *Vitis vinifera* and with MYB transcription factor MYB of *Glycine max* (score- 48 bits).

The sequence was found to have four ORFs with the longest one 246bp in length. The Interproscan result indicated the presence of four transmembrane helices with no conserved domain.

Clone 3 [PNK 3(1)]

The sequence data obtained for the clone 3 was of 857bp in size and vector screening indicated vector sequence from 1 to 72bp. The details of results obtained are presented in Table 16 and Fig. 4. After vector and adaptor screening the total sequence obtained for further analysis was 785bp as detailed below:

Vector/Adaptor edited Sequence

5’GCGTGGTCGCGGCCGAGGTACAAACCATTATTCGCTTCATATCATGA
AACAAATAAAGAGGAAATTTATTCTTCTTATTCTAATTCCACCTTACTA
ACATTATTAGCTAAGGCACCAAATATGGTCATATCCATGATCACACTT
GTGACATAATATTAAAGCTATCCATTTGATGGCTAGCATCGATCACTG
GGCAACCACCATTTCGATGGGGTTGTCCTCCACCTCCAAAGTGTCGGT

Table 16 Results of sequence data analysis for the Clone 3 [PNK 3(1)]

1. **Initial Sequence length** **:** 857
2. **Vector sequence** **:** 1-72
3. Sequence length after Vector/Adaptor Screening : 785
4. Blastn Results:

Accession No.	Description	Q.Coverage	Evalue	Max. Id.
AC112236.3	*Homo sapiens* BAC clone RP 11_308 D13	4%	0.056	88%
AC210335.1	*Populas trichocarpa* clone POP024-E12	6%	0.68	79%
AM4229093.1	*Danio rerio* partial mRNA lecithin retinol acetyltransferase	3%	0.68	100%

5. Blastx Results:

Accession No.	Description	Evalue	Score (Bits)
NP_564264.1	RNS3 (Ribonuclease 3) endoribonuclease (*Arabidopsis thaliana*)	1e-10	70
ABR25970.1	ribonuclease 3 precursor (*Oryza sativ*a)	2e-10	70.1
AAL33776.1	drought induced S like ribonuclease (*Oryza sativa*)	2e-10	70.1

6. ORFs Available:

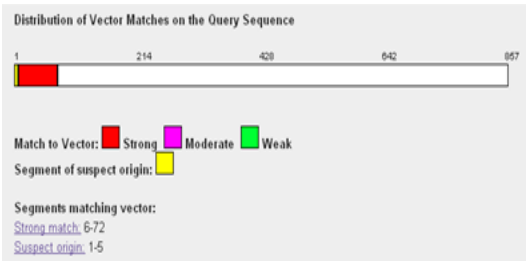
ORF Location	ORF length	Frame
187-615	429	-3

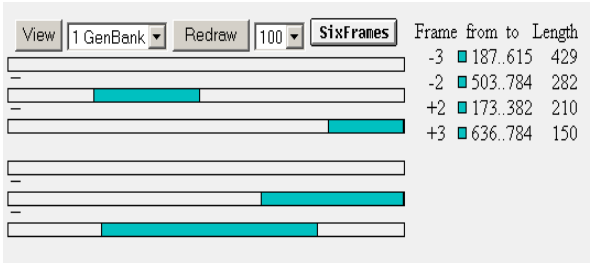
7. CDD available:

Accession no.	Description
c/00208	RNase –T2 Ribonuclease

8. Interproscan scan results:

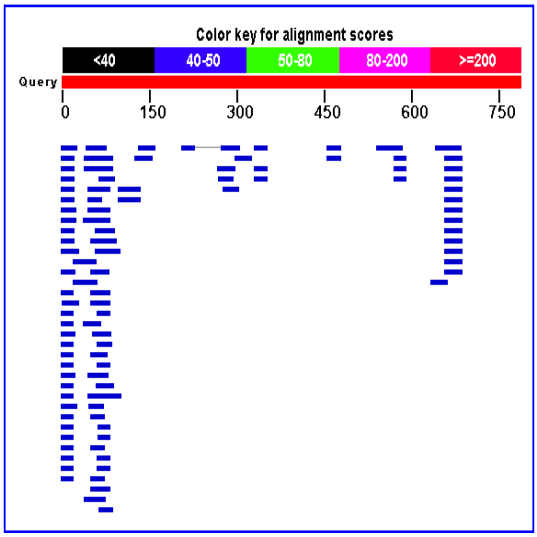
Database entry	Description
G3DSA;3.90.730.10	No description
PTHR11240	Ribonuclease T2
PF00445	Ribonuclease T2

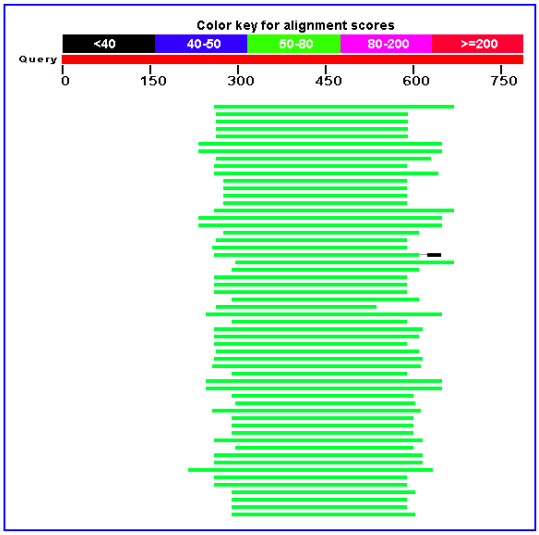




VecScreen output

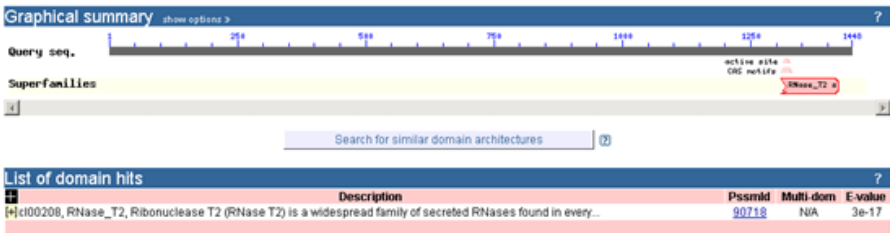
Open reading frames in the cloned sequence





Blastn output

Blastx output



Conserved Domain

Interproscan result

Fig. 4 Results of sequence data analysis for the Clone 3 [PNK 3(1)]

GCTATTTGGCAGCACATCTAGCTTGACAGACGCCGGACAGGTCGAGCT
CCGCGGCGTCGGGCAGGTGATGAATTTGGTGGCGGTGATGTCCACACA
CAACCTCACATTCATGATCCTCTCCAACATGACCGAGCCGCAGTGAAG
CACCGCCGTGTTCACTCCCAATCCTTGGTTGATAGCGTTCACCACATCG
ACGGTCAGGTATTTGTTGGTGTGAGATGGCCTGATACCTCTTCTAGAG
AGGAGATAGAGGAGGTTCGCCTTATCGCGAAGTGCAAGTGCTCTGGA
GAAGTAGTCGCTCACCGTCAACCAGGTGCAGGGGCCGTTGTCTTTCCA
AGTCTGCTCCCACGCCACCATGTTGTTGTCGTTGAGCATGCAGCCGAG
CTCGGCCAATTCTGGTTGAGACTTCTATGATATTTTGGAGAGCCTTGGG
ATCGAAGGAATTTAGAGCATTGATGCTGCGATCAAATTCCTTGCCAAA
ACCGGACGACAAGTCGTCTTTTTGGTCCAGGTGGAGGGAGCAGCATCG
CCTTGCTGGGGGCAGA**3'**

Blastn results indicated that 4 percent of the sequence showed 88 percent homology with *Homo sapiens* clone RP11_308. Blastx result indicated that the sequence had homology (score- 70 bits) with drought induced S like Ribonuclease of *Oryza sativa* and with Ribonuclease of *Arabidopsis thaliana* (score- 70 bits).

The sequence was found to have four ORFs with the longest one 429bp in length. The Interproscan result indicated the presence of domains for Ribonuclease T2. The sequence was found to have one conserved domain for the protein RNase_T2 Ribonuclease.

Clone 4 [PNK 2(1)]

The sequence data obtained for the clone 4 was of 850bp size and vector screening indicated vector sequence from 1 to 74bp and from 603 to 850bp. The details of results obtained are presented in Table 17 and Fig. 5. After vector and adaptor screening the total sequence obtained for further analysis was 492bp as detailed below,

Table 17 Results of sequence data analysis for the Clone 4 [PNK 2(1)]

1. Initial Sequence length **:** 850
2. Vector sequence **:** 1-74, 603-850
3. Sequence length after Vector/Adaptor Screening : 492
4. Blastn Results:

Accession No.	Description	Q.Coverage	Evalue	Max. Id.
BT013878.1	*Lycopersicon esculentum* clone 132858F mRNA	98%	6e-88	75%
EF146919.1	*Populas trichocarpa* clone WSO 1223 unknown mRNA	93%	2e-86	75%
NM_102939.3	*Arabidopsis thaliana* secretory carrier membrane protein (SCAMP)	92%	6e-75	74%

5. Blastx Results:

Accession No.	Description	Evalue	Score (Bits)
ABK94959.1	unknown (*Populas trichocarpa*)	7e-77	288
NP_174485.1	Secretory carrier membrane protein family (*Arabidopsis thaliana*)	1e-77	281
CAO63295.1	unnamed protein (Vitis vinifera)	1e-74	281

6. ORFs Available:

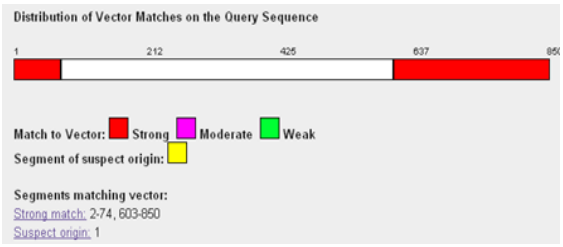
ORF Location	ORF length	Frame
101-491	392	+2

7. CDD available:

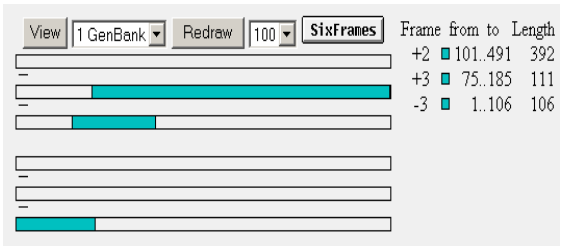
Accession no.	Description
pfam04144	secretory carrier membrane protein family

8. Interproscan scan results:

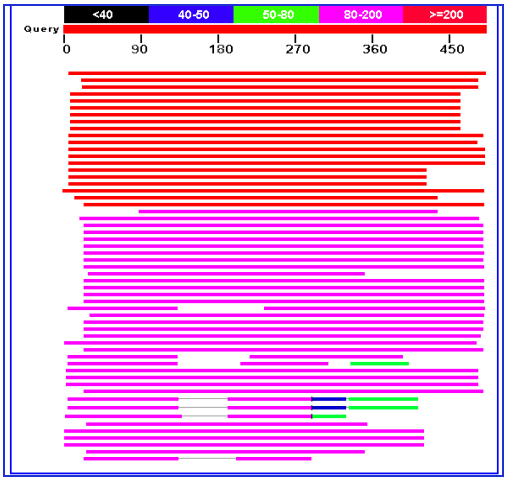
Database entry	Description
PS00652	Cystein rich region
PTHR10687, PF04144, PTHR10687.SF2	SCAMP
tmhmm	Transmembrane helices (8)



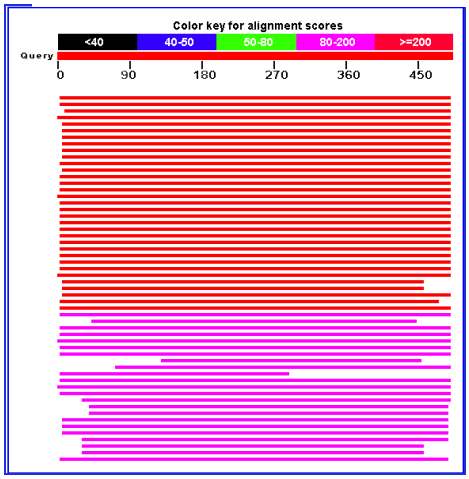
VecScreen output



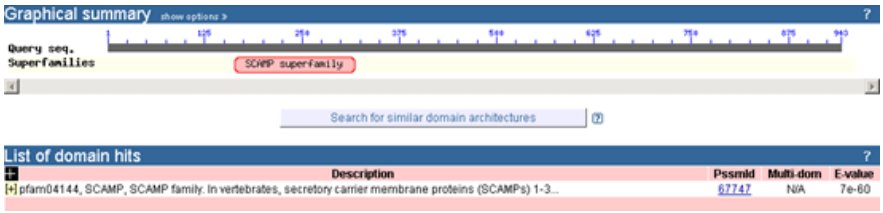
Open reading frames of cloned sequence



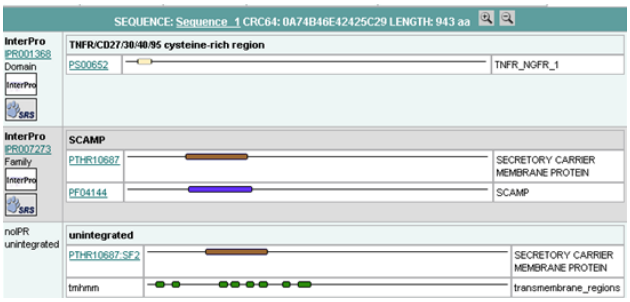
Blastn output



Blastx output



Conserved Domain



Interproscan result

Fig. 5 Results of sequence data analysis for the Clone 4 [PNK 2(1)]

Vector/Adaptor edited Sequence

5’AACACGAGCTGGTGTGCCAGTCAATGACAAGAATTGGCCTCCCTTCT
TTCCTGTTATCCATCATGACATAACCAATGAGATACCAGCCCATGTTCA
GAGGTTGCAGTATTTGGCATTTGCAAGTTGGTTGGGTATTGTCTTTTGC
CTTGTGTTTAACATTGTTGCGATCATTGTCTATTGGATAAGAGGAGGA
GGTGTTAGGATCTTTCTCCTTGCAGTTATCTATGCATTATCTGGATGTC
CTCTTTCATATGTTCTGTGGTATAGGCCTCTTTATCGGGCTATGAGGAC
TGACAGTGCTCTGAAGTTCAGCTGGTTTTTCCTGTTTTATCTGCTTCAC
ATAGGATTTTGCATTTTTGCAACAATCGCCCCTCCAGTAGTTTTTCGTG
GGAAATCATTGACAGGCATACTTGCTGCAATTGATGTGATCTCAGACA
GCACGTTGGCTGGGATATTTTACTTCGTTGGGGCTGGATTGTTTTGTCT
TGAAGT**3’**

Blastn results, indicated that 98 percent of the sequence showed 75 percent homology with *Lycopersicon esculentum* clone 132858F mRNA. Blastx result showed homology of the sequence (score-281 bits) with Secretory carrier membrane protein family of *Arabidopsis thaliana*.

The sequence was found to have three ORFs with the longest one 392bp in length. The Interproscan result indicated the presence of one Cystein rich region, domains for secretory carrier membrane protein family and eight transmembrane helices. The sequence was found to have one conserved domain for the protein secretory carrier membrane protein family.

Clone 5 [PNK 1(2)]

The sequence data obtained for the clone 5 was of 872bp size and vector screening indicated vector sequence from 1 to 49bp and from 414 to 872bp. The

details of results obtained are presented in Table 18 and Fig. 6. After vector and adaptor screening the total sequence obtained for further analysis was 320bp as detailed below,

Vector/Adaptor edited Sequence

5'CGTGGTCGCGGCCGAGGTCATGGCCGAGGCCGAGGACGCGGCGGGC
GGGGAAGATCAGCTGGTCCTGTGCAAGCAGCTGTTTGACTGCATGTGG
AAGGAATTAGTGGTGCTAATGGAAAGAAGGATGCCTGCTCTGCTCCAA
ATCTGTTGCGTTATGTTAGTTTAACAAAGTGAAGTTATTACTAATTATT
GTTTAGTAGTCGTAATTTGCATGAGTTAAGCTGTCATGACAAAGGCAT
TTGCGCGGTTTGGTAGTCTTTGTTTTTTGCAGGTAGGTTAAGTCCATTA
TTCGTTTTAGTTGATCACAACTTTTCGGATAAATTTGTATAATGTGAAT
ACTTCTTTATTTGGAATAATAGAGCAGTACCTGCCCGGGCGGCCGCTC
GA**3'**

Blastn and Blastx results, showed homology of the sequence with several fatty acid desaturase in various plants. Blastn result indicated that 35 percent to 39 percent of the sequence showed 75 percent homology with Fatty acid desaturase mRNA in *Hevea, Persea, Glycine max* etc. Blastx result showed homology of the sequence (score-78 bits) with Microsomal oleate desaturase in *Arachis, Glycine* etc.

The sequence was found to have three ORFs with the longest one 252bp in length. The Interproscan result indicated the presence of five transmembrane helices and did not have any conserved domain.

Clone 6 [PNK 10(2)]

The sequence data obtained for the clone 6 was of 528bp size and vector screening indicated no vector and adaptor sequences. The details of results

Table18 Results of sequence data analysis for the Clone 5 [PNK 1(2)]

1. Initial Sequence length **:** 872
2. Vector sequence **:** 1-49, 414-872
3. Sequence length after Vector/Adaptor Screening : 320
4. Blastn Results:

Accession No.	Description	Q.Coverage	Evalue	Max. Id.
DQ023609.1	*Hevea brasiliensis* omega 6 fatty acid destaurase (FAD) mRNA	39%	2e-15	76%
AY057406.1	*Persea americana* delta-12 fatty acid destaurase mRNA	35%	7e-15	77%
DQ532370.1	*Glycine max* microsomal oleate desaturase FAD2-1B	39%	1e-12	74%

5. Blastx Results:

Accession No.	Description	Evalue	Score (Bits)
AAF82293.1	Microsomal oleate desaturase (*Arachis hypogea*)	2e-13	78.2
AAX14399.1	Oleate desaturase (*Arachis monticola*)	2e-13	78.2
ABF84062.1	Microsomal oleate desaturase (*Glycine max*)	2e-12	75.1

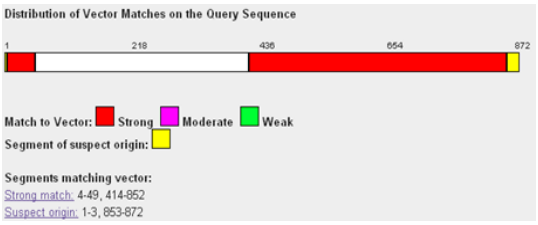
6. ORFs Available:

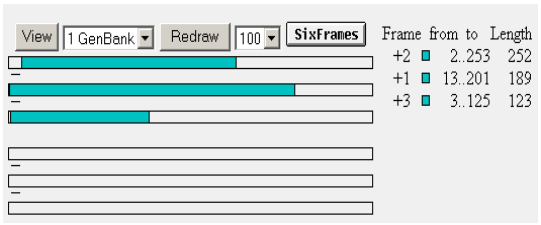
ORF Location	ORF length	Frame
2-253	252	+2

7. CDD available : No CDD

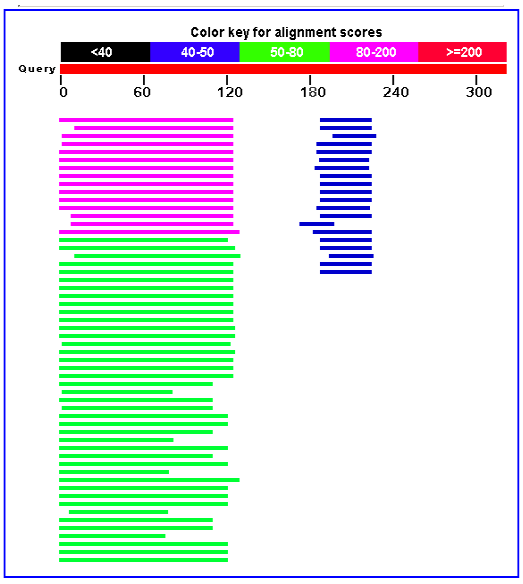
8. Interproscan scan results:

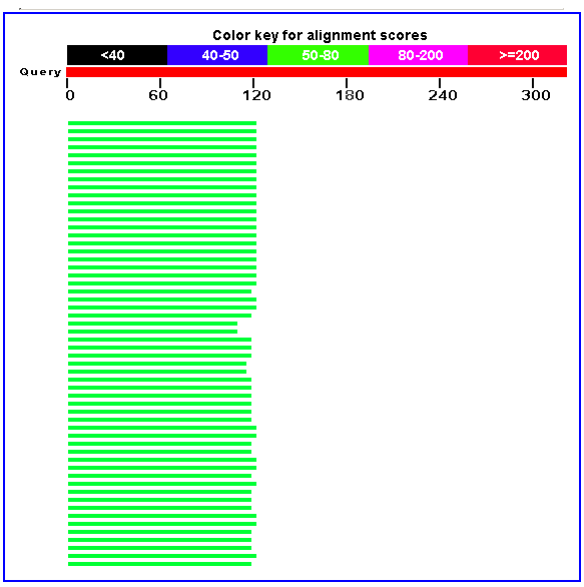
Database entry	Description
tmhmm	Transmembrane helices (5)





VecScreen output **Open reading frames of cloned sequence**





Blastn output **Blastx output**

Interproscan result

Fig. 6 Results of sequence data analysis for the Clone 5 [PNK 1(2)]

obtained are presented in Table 19 and Fig. 7. The total sequence obtained for further analysis was 528bp as detailed below,

Vector/Adaptor edited Sequence

5’TTGACGTGGTCGCCCGCGGGAGGTCAGGTTGCCGGCGATCTTTCTTG
GGTGGTGAGAGGTAGTATTCTTGAGCCATGATCTCCAAATAGAGCTGA
TCCTTGAGCTTCCTAGGATGGTGAAAGCTCGCAGAGACCCAGAGAGGC
AGAAGAAGAAGAAGAAGATCAGACAGCTTATTGCCTAGAATGGCTAT
TCTTACTCAAGCCAAAAGAGTTTCAATTTCTCTCCCACAGCTTAGCATC
GTGCTCAGCACGTCTTCACACAAAAGCAGCGACGGCGGAAACCACGG
CAGTGCCCACCGGGGAAGCCCTCTGTCTGGCAGACACTGGCGCAGTTG
CTGTCCCTCACACACGGACCTTTGAACCTGTGGCTCTGTGACTCGCAG
ATCCTTGCCTCGGCAAAGCTCGGCCCAACATTTCCAAGTGGACATGAT
CAGCAAAACCATGATAAAAATGGCTGGGAACAGACGTGATCACCATT
AGTCCTGAAAGCTAGAATCAGAAGCCGAGGATTTGAAGACCTGCCCG
GGGG3’

Blastn results, indicated that 26 percent of the sequence showed 80 percent homology with *Solanum pimpeinellifolium cv* LA1589 clone and also with several gamma thionins in various plants like *Petunia, Castanea* etc. Blastx result showed homology of the sequence (score-70 bits) with several gamma thionin and defensin proteins in plants like *Castanea, Nelumbo* etc.

The sequence was found to have five ORFs with the longest one 246bp in length. The Interproscan result indicated the presence of domains for gamma thionin, gamma purothionin, knot1 proteins and five transmembrane helices. The sequence was found to have one conserved domain for gamma thionin protein family.

Table 19 Results of sequence data analysis for the Clone 6 [PNK 10(2)]

1. Initial Sequence length **:** 528
2. Vector sequence **:** No vector sequence
3. Sequence length after Vector/Adaptor Screening : 528
4. Blastn Results:

Accession No.	Description	Q.Coverage	Evalue	Max. Id.
EF094941.1	*Solanum pimpeinellifolium cv* LA1589 clone	26%	5e-26	80%
L27173.1	*Petunia infata* gamma thionin homolog gene	26%	5e-26	80%
AF417297.1	*Castanea sativa* putative gamma thionin mRNA	26%	2e-23	78%

5. Blastx Results:

Accession No.	Description	Evalue	Score (Bits)
AAL15885.1	Putative gamma thionin (*Castanea sativa*)	5e-11	70.5
ABN46979.1	Defensin (*Nelumbo nucifera*)	3e-10	67.8
CAO46721.1	Unnamed protein (*Vitis vinifera*)	7e-10	66.6

6. ORFs Available:

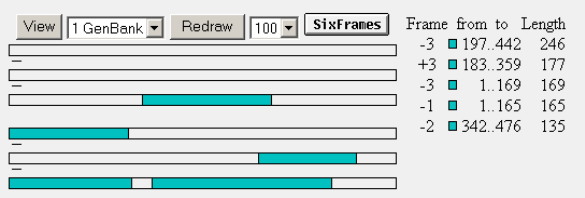
ORF Location	ORF length	Frame
197-442	246	-3

7. CDD available:

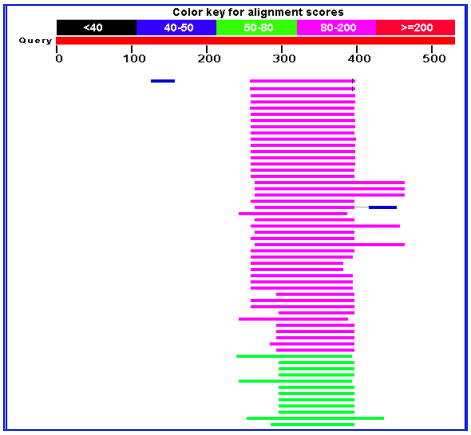
Accession no.	Description
pfam 00304	Gamma thionin family

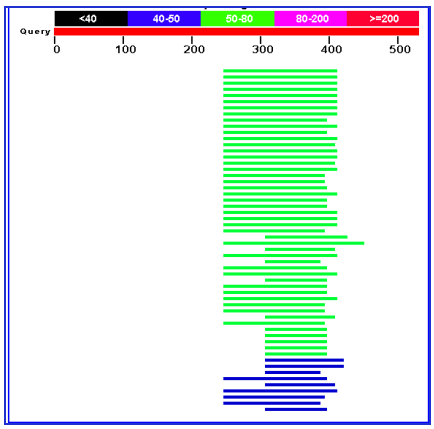
8. Interproscan scan results:

Database entry	Description
SM00505	knot 1 protein
PF00304, PS00940	Gamma thionin
PR00288	Gamma purothionin
tmhmm	Transmembrane helices (3)

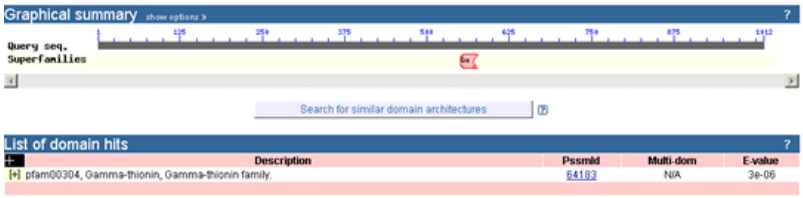


Open reading frames in the cloned sequence

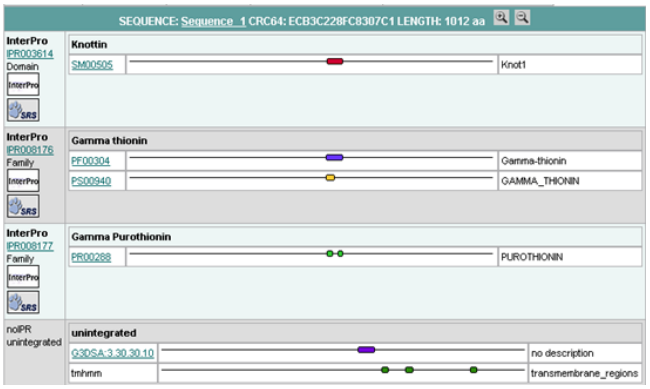




Blastn output **Blastx output**



Conserved Domain



Interproscan result

Fig. 7 Results of sequence data analysis for the Clone 6 [PNK 10(2)]

Clone 7 [PNK 4(2)]

The sequence data obtained for the clone 7 was of 789bp size and vector screening indicated vector sequence from 1 to 82bp. The details of results obtained are presented in Table 20 and Fig. 8. After vector and adaptor screening the total sequence obtained for further analysis was 690bp as detailed below:-

Vector/Adaptor edited Sequence

5'ACTTTCAATCAGGAGCAGAAAAAGATGGGTAACTTTTTCCGAGTGAC
CTGCATTTTGAATAATGCAGCTTGCAAACTGAAACTGAATGAATTCCG
GGAAGCTGCGAAGCTCTGTGCAAAGGCTTTGCAATTAGAGCCTGACAA
CGTGAAGGCTCTCTACCGTAGAGCTCAGTCTTACATTGAGACAAGTGA
TCTGGACTTGGCAGAAATAGACTTATTGGCTGCAAAGGAAATTGAACC
AGACAATGAGCCATTAAATCAACTACTGGAGAGAGTGAAAAAACTAA
AGACACGAGCCAGTAAAATGGATATGAAATTTTACGCAAACATGCTG
GCAAAGGTTAACATTGCACACTGTAAATAACTCAGAAATATCTTTATA
CTGCTCATTTTTGTTGAGTTAAAACAGATCTGATTAGGGTTGGAAAAA
TAAGCATTGAAGAGATTGTTTGCCTCAGCGGCTCTGTATCATTCTATAG
GCGAGAGCTTTTAATTTCTTTTGTGATTAGTGATAAAAGAAGGGACGA
GATAAATAATTTGGTGGGCATATGTTTGGAATGTATTCTGCATGATGA
GCTGTTGTTGATAAATGCTTGAAAGGGATGATGATATGTATAATAATA
CACAGGCTGCTGGGAGTATATATATGTAACTTGTACCTGCCCGGGCGG
CGCTCGAATCAAAAGGGGCC**3'**

Blastn results, indicated that 27 percent of the sequence showed 70 percent homology with *Picea sitchensis* clone WSO299 B13 unknown mRNA and 73% with *Oryza sativa* FK506 binding protein mRNA. Blastx result showed homology of the sequence (score-70 bits) with Peptidylprolyl isomerase FK506 binding protein in *Arabidopsis thaliana*.

Table 20 Results of sequence data analysis for the Clone 7 [PNK 4(2)]

1. Initial Sequence length **:** 789
2. Vector sequence **:** 1-82
3. Sequence length after Vector/Adaptor Screening: 690
4. Blastn Results:

Accession No.	Description	Q.Coverage	Evalue	Max. Id.
EF085144.1	*Picea sitchensis* clone WSO299 B13 unknown mRNA	27%	2e-13	70%
AF140495.2	*Oryza sativa* FK506 binding protein mRNA	21%	2e-13	73%
YO7636.1	*T. aestivum* mRNA for peptidylprolyl isomerase	22%	2e-12	73%

5. Blastx Results:

Accession No.	Description	Evalue	Score (Bits)
EAY93685.1	Hypothetical protein OsI_014918 (*Oryza sativa*)	4e-20	101
BAB02082.1	Peptidylprolyl isomerase FK506 binding protein	2e-10	70.1
NP_001118695.1	ROF1 (ROTAMASE FKBP1) (*Arabidopsis thaliana*)	3e-19	98.6

6. ORFs Available:

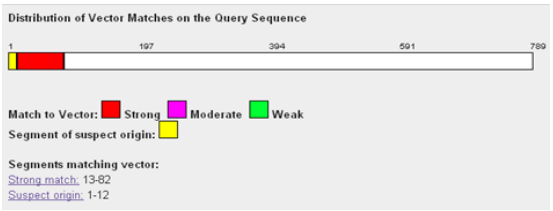
ORF Location	ORF length	Frame
25-363	339	+1

7. CDD available:

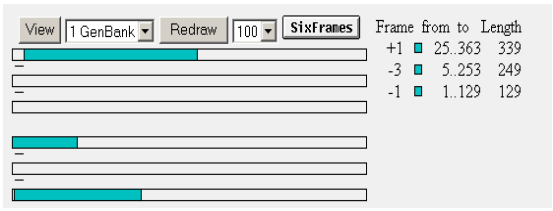
Accession no.	Description
cd00189	TPR Tetratricopeptide repeat domain

8. Interproscan scan results:

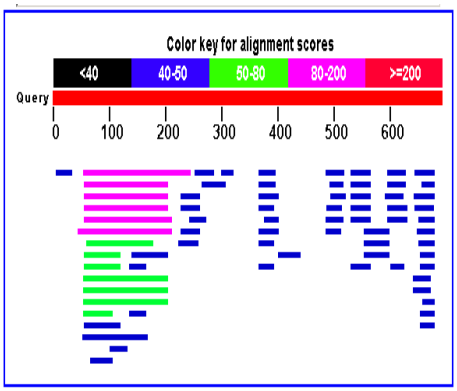
Database entry	Description
PTHR10516	FK506 binding protein
PF00515, SM00028,	Tetratricopeptide TPR-1, TPR-2
tmhmm	Transmembrane helices (5)



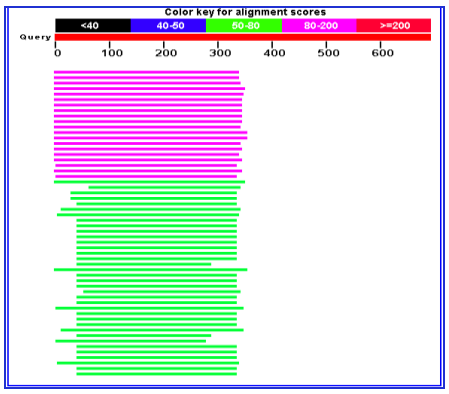
VecScreen output



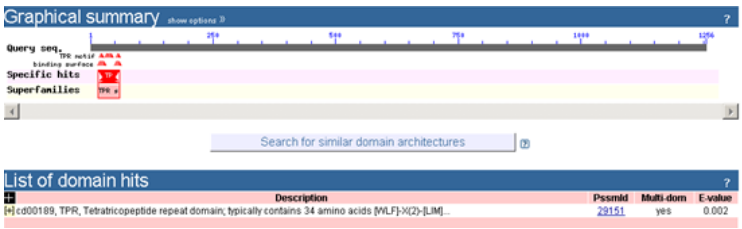
Open reading frames of cloned sequence



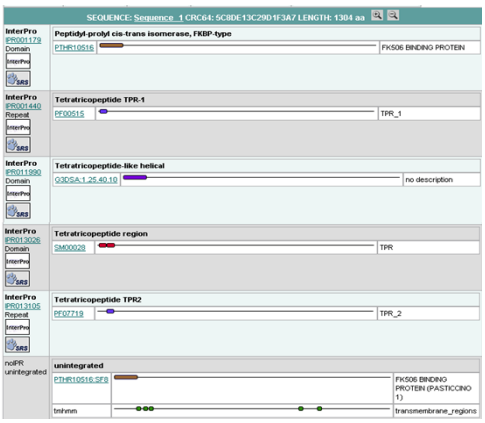
Blastn output



Blastx output



Conserved Domain



Interproscan result

Fig. 8 Results of sequence data analysis for the Clone 7 [PNK 4(2)]

The sequence was found to have three ORFs with the longest one 339bp in length. The Interproscan result indicated the presence of domains for Peptidylprolyl cis-trans isomerase, FK506 binding protein, Tetratricopeptide and five transmembrane helices. The sequence was found to have one conserved domain for Tetratricopeptide repeat domain.

Clone 8 [PNK 14(1)]

The sequence data obtained for the clone 8 was of 827bp in size and vector screening indicated vector sequence from 531 to 628bp and 684 to 744. The details of results obtained are presented in Table 21 and Fig. 9. After vector and adaptor screening the total sequence obtained for further analysis was 645bp as detailed below,

Vector/Adaptor edited Sequence

5'CCCAACCATTTACTTTAACAGCAGAAAAATGTGGATTTCAAGAACTT
GGAGCTGAAACTACTACTGGTGGGACCAAAATTGTGGGCACTAATGTG
CAGTCTCCAACAGTAAAGAAGGCTCAGCCAATGGAACCTTCACAACCA
GATAGCACCAAGCATGGTGATGAAGAGGATGCTTGCTCTGTTGCTTCT
TCATAAATTCATGCTACCTTTTTGCAGCCAATGAAACCTTCACAACCAG
ATAACACCAAGCATGGTGATGAAGAGGATGCTTACTCTGTTGCTTCTT
TCACTGCTGCATCAATTCAAGCAATCAAATCAAGGATTATTGCCGCAA
GTGCTCGAGTATTTAGGGTCACTGAACGTGCAGAGAAGCGGAAAGAA
TTTTACCGAAAGCTAGAAGAGAAATACCAAGCTCTAGAAGCTGAAAA
AAACCAGTCTGAAGCAACGACCAAGATTTTTGATTTTTTCAGATTTGTT
TTGTATGGGTAAATGTGTGTCTCTGTAGTCGTATCTGGTCATTAGCTTG
TTTCCTGGTGTTGAAAATTTGGTTATTCCGGTCAGAAGTGATTAATCCA
AATTTATTGGNTTGGGCNCCATGGCGGTTTCAGTGGGGAAACTGTCGG
GCCTTGCTNAGGAATCGGCA**3'**

Table 21 Results of sequence data analysis for the Clone 8 [PNK 14(1)]

1. Initial Sequence length **:** 827
2. Vector sequence **:** 531-628, 684-744
3. Sequence length after Vector/Adaptor Screening: 645
4. Blastn Results:

Accession No.	Description	Q.Coverage	Evalue	Max. Id.
EF147721.1	*Populus trichocarpa* clone WSO1241 unknown mRNA	36%	2e-13	68%
CU229002.1	*Populus* EST from severe drought stressed leaves	36%	2e-13	68%
AY208876.1	*Brassica napus* seed specific protein Bn15D14A mRNA	42%	6e-13	88%

5. Blastx Results:

Accession No.	Description	Evalue	Score (Bits)
ABK93119.1	unknown (*Populas trichocarpa*)	1e-14	83.2
AAP37969.1	Seed specific protein Bn15D14A (*Brassica napus*)	2e-12	76.3
ABF99369.1	Targeting protein for Xklp2 [*Oryza sativa* (japonica cultivar-group)]	5e-10	67.8

6. ORFs Available:

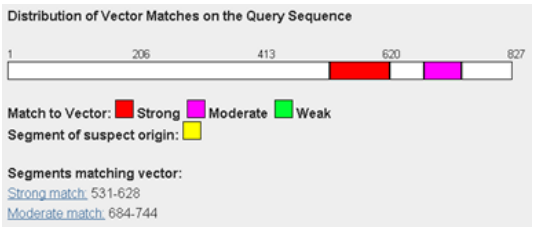
ORF Location	ORF length	Frame
221-491	270	+3

7. CDD available:

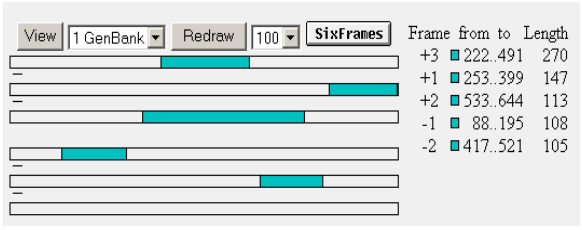
Accession no.	Description
pfam 06886	TPX2, Targeting protein for Xklp2

8. Interproscan scan results:

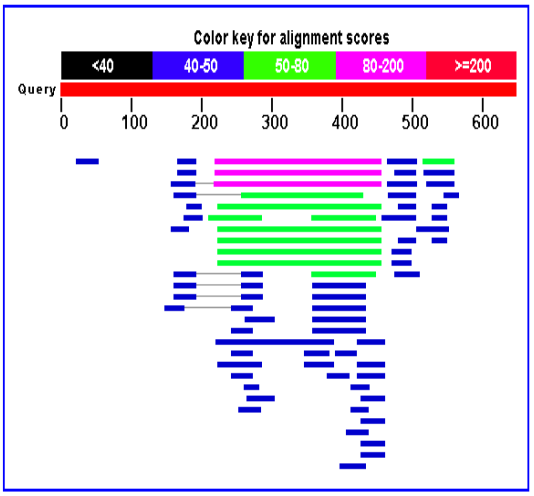
Database entry	Description
PF06886	Targeting for Xklp2 ,TPX2 family
tmhmm	Transmembrane helices (7)



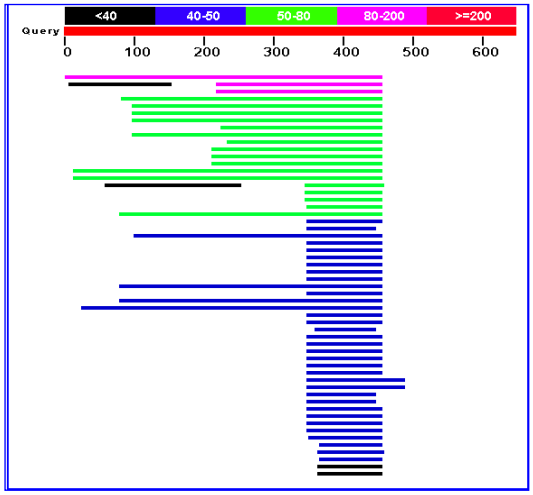
VecScreen output



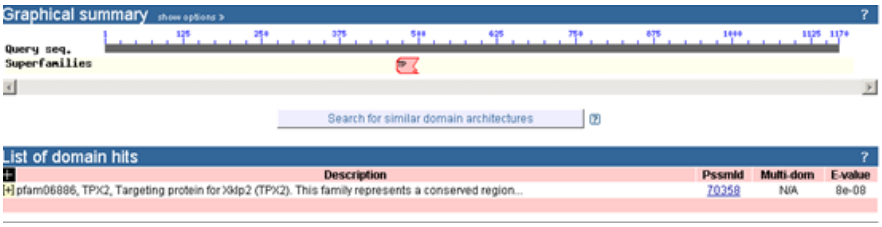
Open reading frames in the cloned sequence



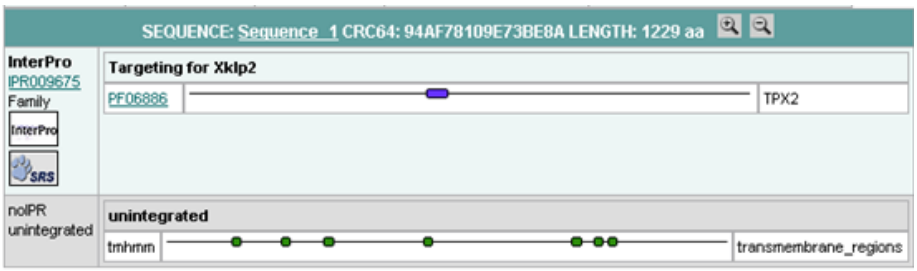
Blastn output



Blastx output



Conserved Domain



Interproscan result

Fig. 9 Results of sequence data analysis for the Clone 8 [PNK 14(1)]

Blastn results, indicated that 36 percent of the sequence showed 68 percent homology with *Populas trichocarpa* clone WSO1241 unknown mRNA and with *Populas* EST from severe drought stressed leaves. Blastx result showed homology of the sequence (score-83.2 bits) with unknown protein from *Populus*, Seed specific protein Bn15D14A from *Brassica napus* and Targeting protein for Xklp2 from *Oryza sativa* (japonica cultivar-group).

The sequence was found to have five ORFs with the longest one 270bp in length. The Interproscan result indicated the presence of domain for targeting protein for Xklp2, TPX2 family and seven transmembrane helices. The sequence was found to have one conserved domain for TPX2, targeting protein for Xklp2.

Clone 9 [PNK 1(3)]

The sequence data obtained for the clone 9 was of 857bp in size and vector screening indicated vector sequence from 1 to 84bp and 395 to 857bp.

The details of results obtained are presented in Table 22 and Fig. 10. After vector and adaptor screening the total sequence obtained for further analysis was 292bp as detailed below,

Vector/Adaptor edited Sequence

5'ATGGCTGTCGACAAGAAAGTAGCTGATGGATTGCTGAGACTTATACT
ACTGAAAGGGCCTCTTGGTAGCTGCGTTTTTACCGGTGATTATGACAG
AAAAGCTCTTGATGAAACTCTTCATGCATTCTGCAGTAGATGATACTG
GTTCTACAGTCAGATTTGTGTGATTCTGGAAGCATTTCTTGCAATCATG
CTTATTTCGCAGCCTTGTCCATAGATATGAGGGTCAAAGATTTTACAA
GACTTACTGTTTTACTAAAACCATCTGTGTACCTGCCCGGGCGGCCGCT
CGA**3'**

Table 22 Results of sequence data analysis for the Clone 9 [PNK 1(3)]

1. **Initial Sequence length** **:** 857
2. **Vector sequence** **:** 1-84, 395-857
3. Sequence length after Vector/Adaptor Screening: 292
4. Blastn Results:-

Accession No.	Description	Q.Coverage	Evalue	Max. Id.
CR936327.2	*Medicago truncatula* chromsome 5 clone mte-68q1	41%	1e-24	81%
NM001070337.1	*Oryza sativa* Oso9q0539100	49%	4e-24	80%
DQ166522.1	*Fagus sylvatica* putative 3-dehydroquinate synthase mRNA	42%	4e-22	80%

5. Blastx Results:

Accession No.	Description	Evalue	Score (Bits)
EAZ09909.1	Hypothetical protein OsI_031141 (*Oryza sativa*)	6e-07	56.6
NP_001063802.1	Oso9q0539100 *Oryza sativa*	6e-07	56.6
ABA54866.1	Putative 3-dehydroquinate synthase (*Fagus sylvatica*)	4e-06	53.9

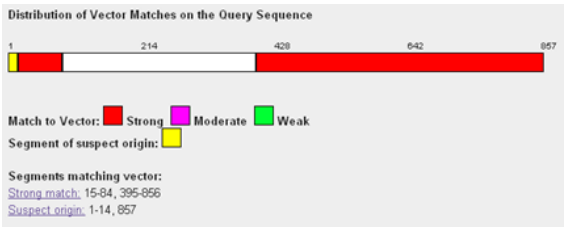
6. ORFs Available:

ORF Location	ORF length	Frame
135-291	158	+3

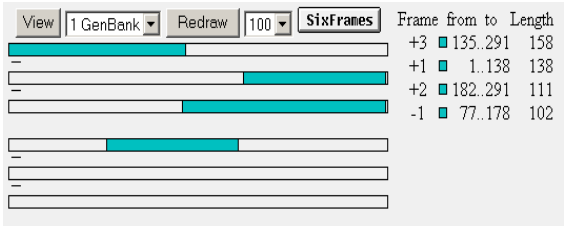
7. CDD available : No CDD

8. Interproscan scan results:

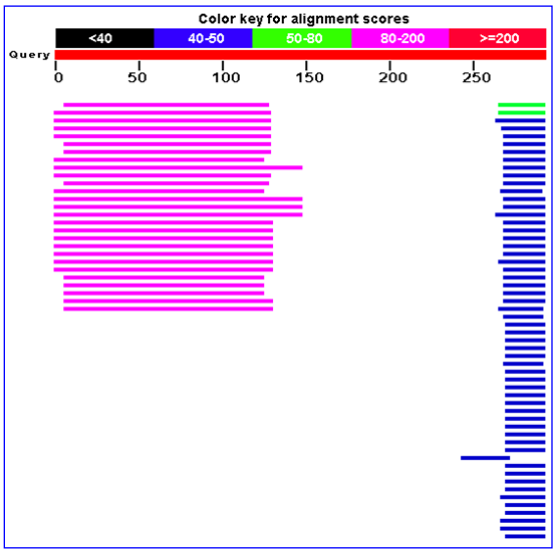
Database entry	Description
signal p	Signal peptide (1)
tmhmm	Transmembrane helices (1)



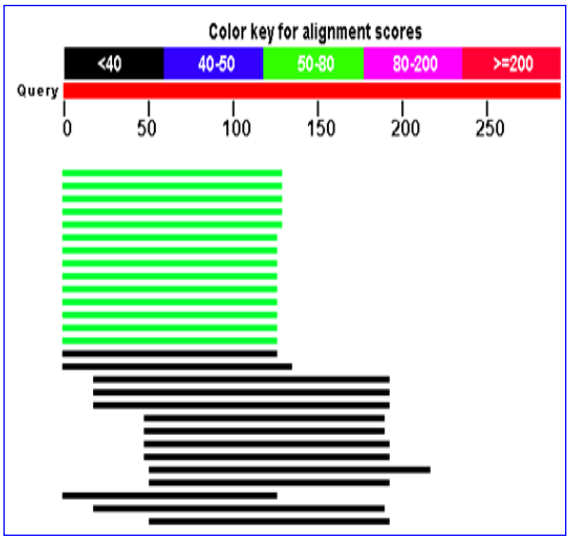
VecScreen output



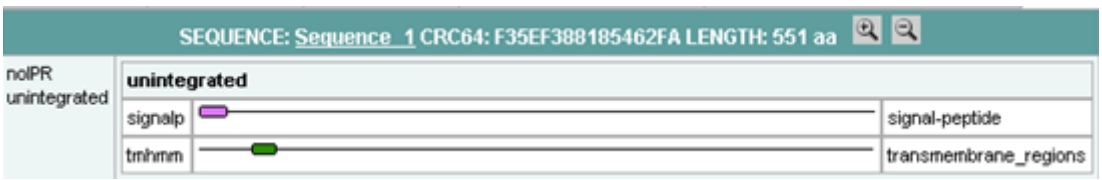
Open reading frames in the cloned sequence



Blastn output



Blastx output



Interproscan result

Fig. 10 Results of sequence data analysis for the Clone 9 [PNK 1(3)]

Blastn results, indicated that 41 percent of the sequence showed 81 percent homology with *Medicago truncatula* chromosome 5 clone mte-68q1 and 80 percent with *Fagus sylvatica* putative 3-dehydroquinate synthase mRNA. Blastx result showed homology of the sequence (score-56.6 bits) with hypothetical protein OsI_031141 from *Oryza sativa*, and Putative 3-dehydroquinate synthase from *Fagus sylvatica*.

The sequence was found to have four ORFs with the longest one 158bp in length. The Interproscan result indicated the presence of one signal peptide and one transmembrane helix and did not have any conserved domain.

Clone 10 [PNK 5(2)]

The sequence data obtained for the clone 10 was of 851bp size and vector screening indicated vector sequence from 1 to 82bp and 410 to 851bp. The details of results obtained are presented in Table 23 and Fig. 11. After vector and adaptor screening the total sequence obtained for further analysis was 287bp as detailed below,

Vector/Adaptor edited Sequence

5'ACAGAAAGAGCTTGCTGAGACTGAATTTGATGGCTACTGTGAAGGT
GAACTAGTAAAGGTCACACTTTCTGGGAATCAACAGCCCGTTAGGACT
GAAATTACTGAGGCTGCCATGGAACTAGGCGCTGATAAGCTCTCTCTG
TTGATTACTGAAGCATATAAGGATGCACACCAGAAGAGTGTGCAGGCC
ATGAAGGAGAGAATGAGTAATCTAGCACAGAGTTTGGGAATGCCTCCT
GGTTTGAGCGATGGTTTGAAGTAGTGCTAGAAAAAAAAATGCAAATGT
A**3'**

Blastn results, indicated that 83 percent of the sequence showed 79 percent homology with *Picea sitchensis* clone WSO2742 G13 unknown mRNA and 76

Table 23 Results of sequence data analysis for the Clone 10 [PNK 5(2)]

1. Initial Sequence length **:** 851
2. Vector sequence **:** 1-82, 410-851
3. Sequence length after Vector/Adaptor Screening: 287
4. Blastn Results:

Accession No.	Description	Q.Coverage	Evalue	Max. Id.
EF086744.1	*Picea sitchensis* clone WSO2742 G13 unknown mRNA	83%	9e-51	79%
AK224366.1	*Oryza officinallis* cDNA clone CCP06F01	81%	5e-48	78%
EF051318.1	*Gymnadenia conopsea* expressed DUF 149 protein like mRNA	89%	6e-47	76%

5. Blastx Results:

Accession No.	Description	Evalue	Score (Bits)
ABK26000.1	Unknown (*Picea sitchensis*)	9e-37	155
CAN79971.1	Hypothetical protein (*Vitis vinifera*)	4e-36	153
NP_565561.1	Unknown protein (*Arabidopsis thaliana*)	9e-34	145

6. ORFs Available:

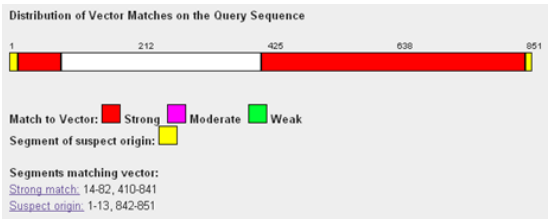
ORF Location	ORF length	Frame
113-262	150	+2

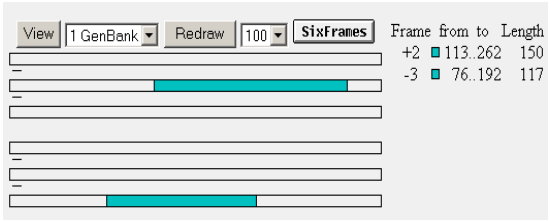
7. CDD available:

Accession no.	Description
c/00494	DUF 149, Uncharacterized YbaB family

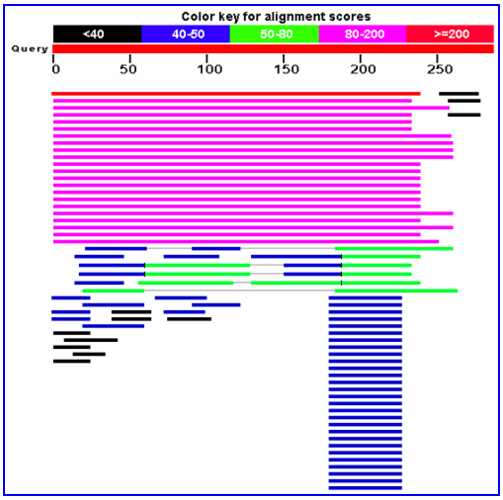
8. Interproscan scan results:

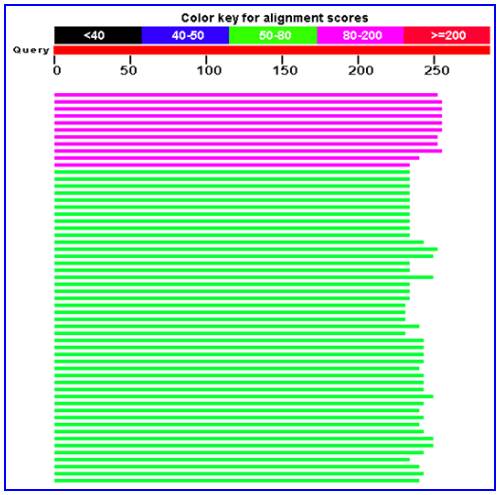
Database entry	Description
G3DSA;3.30.1310.10	Conserved hypothetical protein
PF02575	DUF 149
TIGR00103	TIGR00103: Conserved hypothetical protein



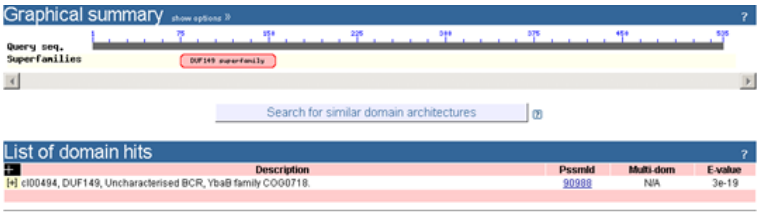


VecScreen output **Open reading frames in the cloned sequence**

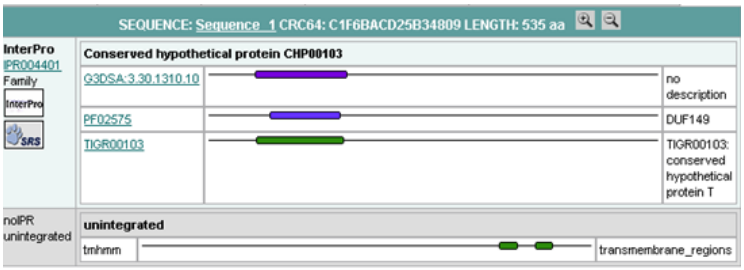




Blastn output **Blastx output**



Conserved Domain



Interproscan result

Fig. 11 Results of sequence data analysis for the Clone 10 [PNK 5(2)]

percent with *Gymnadenia conopsea* expressed DUF 149 protein like mRNA. Blastx result showed homology of the sequence (score-155 bits) with unknown protein from *Picea sitchensis* and hypothetical protein from *Vitis vinifera*.

The sequence was found to have two ORFs with the longest one 150bp in length. The Interproscan result indicated the presence of domains for conserved hypothetical protein and DUF 149 protein. The sequence was found to have one conserved domain for DUF 149, uncharacterized YbaB family.

Clone 11 [PNK 12(1)]

The sequence data obtained for the clone 11 was of 683bp size and vector screening indicated absence of vector and adaptor sequences. The details of results obtained are presented in Table 24 and Fig. 12. The total sequence obtained for further analysis was 628bp as detailed below,

Vector/Adaptor edited Sequence

5'GCGTGGTCGCGGGCGAGGCCAGAGTGAAATTTGTTACAAGTAAAAC
TTCACAACATAGGTGCAAGACTTGTTGGTGTCGACCAATTTGGGAACA
AATATTATGAGAAACTTGAAGGCGTGATGTATGGGAGACACAGATGG
GTGGAATATGCAAAAAAGGATCGCCACAATGCTTCTCAAGTGCCTCCT
GAGTGGCATGGCTGGCTGCATAACATTACTGATCCTACTGGTGATGAA
TTGTTGATGCTCAGACCAAAGAGATATGGGGTGGCGCACAGAGAGAA
CTTTTCAGGAGAGGGAGAAGAATACATCCACCATTCAAAGGGTCACGC
ACTCAATCCAGATCAGAAAGACTGGACAAGATATCAGCCGTGGCAGC
CTGCAGAGAAGTCTTAGGAAATCAAAAGAAAATGTGGATGGGCGTAA
GCTTTGTTGTTTGTTTCTTAAATTTATTCGTACCTGCCCGGGGCGGCCG
TCTAATCACTTGTGGCGGGCCGCCTGCCACGGTCGACCATTATGGGAA
GAAGCTCCCACCGCGTTGGGATGCATAACTTGCAGTATTTCTAATAGT
GTCACCTTTATAGCCATTGGGCTTAATTCATGATTATTTATGTATTCCG

Table 24 Results of sequence data analysis for the Clone 11 [PNK 12(1)]

1. Initial Sequence length **:** 683
2. Vector sequence **:** No Vector Sequence
3. Sequence length after Vector/Adaptor Screening : 683
4. Blastn Results:

Accession No.	Description	Q.Coverage	Evalue	Max. Id.
AK246302.1	*Solanum lycopersicon* cDNA clone, FCO6BE04	51%	6e-64	76%
CU229918.1	*Populus* EST from mild drought stressed leaves	49%	6e-58	75%
NM_111180.4	*Arabidopsis* NADH-ubiquinone oxidoreductase family	42%	8e-44	74%

5. Blastx Results:

Accession No.	Description	Evalue	Score (Bits)
CAO62196.1	unnamed protein product (*Vitis vinifera*)	1e-53	213
NP_566192.1	NADH: ubiquinone oxidoreductase family protein (*Arabidopsis thaliana*)	3e-53	211
ABK23341.1	unknown (*Picea sitchensis*)	1e-49	199

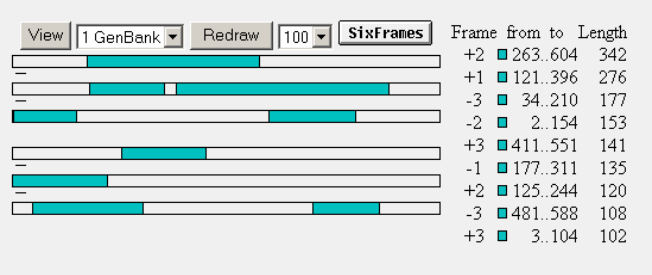
6. ORFs Available:

ORF Location	ORF length	Frame
263-604	342	+2

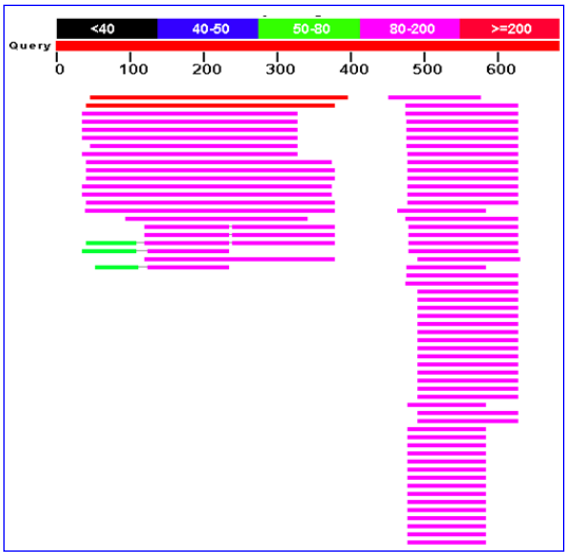
7. CDD available : No CDD

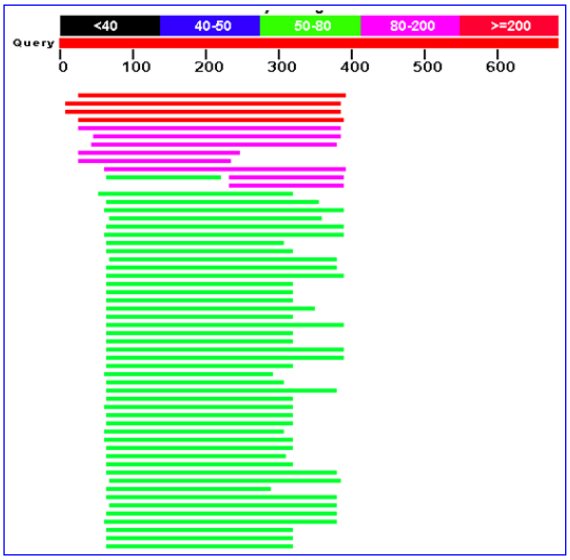
8. Interproscan scan results-

Database entry	Description
PTHR12910,PF05071	NADH ubiquinone oxidoreductase subunit B17.2 family
tmhmm	Transmembrane helices (9)

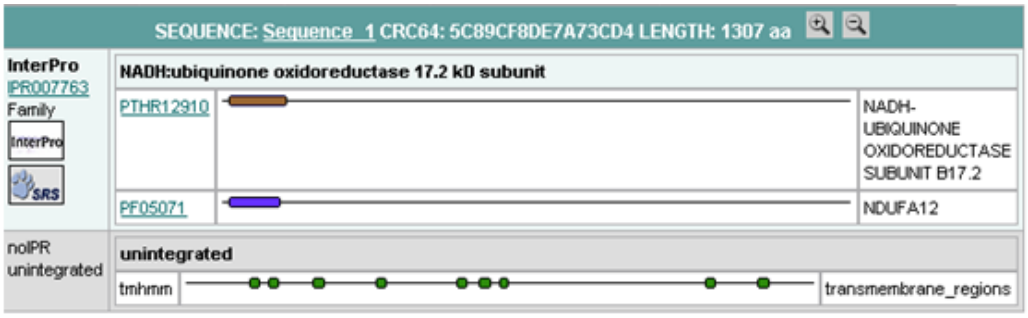


Open reading frames in the cloned sequence





Blastn output **Blastx output**



Interproscan result

Fig. 12 Results of sequence data analysis for the Clone 11 [PNK 12(1)]

GTGTAAAAGGGATACGTTACCATAAACCAAAACATATAGCAAGAACG
AGAAATAAAAGTAAAG **3'**

Blastn results indicated that 51 percent of the sequence showed 76 percent homology with *Solanum lycopersicon* cDNA clone, FCO6BE04, 75 percent with *Populus* EST from mild drought stressed leaves and 74 percent with *Arabidopsis* NADH-ubiquinone oxidoreductase family. Blastx result showed homology of the sequence (score-213 bits) with unnamed protein product from *Vitis vinifera* and NADH: ubiquinone oxidoreductase family protein from *Arabidopsis thaliana*.

The sequence was found to have nine ORFs with the longest one 342bp in length. The Interproscan result indicated the presence of domain for NADH ubiquinone oxidoreductase subunit B17.2 family and nine transmembrane helices and did not have any conserved domain.

Clone 12 [PNK 3(2)]

The sequence data obtained for the clone 12 was of 846bp size and vector screening indicated vector sequence from 1 to 75bp and 808 to 846bp.

The details of results obtained are presented in Table 25 and Fig. 13. After vector and adaptor screening the total sequence obtained for further analysis was 694bp as detailed below,

Vector/Adaptor edited Sequence

5'ACTTACATCACAACAGTTTGCCACTACAGAAAACCGCGGATGTAGAT
CTCAGAAGTTTCCTACTGCAGTAGCGGCACCCTACGTTCCTGTGCGGC
ACTACCAGAATGTTTATCATGGGATGGCCCCACCTGTAACTGTAAGAA
CGGCAGTTCCTGTGTTTTCAGCACCTCCTCATCCACCAACACCTGGCTG
CTGTGCTCCTCCAGGAATGGGGCCGGGAGTGCGAATGGCTCCAGCTGT
GAATATAAGATCTGTTGTGCCAGTATTTGCAGCACCTCCATCCGTGGTC

Table 25 Results of sequence data analysis for the Clone 12 [PNK 3(2)]

1. Initial Sequence length **:** 846
2. Vector sequence **:** 1-75, 808-846
3. Sequence length after Vector/Adaptor Screening : 694
4. Blastn Results:

Accession No.	Description	Q.Coverage	Evalue	Max. Id.
AM443687.2	*Vitis vinifera* contig VV78X152081.5	15%	3e-05	71%
BX819144.1	*Arabidopsis thaliana* full length cDNA complete sequence	8%	1e-04	81%
NM128398.3	*Arabidopsis thaliana* DRB2 (DSRNA binding protein 2)	8%	0.001	79%

5. Blastx Results:

Accession No.	Description	Evalue	Score (Bits)
CAN80689.1	Hypothetical protein (*Vitis vinifera*)	0.007	44.3
YP_001288224.1	Conserved proline rich protein (*Mycobacterium tuberculosis*)	0.14	40.1
ZP_00878206.1	COG0443: Molecular chaperone (*Mycobacterium tuberculosis*)	0.14	40.0

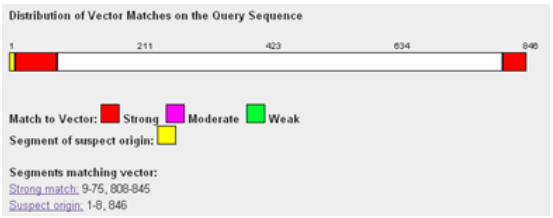
6. ORFs Available:

ORF Location	ORF length	Frame
119-577	459	+2

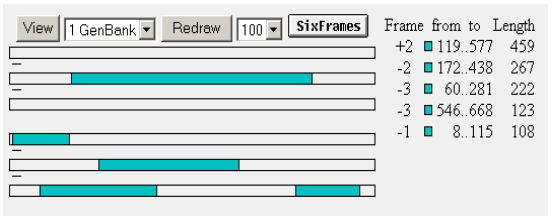
7. CDD available : No CDD

8. Interproscan scan results:

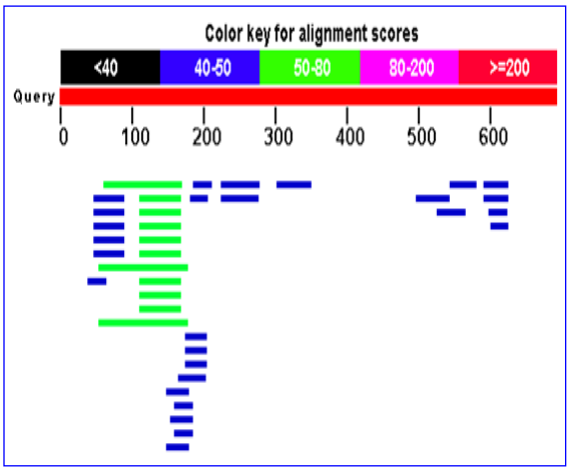
Database entry	Description
tmhmm	Transmembrane helices (3)



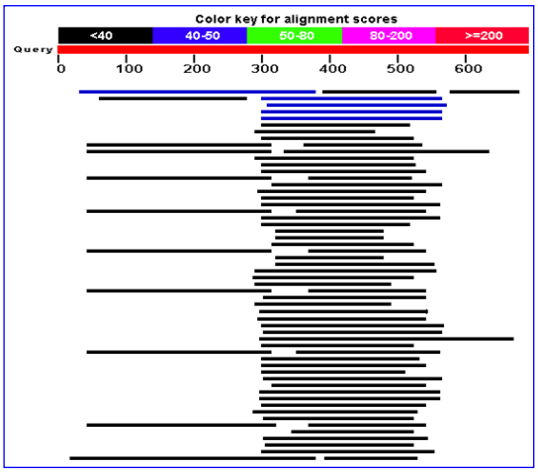
VecScreen output



Open reading frames in the cloned sequence



Blastn output



Blastx output

Interproscan result

Fig. 13 Results of sequence data analysis for the Clone 12 [PNK 3(2)]

AGAATTGAGGACCCCCCATCAGTTTTTGCTGCTCCTACAACACTGGCA
AGGTCATCCATAAAGATCGAGGAGAAGGGTAATCCCACTTCATCAACA
GCAGTTATAGCTCCACCTCCGCCCACACAGACTTCAGTGAAGACGGAT
GACATTACCAATTTGCCAGGGCCTTCCGTTTCCGCAGCTCCTCACTCTG
TCCAGCCATCGGTTATAGTAGAAGAAACTAGCATCCCTGCTCCAAAGA
ACATGCAAAACCCAGTCATACAGAACCTACAACAGCTAAAGATATGA
TGAGGAGGTTGTAAGTTGGAAAATGATTCTCTTTCTCCGTGCTGTTCCT
TAACGTATTTGTTTGGTTTTTCCGATGTATGTCGTAAATCATCCTGCGT
AGTTAGCTTATGGTAGTAC**3'**

Blastn results, indicated that 15 percent of the sequence showed 71 percent homology with *Vitis vinifera* contig VV78X152081.5 and 79 percent with *Arabidopsis thaliana* DRB2 (DSRNA binding protein 2). Blastx result showed homology of the sequence (score-44.3 bits) with Hypothetical protein from *Vitis vinifera*, conserved proline rich protein and molecular chaperone from *Mycobacterium tuberculosis*.

The sequence was found to have five ORFs with the longest one 459bp in length. The Interproscan result indicated the presence of three transmembrane helices but did not have any conserved domain.

Clone 13 [PNK 4(3)]

The sequence data obtained for the clone 13 was of 826bp size and vector screening indicated vector sequence from 1 to 49bp. The details of results obtained are presented in Table 26 and Fig. 14. After vector and adaptor screening the total sequence obtained for further analysis was 755bp as detailed below,

Table 26 Results of sequence data analysis for the Clone 13 [PNK 4(3)]

1. Initial Sequence length **:** 826
2. Vector sequence **:** 1-49
3. Sequence length after Vector/Adaptor Screening : 755
4. Blastn Results:

Accession No.	Description	Q.Coverage	Evalue	Max. Id.
AM433733.1	*Vitis vinifera*, whole genome sequence	31%	7e-13	75%
AC020891.8	*Homo sapiens* chromosome 15 clone RP11-108K3	5%	0.015	89%
AK246858.1	*Solanum lycopersicon* cDNA clone FC26BM11	3%	0.19	93%

5. Blastx Results:

Accession No.	Description	Evalue	Score (Bits)
CA061795.1	Unnamed protein product (*Vitis vinifera*)	2e-13	79.3
AAG51624.1	Putative phorbol ester/diacyl glycerol binding protein (*Arabidopsis thaliana*)	5e-06	55
NP_177903.4	PHD finger family protein (*Arabidopsis thaliana*)	5e-06	55.1

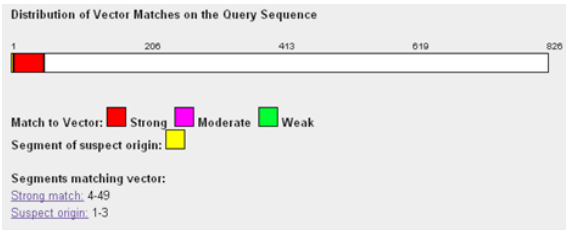
6. ORFs Available:

ORF Location	ORF length	Frame
329-616	288	+2

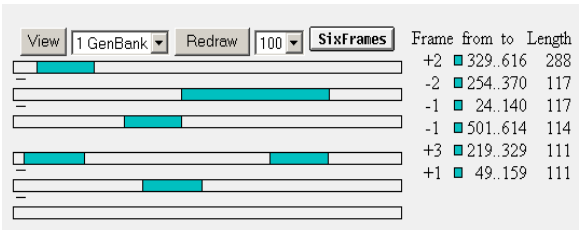
7. CDD available : No CDD

8. Interproscan scan results:

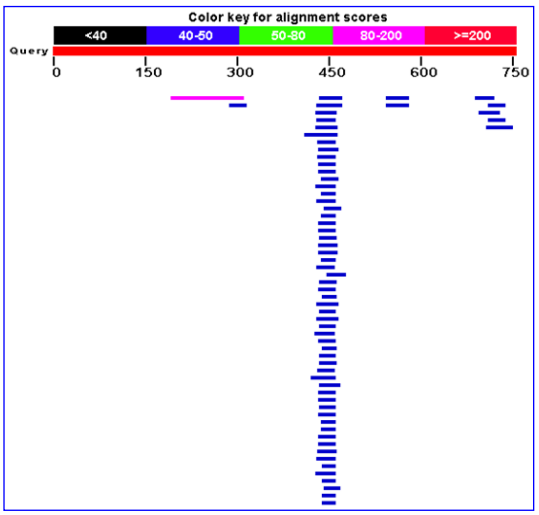
Database entry	Description
signal p	Signal peptide



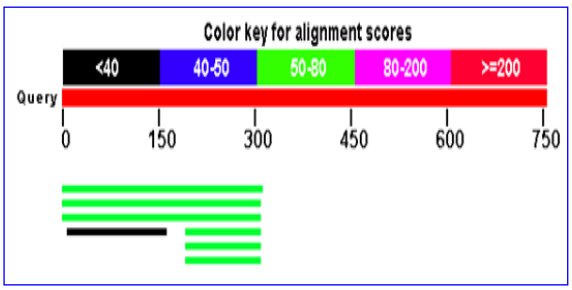
VecScreen output



Open reading frames in the cloned sequence



Blastn output



Blastx output

Interproscan result

Fig. 14 Results of sequence data analysis for the Clone 13 [PNK 4(3)]

Vector/Adaptor edited Sequence

5'ACAGGAGAAAAAGTGATAGAATTTTATCTTCTGGAAAGCAACTCCTT
CATGGGCTTACTTCTGTTCACTCTAAAAATGCTGGGGTGGACGTTGAA
AAGAAGTCCAAGGCTAGAAAGGACAAAGCTGGAATTTTGTGTCATGC
ATCTTATGCTGCTATAAAGCAATATGGTTCACTCCCGTCTACTTGAATT
CTTCACATTGAGACTTTGCAGAAAGAAATGATTATGACATCCGATCAG
GCTTCTAACCAAAATCAATGGTTACCTAAAGGTTTTGCGTATGTTCCAA
TAGGTTGCTTATCGAAGGAGAAGCAAGAACCTGATGGATAATGTCTCC
CCTTCAATGGGCGACTTGTTTGCACTTTCATCATGACTCCCAATTTTGC
TCAAGCATATTGTTTGCCGCAATGGTGGAGCTTCAATGCTGTGGTAAC
TGTTTGATTAAAAGCAAAAAAAAAAAAAACCAGATCAATTGGTCAGC
TGAGTTTTTGATGGAGCTCCTCAAGCTCCTGTTACTGCAGAGAAATGA
CAAGGCCAGGCTATCCTGCAGGATTTCTGGGGGTGGCTTTGCAAAATG
TTCATTGCCAGCGGAAAGAGAGACCAGGGTGGTGTCATGATTTTTAAC
AGAACGTTAGTTGAATAGCTATGTTTGGCCTGTCCAATGGAGATGAGA
TTTTACTCTGATACTTGTATATTAGGAAAGCTGGTGGCTTTCTTTTCTGT
AGAATCTGTAAATTAGACCGTTGTATTGTGTCG**3'**

Blastn result indicated that 31 percent of the sequence showed 75 percent homology with *Vitis vinifera*, whole genome sequence. Blastx result showed homology of the sequence (score-79.3 bits) with unnamed protein product from *Vitis vinifera*, and Putative phorbol ester/diacyl glycerol binding protein from *Arabidopsis thaliana*.

The sequence was found to have six ORFs with the longest one 258bp in length. The Interproscan result indicated the presence of one signal peptide and did not have any conserved domain.

Clone 14 [PNK 2(3)]

The sequence data obtained for the clone 14 was of 524bp size and vector screening indicated vector sequence from 1 to 51bp. The details of results obtained are presented in Table 27 and Fig. 15. After vector and adaptor screening the total sequence obtained for further analysis was 533bp as detailed below:-

Vector/Adaptor edited Sequence

5'CGTGGTCGCGGCCGAGGTACAAATTCAGCACCGACATTCCCCAATAT
ATGAAATTGTAACAGAAGCACCATCTTGCGTAGAGCAGGAGGATGAG
CTCAGTTCCACTGGAAAACCAGTCCACATCTGCTATATCCTTGAACATC
TCTGAATCTATACACTTCGTTGCAGAATTGCGCAACAAACTGATTGCG
AGAAAGGCTATCCACCCCTTGCGAAAGATAAGGAAAACATATCTAAA
AAGACTCCTATCAACCGATTCTTAAGCATATTGCAATCAACCACTCTAT
GCTAAACCCTGTCAGAATCAAAAAGAAGTTTCACCTTACAGTTCTGCA
GTCCACTAATACCCCACAACAGAAATTCAATAGTCCATATGATACTTG
TCATAAAAAGGAAGCCTAGTAAAACTCTTGCCCTCTTAAGCCAGTTCC
TAGATGAGATTCCATATGTTGATCATGATCAAACTTCATTGTCCGTCTG
TTACCACTAAAAAGTGCACACAAGACTTGGAGGTAACTGCGTGTACCT
GCCCG**3'**

Blastn results, indicated that 5 percent of the sequence showed 93 percent homology with *Homo sapiens* BAC clone RP11-517 F1, 100% with *Agaricus bisporus* partial mRNA for putative inorganic phosphate and 92% with *Vigna unguiculata* partial mRNA for putative ATP synthase. Blastx result showed homology of the sequence (score-34.7 bits) with Conserved hypothetical protein from *Trichomonas vaginalis*.

Table 27 Results of sequence data analysis for the Clone 14 [PNK 2(3)]

1. Initial Sequence length **:** 584
2. Vector sequence **:** 1-51
3. Sequence length after Vector/Adaptor Screening : 533
4. Blastn Results:

Accession No.	Description	Q.Coverage	Evalue	Max. Id.
AC104698.2	*Homo sapiens* BAC clone RP11-517 F1	5%	0.45	93%
AJ534357.1	*Agaricus bisporus* partial mRNA for putative inorganic phosphate	4%	0.45	100%
AM748447.1	*Vigna unguiculata* partial mRNA for putative ATP synthase	5%	5.5	92%

5. Blastx Results:

Accession No.	Description	Evalue	Score (Bits)
XP_001580995.1	Conserved hypothetical protein (*Trichomonas vaginalis*)	3.0	34.7

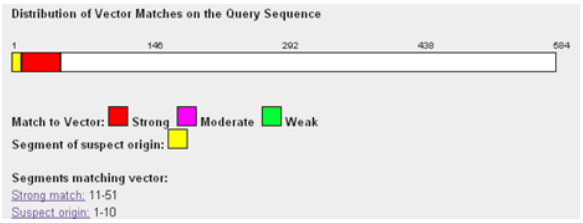
6. ORFs Available:

ORF Location	ORF length	Frame
2-181	180	-2

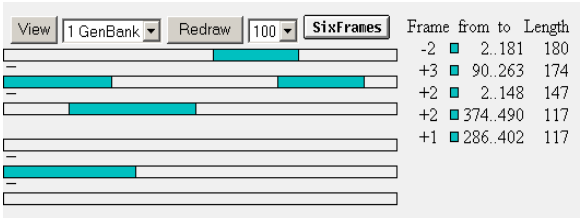
7. CDD available : No CDD

8. Interproscan scan results:

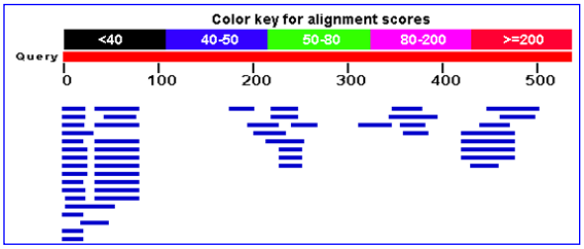
Database entry	Description
tmhmm	Transmembrane helices (2)



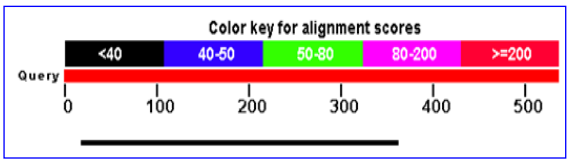
VecScreen output



Open reading frames in the cloned sequence



Blastn output



Blastx output

Interproscan result

Fig. 15 Results of sequence data analysis for the Clone 14 [PNK 2(3)]

The sequence was found to have five ORFs with the longest one 180bp in length. The Interproscan result indicated the presence of two transmembrane helices but did not have any conserved domain.

Clone 15 [PNK 7(2)]

The sequence data obtained for the clone 15 was of 711bp size and vector screening indicated vector sequence from 462 to 564bp. The details of results obtained are presented in Table 28 and Fig. 16. After vector and adaptor screening the total sequence obtained for further analysis was 585bp as detailed below,

Vector/Adaptor edited Sequence

5'TTGCAGTGGTCGCCGCGGGAGGGATGATATCCGAATCATACACTAA
GGTGTTTGATTCTGAGAGAGATGCCAAGAGAAGCCAACAGGATGATTC
GAGATCATATGGTAAACGAGATGATTCCGAGAGAGATGCTAAGAGGA
GCGGACAGGATGATTCAAGATCATACGGTAAGCGTGACAACTATCGTA
AATTCGATACCGAGAGCGCCAGGCGATAGGCCTTGTGAGAGAGACCA
ACGATATGCCTACTCAGTTATATTGTGCTCTCTGTTTAAGACTATCTGG
AATGTGCATTTAATATGCTTGATTGAATGTTGCATAATGTGGTTACAGT
GGTAGCCTTGGGGCAAGTAGCTGTAATCTAAACTTCGTAGTATTTCCT
GTAAGATGTGTTGCTACTCGAGCTTGATTTTCAAGTTTTCTAGTGCTAC
TACAATGTTCATTGGTCATACTGTTACCTGAGTAGCAGAATAAGATTA
TCCGATCACAAACCACACCAGNCNTAAGACCCAGAAAAGTTAAAAAA
AAACCNGAGGTGACAATGAATAGAAAAAAACCAAAAAGGCTANTCNA
CAATCAATAAAC**3'**

Blastn results indicated that 6 percent of the sequence showed 85 percent homology with *Vitis vinifera* contig VV78X216460.7 whole genome, 92% with Human DNA sequence from clone RP3-363L9 and 88% with *Lotus japonicus* genomic DNA chromosome 1 clone: Lit18M13. Blastx result showed homology

Table 28 Results of sequence data analysis for the Clone 15 [PNK 7(2)]

1. Initial Sequence length **:** 711
2. Vector sequence **:** 462-564
3. Sequence length after Vector/Adaptor Screening : 585
4. Blastn Results:

Accession No.	Description	Q.Coverage	Evalue	Max. Id.
AM432430.2	*Vitis vinifera* contig VV78X216460.7 whole genome	6%	1.7	85%
Z82205.1	Human DNA sequence from clone RP3-363L9	4%	1.7	92%
AP010411.1	*Lotus japonicus* genomic DNA chromosome 1 clone:Lit18M13	5%	1.7	88%

5. Blastx Results:

Accession No.	Description	Evalue	Score (Bits)
XP_001612750.1	Hypothetical protein(*Plasmodium vivax*)	0.002	45.8
XP_001134599.1	RNA binding region containing protein (*Dictyostelium*)	0.0494	40.0

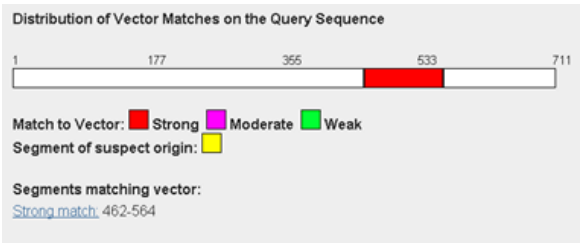
6. ORFs Available:

ORF Location	ORF length	Frame
64-339	273	+1

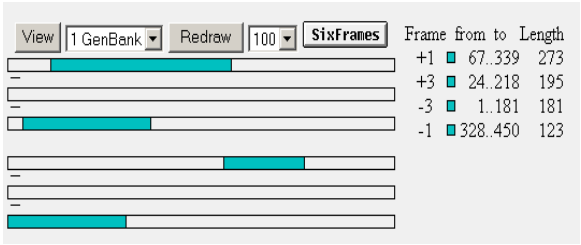
7. CDD available : No CDD

8. Interproscan scan results:

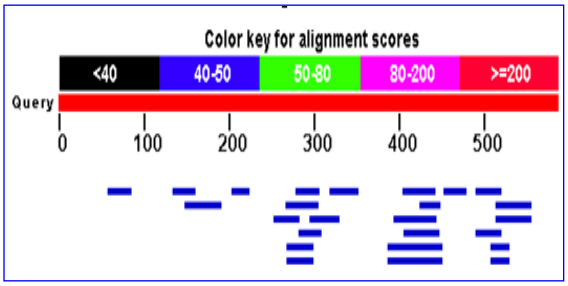
Database entry	Description
tmhmm	Transmembrane helices (1)



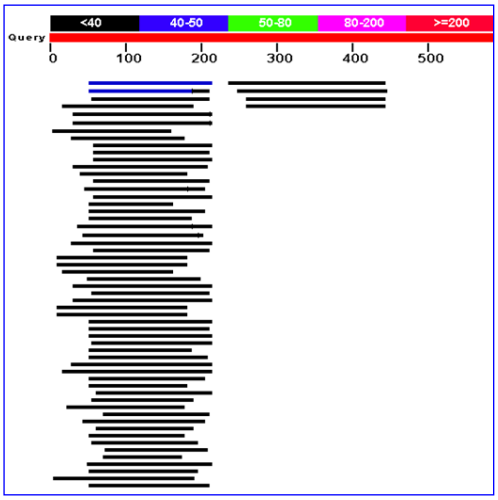
VecScreen output



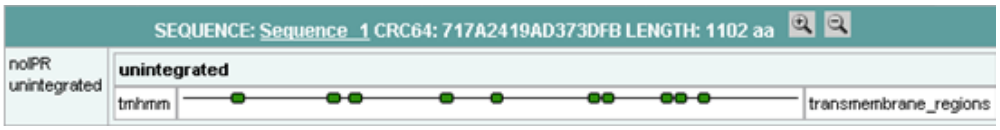
Open reading frames in the cloned sequence



Blastn output



Blastx output



Interproscan result

Fig. 16 Results of sequence data analysis for the Clone 15 [PNK 7(2)]

of the sequence (score-45.8 bits) with Hypothetical protein from *Plasmodium vivax* and RNA-binding region-containing protein (RNP-1) from *Dictyostelium discoideum*

The sequence was found to have four ORFs with the longest one 273bp in length. The Interproscan result indicated the presence of one transmembrane helix. The sequence did not have any conserved domain.

Clone 16 [PNK 3(3)]

The sequence data obtained for the clone 16 was of 831bp size and vector screening indicated vector sequence from 1 to 49bp and 352 to 831. The details of results obtained are presented in Table 29 and Fig. 17. After vector and adaptor screening the total sequence obtained for further analysis was 259bp as detailed below,

Vector/Adaptor edited Sequence

5'GTTGTGTTACTTAGGTTAAGCATTGATGGCTTCCACAAATAAAACAA
AAACAGCCGAGCAGCATATATTTTGGATATTGGAAATTTTGGAAGCTT
GCTGTGGCTTATATGGAATGGTTGTTTTTTTGTGCTTTATGTTCCAACG
GGGAGTCAGGTGTGGAGTGAAGGGCCATGTTAGCCATTGTAATGTTAA
TTTGTGAATATTGGCAAAAATGGATATTGTAATTTTTGGGATACATTCA
ACCATTTTTTGTGAATTG**3'**

Blastn result indicated that 13 percent of the sequence showed 88 percent homology with *Caenorahabditis brigasae* cosmid CB014E04, complete sequence. Blastx result showed homology of the sequence (score-33.1 bits) with Polyprotein from Human echovirus.

Table 29 Results of sequence data analysis for the Clone 16 [PNK 3(3)]

1. Initial Sequence length **:** 831
2. Vector sequence **:** 1-49, 352-831
3. Sequence length after Vector/Adaptor Screening : 259
4. Blastn Results:

Accession No.	Description	Q.Coverage	Evalue	Max. Id.
AC084444.1	*Caenorahabditis brigasae* cosmid CB014E04, complete sequence	13%	0.20	88%
AC008955.6	Homo sapiens Chromosome 5 clone CTD-2341N.17	11%	0.20	93%
AM471191.2	Vitis vinifera contig VV78X204695.7 whole genome	16%	0.72	82%

5. Blastx Results:

Accession No.	Description	Evalue	Score (Bits)
CAJ88938.1	Polyprotein (Human echovirus)	6.7	33.1

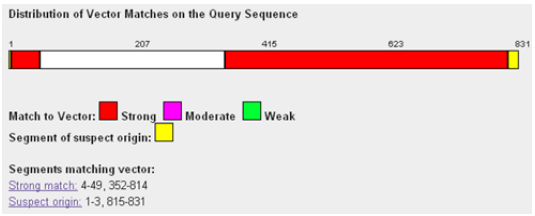
6. ORFs Available:

ORF Location	ORF length	Frame
16-246	231	-2

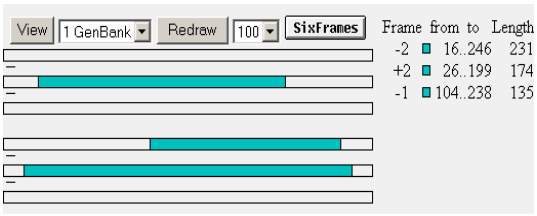
7. CDD available : No CDD

8. Interproscan scan results:

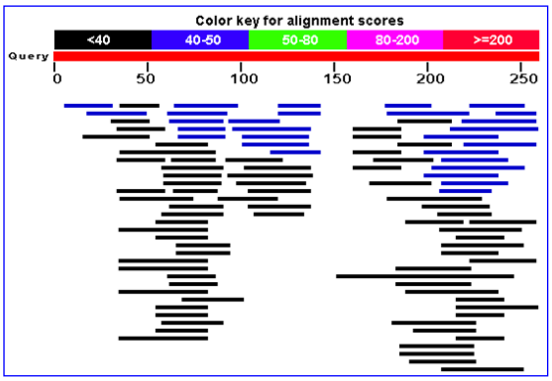
Database entry	Description
PS00596	High potential iron-sulphur protein
Signal p	Signal peptide
tmhmm	Transmembrane helices (5)



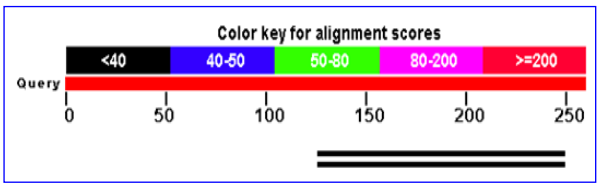
VecScreen output



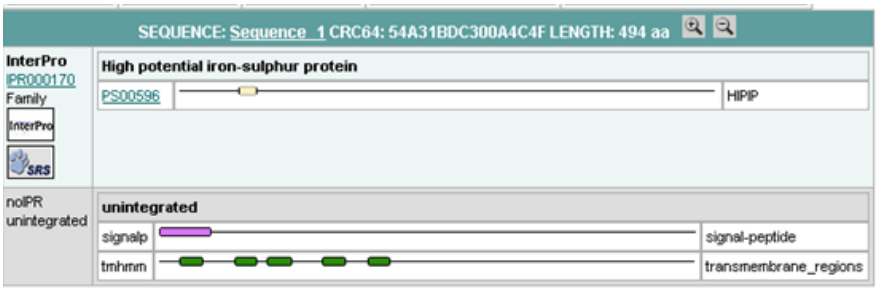
Open reading frames in the cloned sequence



Blastn output



Blastx output



Interproscan result

Fig. 17 Results of sequence data analysis for the Clone 16 [PNK 3(3)]

The sequence was found to have three ORFs with the longest one 231bp in length. The Interproscan result indicated the presence of one domain for high potential iron-sulphur protein, one signal peptide and five transmembrane helices but did not have any conserved domain.

Clone 17 [PNK 6(1)]

The sequence data obtained for the clone 17 was of 839bp size and vector screening indicated vector sequence from 1 to 49bp and 709 to 839. The details of results obtained are presented in Table 30 and Fig. 18. After vector and adaptor screening the total sequence obtained for further analysis was 616bp as detailed below,

Vector/Adaptor edited Sequence

5'ATGAGGGGAACTAGCGTAGTGTTGTTATAGGCGAACCTTTTATGACT
TTGATCTATCATATGGAGGACTGTATGATTTTTATCTGCTCTTGATCGA
GTTTGACATAAATGCGCTCTTTAATAACTTCGATTACCTAATATTACGT
CTTTTATTCCTTGGATACATTGTTTTGGCTTTTGATGTTCTGTATTATTG
CGTGCTGCACTGTTTCTATGGTTTGCTTCCAATGAAGAAGTGCAGGCC
ATTGCACCTCTGAAGATATTGTCTATTGCGGGTATTGTTAAAGGGAGG
ACTATATTCTTGTAATATTTAGTTGCACATTTTTTATCAAATAGTTAATT
TTAGTATATATTGCTTTACTTTTATTCCGATTGACATGGTATTTTGAAGT
GCTTGCTATTTTGCAGGATCAGAGGTTGTTTTTCATTGAAGCATCTGAA
ACTATTCTATGGATTATTTAACTCGAAGTTCATGTGTCTGAAAGCTGAT
TTTATCGTTATGCAATTTGCAGGCAAAAACTAGTGGTCGAATCCTCTTG
GAGCTGCTAAGACGAGCTGCCGCAGAAATTTCCTAATGGGTGCTAATT
TCCAGTTTGAGACGAGGTTGACAATTGAAG**3'**

Blastn result indicated that 6 percent of the sequence showed 85 percent homology with *Pan troglodytes* BAC clone CH251-354J15. Blastx result showed

Table 30 Results of sequence data analysis for the Clone 17 [PNK 6(1)]

1. Initial Sequence length **:** 839
2. Vector sequence **:** 1-49, 709-839
3. Sequence length after Vector/Adaptor Screening : 616
4. Blastn Results:

Accession No.	Description	Q.Coverage	Evalue	Max. Id.
AC195641.1	*Pan troglodytes* BAC clone CH251-354J15	6%	0.15	85%
AC112180.2	*Homo sapiens* Chromosome 5 clone RP11_130F17	8%	0.15	78%
AM482517.2	*Vitis vinifera* contig VV78X047649.9 whole genome sequence	6%	0.53	87%

5. Blastx Results:

Accession No.	Description	Evalue	Score (Bits)
NP_506926.1	Seven TM receptor family protein (str-9) *Caenorhabditis elegans*	0.40	38.1
YP_273232.1	Glycosyl transferase group 2 family protein (*Psuedomonas syrin*gae)	3.4	35.0
ABK57986.1	Cytochrome oxidase subunit 1(*Prorocentrum donghaiense*)	4.4	34.7

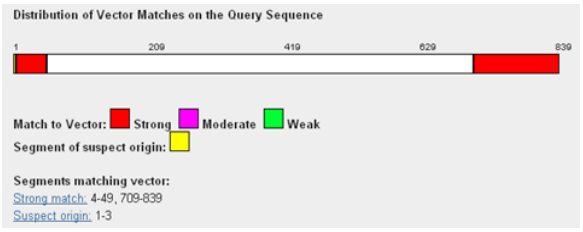
6. ORFs Available:

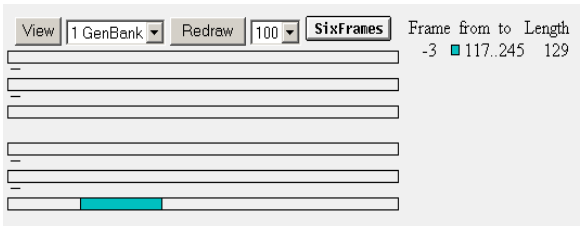
ORF Location	ORF length	Frame
117-245	129	-3

7. CDD available : No CDD

8. Interproscan scan results:

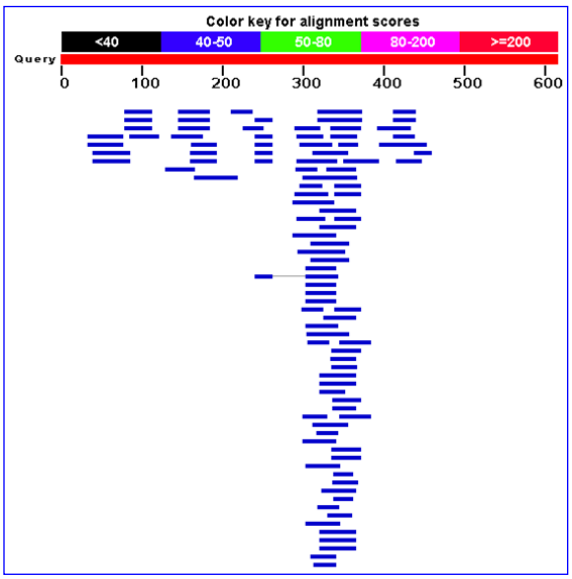
Database entry	Description
signal p	Signal peptide
tmhmm	Transmembrane helices (11)

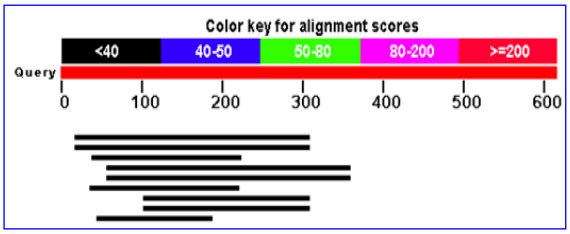




VecScreen output

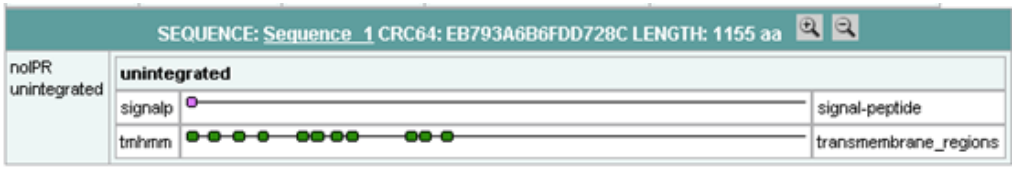
Open reading frames in the cloned sequence





Blastn output

Blastx output



Interproscan result

Fig. 18 Results of sequence data analysis for the Clone 17 [PNK 6(1)]

homology of the sequence (score-38.1 bits) with Seven TM receptor family protein (str-9) from *Caenorhabditis elegans* and Glycosyl transferase group 2 family protein from *Psuedomonas syrin*gae.

The sequence was found to have one ORF with the longest one 129bp in length. The Interproscan result indicated the presence of one signal peptide and eleven transmembrane helices but did not have any conserved domain.

Clone 18 [PNK 4(1)]

The sequence data obtained for the clone 18 was of 890bp size and vector screening indicated vector sequence from 1 to 73bp and 461 to 890. The details of results obtained are presented in Table 31 and Fig. 19. After vector and adaptor screening the total sequence obtained for further analysis was 348bp as detailed below,

Vector/Adaptor edited Sequence

5'ACTGCTCTATTATTCCAAATAAAGAAGTATTCACATTATACAAATTT
ATCCGAAAAGTTGTGATCAACTAAAACGAATAATGGACTTAACCTACC
TGCAAAAAACAAAGACTACCAAACCGCGCAAATGCCTTTGTCATGACA
GCTTAACTCATGCAAATTACGACTACTAAACAATAATTAGTAATAACT
TCACTTTGTTAAACTAACATAACGCAACAGATTTGGAGCAGAGCAGGC
ATCCTTCTTTCCATTAGCACCGCTAATTCCTTCCACATGCAGTCAAACA
GCTGCTTGCACAGGACCATCTGATCTTCCCCGCCCGCCGCGTCCTCGGC
CTCGGCCATGA**3'**

Blastn result indicated that 9 percent of the sequence showed 90 percent homology with Zebrafish DNA sequence from clone CH211-212C13. Blastx result showed homology of the sequence (score-33.1 bits) with Zinc finger in N recognin family protein from *Tetrahymena thermophila*.

Table 31 Results of sequence data analysis for the Clone 18 [PNK 4(1)]

1. Initial Sequence length **:** 890
2. Vector sequence **:** 1-73, 461-890
3. Sequence length after Vector/Adaptor Screening : 348
4. Blastn Results:

Accession No.	Description	Q.Coverage	Evalue	Max. Id.
BX548078.9	Zebrafish DNA sequence from clone CH211-212C13	9%	0.082	90%
CP000943.1	*Methylobacterium spp* 4-46, complete genome	9%	0.29	90%
EU012337.1	*Arabidopsis thaliana* clone fosmid UK3 chr5 fos37 sequence	11%	1.00	96%

5. Blastx Results:

Accession No.	Description	Evalue	Score (Bits)
XP_001008157.1	Zinc finger in N recognin family protein (*Tetrahymena thermophila*)	6.7	33.1

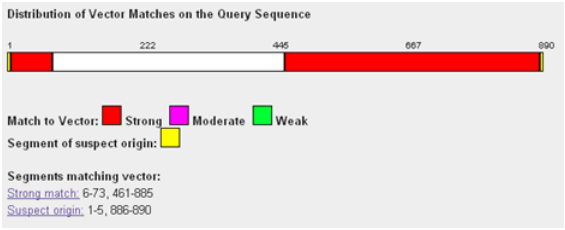
6. ORFs Available:

ORF Location	ORF length	Frame
200-346	147	-3

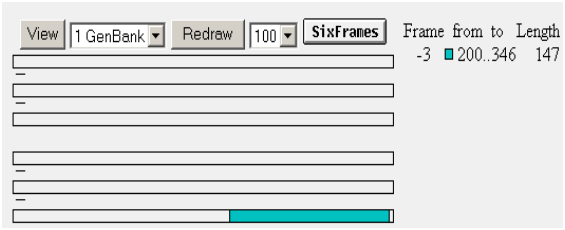
7. CDD available : No CDD

8. Interproscan scan results:

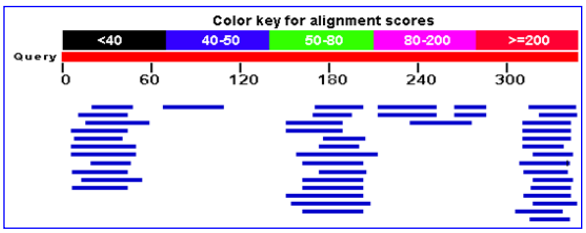
Database entry	Description
tmhmm	Transmembrane helices (2)



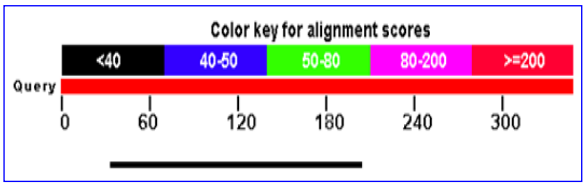
VecScreen output



Open reading frames in the cloned sequence



Blastn output



Blastx output

Interproscan result

Fig. 19 Results of sequence data analysis for the Clone 18 [PNK 4(1)]

The sequence was found to have one ORF with the longest one 147bp in length. The Interproscan result indicated the presence of two transmembrane helices but did not have any conserved domain.

Clone 19 [PNK 2(2)]

The sequence data obtained for the clone 19 was of 910bp size and vector screening indicated vector sequence from 1 to 81bp and 469 to 910. The details of results obtained are presented in Table 32 and Fig. 20. After vector and adaptor screening the total sequence obtained for further analysis was 387bp as detailed below,

Vector/Adaptor edited Sequence

5'CGTGGTCGCGGCCGAGGTCATGGCCGAGGCCGAGGACGCGGCGGGC
GGGGAAGATCAGCTGGTCCTGTGCAAGCAGCTGTTTGACTGCATGTGG
AAGGAATTAGTGGTGCTAATGGAAAGAAGGATGCCTGCTCTGCTCCAA
ATCTGTTGCGTTATGTTAGTTTAACAAAGTGAAGTTATTACTAATTATT
GTTTAGTAGTCGTAATTTGCATGAGTTAAGCTGTCATGACAAAGGCAT
TTGCGCGGTTTGGTAGTCTTTGTTTTTTGCAGGTAGGTTAAGTCCATTA
TTCGTTTTAGTTGATCACAACTTTTCGGATAAATTTGTATAATGTGAAT
ACTTCTTTATTTGGAATAATAGAGCAGTACCTGCCCGGGCGGCCGCTC
GA**3'**

Blastn result indicated that 11 percent of the sequence showed 86 percent homology with *Monosiga brevicollis* MX1 predicted protein MONBRDRAFT 33160 and 100 percent with Nidula niveotomentosa partial mRNA for glucose 6-phosphate -1-dehydrogenase. Blastx result showed homology of the sequence (score-33.1 bits) with Zinc finger in N recognin family protein from *Tetrahymena thermophila*.

Table 32 Results of sequence data analysis for the Clone 19 [PNK 2(2)]

1. Initial Sequence length **:** 910
2. Vector sequence **:** 1-81, 469-910
3. Sequence length after Vector/Adaptor Screening : 387
4. Blastn Results:

Accession No.	Description	Q.Coverage	Evalue	Max. Id.
XM001747283.1	*Monosiga brevicollis* MX1 predicted protein MONBRDRAFT 33160	11%	6e-04	86%
AM497810.1	Nidula niveotomentosa partial mRNA for glucose 6-phosphate -1-dehydrogenase	7%	0.002	100%
CT028662.1	Poplar cDNA sequences	6%	0.026	100%

5. Blastx Results:

Accession No.	Description	Evalue	Score (Bits)
XP_00108157.1	Zinc finger in N recognin family protein (*Tetrahymena thermophila*)	6.6	33.1

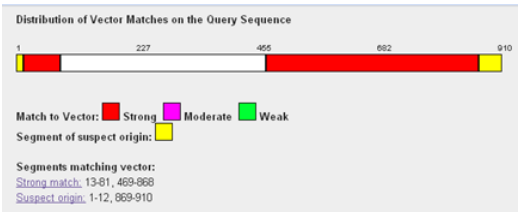
6. ORFs Available:

ORF Location	ORF length	Frame
20-166	147	+2

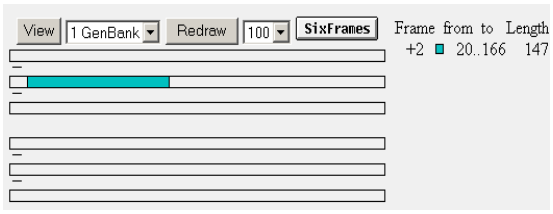
7. CDD available : No CDD

8. Interproscan scan results:

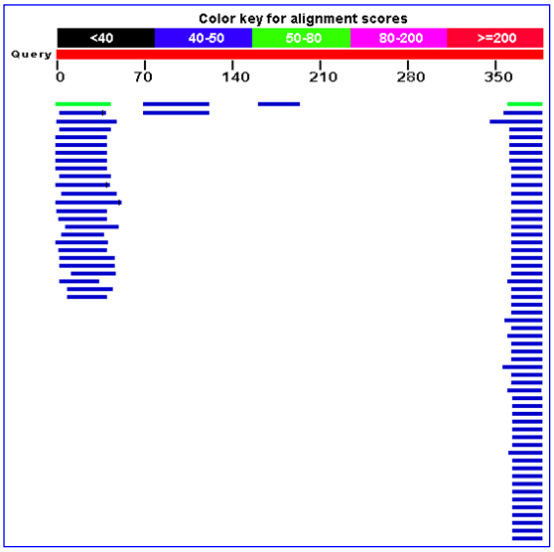
Database entry	Description
tmhmm	Transmembrane helices (5)



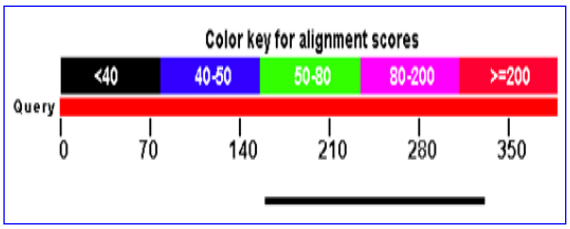
VecScreen output



Open reading frames in the cloned sequence



Blastn output



Blastx output

Interproscan result

Fig. 20 Results of sequence data analysis for the Clone 19 [PNK 2(2)]

The sequence was found to have one ORF with the longest one 147bp in length. The Interproscan result indicated the presence of five transmembrane helices but did not have any conserved domain.

Clone 20 [PNK 5(1)]

The sequence data obtained for the clone 20 was of 877bp size and vector screening indicated vector sequence from 1 to 74bp and 439 to 877. The details of results obtained are presented in Table 33 and Fig. 21. After vector and adaptor screening the total sequence obtained for further analysis was 323bp as detailed below,

Vector/Adaptor edited Sequence

5'ACTAATCTGTGTAGAAATCAGCAAACAGCATTTCCACAAAAACTTCC
TGAACATGACGATTTACAAACAAAATCAAACTCCTGCAACCAATCATC
TTCAAGAACAATCTGCTGTAAAATCCCAGTTCTGGTAAACCTAACCGC
TTGCCGTCCAAGGCCCCCCTGCTGATGGCGTTATGTTTCTTTGCTTTCT
GAACAATAAATTAGCATCGTGAACTGCTGATTCTGCATTCAGATGAAA
GCTTCTCTCACTGCAGTAGGTAACTTTAATTCAGGCTCAAATACACCA
AGTGGGCCAAAATAACTATCAACCATGTCCTTTGG**3'**

Blastn result indicated that 31 percent of the sequence showed 75 percent homology with *Vitis vinifera* contig VV78XO40591.14 whole genome shotgun sequence and 73 percent with *Castanea sativa* wound responsive protein 15.46 mRNA. Blastx result showed homology of the sequence (score-48.9 bits) with Hypothetical protein OSj_031132 from *Oryza sativa*, Putative Enoyl_CoA hydratase from *Oryza sativa* and Wound responsive protein 15.46 from *Castanea sativa*.

Table 33 Results of sequence data analysis for the Clone 20 [PNK 5(1)]

1. **Initial Sequence length** **:** 877
2. **Vector sequence** **:** 74, 439-877
3. Sequence length after Vector/Adaptor Screening : 323
4. Blastn Results:

Accession No.	Description	Q.Coverage	Evalue	Max. Id.
AM428196.2	*Vitis vinifera* contig VV78XO40591.14 whole genome shotgun sequence	31%	2e-08	75%
NM001071867.1	*Oryza sativa* (Japonica cultivar group) Os10q0552900	25%	0.002	74%
AY055745.1	*Castanea sativa* wound responsive protein 15.46 mRNA	27%	0.002	73%

5. Blastx Results:

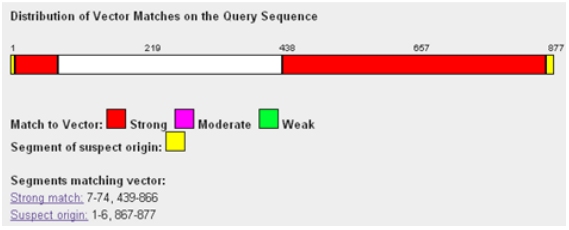
Accession No.	Description	Evalue	Score (Bits)
EAZ16923.1	Hypothetical protein OSj_031132 (*Oryza sativa*)	1e-04	48.9
ABB47969.1	Putative Enoyl_CoA hydratase (*Oryza sativa*)	1e-04	48.9
AAL17694.1	Wound responsive protein 15.46 (*Castanea sativa*)	0.004	43.9

6. ORFs Available:

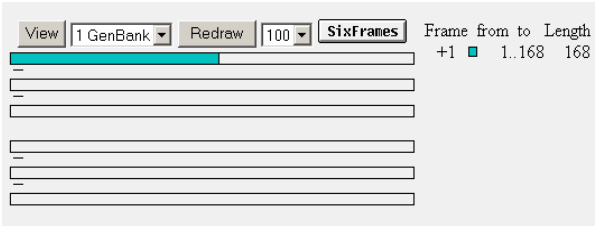
ORF Location	ORF length	Frame
1-168	168	+1

7. CDD available : No CDD

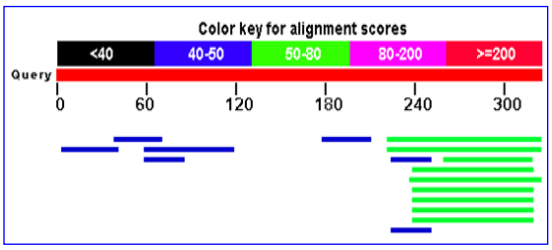
8. Interproscan scan results: No hits reported



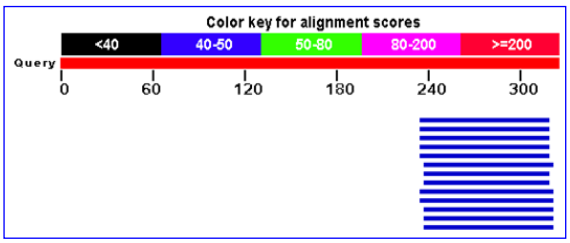
VecScreen output



Open reading frames in the cloned sequence



Blastn output



Blastx output

Fig. 21 Results of sequence data analysis for the Clone 20 [PNK 5(1)]

Table 34 Details of the clones derived from differentially expressed sequences from *P. nigrum* variety Kalluvally

Clone ID	Size (bp)	Homology	Score (bits)	E-value	% homology (Blastn)
PNK 11(2)	521	Heat shock proteins	39.3	0.12	84
PNK 1(1)	453	MYB transcription factor	48.5	2.00E-04	80
PNK 3(1)	785	RNS 3 endoribonuclease	70.0	1.00E-10	*
PNK 2(1)	492	Secretory carrier membrane protein family	281.0	1.00E-77	74
PNK 1(2)	320	Fatty acid desaturase	78.2	2.00E-13	76
PNK 10(2)	528	Putative gamma thionin	70.5	5.00E-11	78
PNK 4(2)	690	Peptidyl prolyl isomerase	70.1	2.00E-10	73
PNK 14(1)	645	Seed specific protein	76.3	2.00E-12	88
PNK 1(3)	292	Putative 3-dehydroquinate synthase	53.9	4.00E-06	80
PNK 5(2)	287	Unknown protein	155	9.00E-37	79
PNK 12(1)	683	NADH-ubiquinone oxido reductase family	211	3.00E-53	74
PNK 3(2)	694	Hypothetical protein	44.3	0.007	71
PNK 4(3)	755	Unnamed protein product	79.3	2.00E-13	75
PNK 2(3)	533	Conserved hypothetical protein	34.7	3.0	*
PNK 7(2)	585	Hypothetical protein	45.8	0.002	*
PNK 3(3)	259	Polyprotein	33.1	6.7	*
PNK 6(1)	616	Seven TM receptor family protein	38.1	0.40	*
PNK 4(1)	348	Zinc finger protein	33.1	6.7	*
PNK 2(2)	387	Zinc finger protein	33.1	6.6	*
PNK 5(1)	323	Hypothetical protein	48.9	1.00E-04	*

***** Percent homology not available for these sequences in Blastn results

Table 35 Accession numbers for the twenty sequences submitted in the database (www.ncbi.nlm.nih.gov/dbEST)

S.No.	Clone ID	Accession no.
1.	PNK 1(1)	FG618572
2.	PNK 2(1)	FG618574
3.	PNK 3(1)	FG618575
4.	PNK 4(1)	FG618576
5.	PNK 5(1)	FG618570
6.	PNK 6(1)	FG618571
7.	PNK 1(2)	FG618573
8.	PNK 2(2)	FG618578
9.	PNK 3(2)	FG618582
10.	PNK 4(2)	FG618583
11.	PNK 5(2)	FG618584
12.	PNK 1(3)	FG618577
13.	PNK 2(3)	FG618579
14.	PNK 3(3)	FG618580
15.	PNK 4(3)	FG618581
16.	PNK 7(2)	FG804679
17.	PNK 10(2)	FG804675
18.	PNK 11(2)	FG804676
19.	PNK 12(1)	FG804677
20.	PNK 14(1)	FG804678

The sequence was found to have one ORF with the longest one 168bp in length. No hits were reported for Interproscan result and no conserved domain was found in the sequence.

The details of all the twenty clones are given in Table 34.

4.12. Submission of sequences to dbEST

The sequences after sequence data analysis were submitted to dbEST. The accession number provided by the databank for all the twenty clones is given in Table 35 and is available in the website www.ncbi.nlm.nih.gov/dbEST.

Discussion

5. DISCUSSION

Black pepper is a spice of great economic importance, as it earns the highest amount of foreign exchange out of the total amount earned through spices. The export of the spice depends upon its production. There are many factors which influence the production among which soil moisture plays an important role. This is true in case of black pepper as 90 per cent of the area under black pepper is rainfed. The crop suffers acute soil moisture shortage from December to May leading to a decrease in the productivity. Hence, it becomes important to identify the genotypes which can not only tolerate water stress but also maintain its production levels. Kalluvally is one such hardy variety of black pepper which can tolerate water stress. Thankamani *et al*. (2003) has found that amongst forty four genotypes of black pepper, Kalluvally is one of the most drought resistant one. Therefore, in the present study, the var. Kalluvally was selected.

During water stress there is induction of genes that can be broadly classified into two groups: those that protect against the stress and those that regulate signal transduction and gene expression associated with other processes (Shinozaki *et al*., 2003). The first group of gene products is involved in cell protection including osmoprotectants, turnover proteins, membrane modifiers and detoxification protein (Shinozaki *et al*., 1996). The second group includes regulatory genes, such as signaling molecules and transcription factors, leading to expression of stress inducible genes and hence, plays a crucial role in the response to the stress (Seki *et al*., 2003; Singh *et al*., 2002).

The present study was aimed to identify the differentially expressed genes using Suppression Subtractive Hybridization (SSH) and thus developing Expressed Sequence Tags (ESTs) in black pepper var: Kalluvally with special reference to drought tolerance.

ESTs are short, complementary DNA sequences usually 200 to 800 nucleotides long that represent the expressed portion of genes. ESTs provide researchers with a quick and inexpensive route for discovering new genes for obtaining data on gene expression and regulation during developmental stages or stress conditions. Several works have been done earlier on EST development for identification of abiotic stress induced genes (Iturriaga *et al*., 2006; Jin *et al*., 2006; Quaggiotti *et al*., 2007; Diab *et al*., 2008), in secondary metabolism (Park *et al*., 2004), study on bud dormancy (Keilin *et al*., 2007), to derive genetic relationships (Guo *et al*., 2007) and in gene discovery and marker development (Luo *et al*., 2005).

5.1. Tolerance of black pepper var. Kalluvally to water stress

The pot culture experiments in the present study indicated variation in soil moisture from 3 to 30 per cent on dry weight basis during the stress period by withholding water upto 96 h. The potted plants showed wilting symptoms on third day after withholding water i.e. when the soil moisture was around 9 per cent. The wilted plants could be recovered by reirrigating them. However, the plants could not be revived if not irrigated within 48 h after wilting.

The growth stage of the plant, the distribution of roots in the soil, type of potting mixture used, the quantity of potting mixture etc. could influence the response of the plants to water stress. However, the observational trial conducted in the present study provided the preliminary data that was useful for carrying out the main project work.

The results obtained in this preliminary study were utilized for collecting leaf samples from water stressed plants.

5.2. Total RNA isolation

A critical step in the successful implementation of RNA based techniques for molecular biology and functional genomic experiments (e.g. SSH and generation of ESTs) is the routine isolation of high quality RNA (Iandolino *et al*., 2004).

RNA isolation presents more challenges than DNA isolation because of the susceptibility of RNA to degradation by ribonucleases (RNases). RNases are very stable and active enzymes which do not generally require cofactors to function. Since, only minute amounts of RNases are sufficient to destroy RNA; extreme caution is needed with 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 the 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. Also gloves were worn throughout the experiment, to avoid RNA degradation from RNase that is present in the working hands.

Another problem in isolating high quality RNA is the interference with aqueous metabolites like phenols and polysaccharides which accumulates in tissues and may be purified along with nucleic acids (Loomiz, 1974; Stokes *et al*., 1990). In the present study normal and 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 in the present study, to isolate high quality RNA, the procedure described by Chomczynski and Sacchi (1987) with some modifications was adopted.

Young, tender leaves from normal and 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 (Babu, 2000).

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 due to precipitation of insoluble lipids and thus was remedied by adding additional chloroform and reextracting.

Isopropanol was added to precipitate RNA which was then followed by ethanol (80%) wash. 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 both the samples when analyzed on agarose gel (Plate 2A). In addition, the samples were quantified spectrohotometrically and their quality and quantity was assessed (Table 12). The ratio for OD_{260}/OD_{280} for both samples was approximately 2.0 indicating pure RNA. The ratio for OD_{260}/OD_{230} was greater than 1.0 indicating pure RNA free from polysaccharides and polyphenols.

5.3. mRNA isolation

PolyA mRNA was isolated from pooled total RNA of normal and water stressed plants using PolyATtract mRNA isolation kit.

The PolyATtract mRNA isolation system utilizes the Magnesphere technology to eliminate the need for oligo (dT) cellulose and its associated problems. With total RNA as starting material, the PolyA mRNA fraction can be isolated free of other nucleic acid contamination.

In the present study, one reaction each was carried out for normal and water stressed total RNA. The volume of total RNA in both the samples was made to 500 µl with RNase free water. Then, biotinylated oligo (dT) primer was added that hybridized at high efficiency in solution to the 3' poly (A) region present in both the mRNA population. The hybrids were then captured and washed at high stringency using Streptavidin coupled to paramagnetic particles and a magnetic separation stand (Fig. 1). The mRNA was eluted from the solid phase by simple addition of ribonuclease free deionized water.

Both the mRNA samples were concentrated using sodium acetate and isopropanol. Finally, the pellet was dissolved in 4µl of RNase free water which contained approximately 2µg of mRNA required as a starting material for SSH.

The efficiency of the procedure was confirmed by checking the quality and quantity of mRNA thus obtained. For agarose gel separation, a homogenous and uniform smearing pattern is considered to be a criterion for good quality of mRNA generated which shows no contamination with either rRNAs or genomic DNA (Lin *et al*., 1999). Similar results were obtained for both the mRNA samples giving a smear pattern of greater than 0.5 kb in size (Plate 2B). Further, the quality and quantity was confirmed by spectrophotometric readings (Table 13). A

ratio of greater than 1.9 at OD_{260}/OD_{280} and 1.0 at OD_{260}/OD_{230} indicated pure, high quality mRNA.

5.4. Suppression Subtractive Hybridization (SSH)

SSH is a powerful technique that enables researchers to compare two populations of mRNA and obtain clones of genes that are expressed in one population but not in the other. The SSH method is based on a suppression Polymerase Chain Reaction (PCR) effect (Lukyanov *et al*., 1995; Siebert *et al*., 1995) and combines normalization and subtraction in a single procedure (Gurskaya *et al*., 1996). The normalization step equalizes the abundance of cDNA fragments within the target population and the subtraction step excludes sequences that are common to the compared population. This dramatically increases the probability of obtaining low abundance differentially expressed cDNA fragments and it simplifies analysis of the subtracted library (Diatchenko *et al*., 1996; Jin *et al*., 1997).

The PCR select cDNA subtraction kit used in the present study was provided by CLONTECH, USA. It provides almost all the reagents except for PCR amplification. The kit provided by CLONTECH has been efficiently utilized by several workers (deLos Reyes *et al*., 2003; Park *et al*., 2004; Way *et al*., 2005) for SSH.

In the present study, SSH reaction was performed with normal and water stressed cDNA such that the housekeeping genes and others common in both will get hybridized and subtracted out while, only the over expressed/ newly expressed ones will be retained.

5.4.1. Molecular basis of SSH

In order to identify the differentially expressed genes, usually the mRNAs are targeted. From the total RNA isolated, the mRNA was separated using PolyATtract that binds with the Poly (A) tail of mRNA.

The reagents provided in the kit permits synthesis of cDNA which was later utilized for subtractive hybridization. The cDNA, so synthesized from the two different samples act as the 'tester' and 'driver' which are then digested with *Rsa*I – a four base cutting restriction enzyme that yields, blunt ends. The tester cDNA (in the present study, samples from stressed plants) is divided into two portions and each ligated with different adaptors. Since, the adaptors do not contain a phosphate group, only one strand of each adapter attaches to the 5'ends of cDNA. The two adaptors have stretches of identical sequence to allow annealing of the PCR primer once the recessed ends have been filled in.

Two hybridizations are then performed and in the first hybridization, an excess of 'driver' is added to each 'tester' sample. The samples are then heat denatured and allowed to anneal. The common cDNA in the driver and tester samples would anneal, thus differentiating the differentially expressed ones. During the second hybridization, the two primary hybridization samples are mixed together without denaturing. The subtracted single stranded tester cDNAs reassociate and the driver cDNA further added at this step helps to enrich differentially expressed sequences.

The PCR reaction is performed with the nested primers to exponentially amplify the differentially expressed sequences. The second PCR helps to enrich the differentially expressed sequences by reducing the background PCR products. Such cDNAs can be cloned directly into a T/A cloning vector for producing the subtracted cDNA library.

5.4.2. Control Subtraction

The control reaction was performed to verify the efficiency of reagents provided in the kit and also to estimate the yield and size distribution of synthesized ds cDNA.

The PolyA mRNA from human skeletal muscle was provided in the kit for performing the control reaction. ϕX174/*Hae*III control DNA was additionally added as a test material to act as differentially expressed fragments. The PCR product of the control reaction when checked on agarose gel indicated the presence of ϕX174/*Hae*III fragments in the subtracted sample which was confirmed through comparing with the banding patterns of control subtracted cDNA provided in the kit.

The positive results obtained in the control subtraction clearly validated the reagents provided and procedure adopted for SSH.

5.4.3. Forward Experimental Subtraction

A forward experimental subtraction was carried out with two mRNA populations. One mRNA population, isolated from water stressed plants was expected to have differentially expressed genes and was used as the ‘tester’. The other population of mRNAs, isolated from normal plants containing transcripts common to both populations, constituted ‘driver’. After conversion into double stranded cDNAs, the tester and the driver cDNAs were digested with *Rsa*I enzyme and then the tester cDNA was hybridized with driver cDNAs. As a result, the transcripts present in both tester and driver cDNA hybridizes and gets subtracted out leaving only the differentially expressed ones which was present in the tester (stressed plants).

The resulting subtracted cDNA pool enriches exponentially during PCR amplification due to the presence of specific adaptors that prevents undesirable amplification.

The PCR product when checked on agarose gel indicated the presence of smear ranging from 0.2 to 1.5 kb for subtracted sample and it was greater than 2 kb for unsubtracted, clearly indicating the presence of rare differentially expressed transcripts in subtracted sample. The smear of subtracted sample was later eluted and cloned in pGEMT vector.

5.5. 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 (Plate 7A) was 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 ligated product containing differentially expressed cDNA fragments 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 while 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 (Plate 7B).

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 (Plate 10) along with T7 and SP6 regions of the vector.

5.6. Sequencing of cDNA clones

The cDNA sequences that were obtained by single pass sequencing were referred to as Expressed Sequence Tags (ESTs).

5.7. *In Silico* analysis of the cDNA sequences

Clone 1 [PNK 11(2)]

When the sequence was subjected to Blastn and Blastx homology search, it revealed the identity present with heat shock proteins from different organisms. Initial 30 per cent of the sequence showed over 80 per cent homology with heat shock proteins in different plants like *Malus, Castanea* (Soto *et al*., 1999). Similar results were obtained from Blastx result.

The sequence had two ORFs with the longest one 180 bp in length. Functional aspects of the domains present in the sequence were discovered through 'Interproscan'. The result indicated the presence of heat shock protein domains for HSP-20 and HSP-17 and a small heat shock protein family.

Heat shock proteins are induced during water deficit (Borkird *et al*., 1991) and are involved in refolding of proteins, in order to regain their function or the prevention of protein aggregation (Vierling, 1991). Small HSPs are another type of proteins that are associated with plant desiccation tolerance (Hoekstra *et al*., 2001).

The sequence was found to have one transmembrane helix, the presence of which shows that it may be involved in transportation of some important molecules. The sequence did not have any conserved domain.

Clone 2 [PNK 1(1)]

The sequence had 80 per cent homology with *Glycine max* MYB transcription factor and, with a hypothetical protein of *Vitis* and several other sequences from different organism. MYB proteins are a super family of transcription factors that play regulatory roles in developmental processes and

defense responses in plants (Yanhui *et al.*, 2006). These factors function as transcription activators in the dehydration (Abe *et al*., 1997).

The sequence had four ORFs with longest one 246 bp in length. Four transmembrane helices were present indicating some role of the sequence in the transportation of molecules across the membrane. The sequence did not have any conserved domain.

Although, the sequence lacks any conserved domain, it is clear from the presence of ORFs that the sequence codes for some proteins. These proteins may be involved directly or indirectly in response to water stress. The function of the sequence can only be assigned after cloning full length gene.

Clone 3 [PNK 3(1)]

Blastn result showed homology (88%) with *Homo sapiens* clone and several other sequences from different organisms. But Blastx result indicated that the sequence had good homology (70 bits) with drought induced S like Ribonuclease protein from *Oryza* (Salekdeh *et al*., 2002).

Ribonucleases are group of enzymes that degrades RNA. Eneas *et al*. (2008) have found that RNase present in cotyledon hydrolyzes seed storage RNA during germination and seedling establishment which may have a protective role against stress during later part of establishment.

The Interproscan result showed presence of one domain for protein RNase T_2 – Ribonuclease. The sequence had four ORFs with longest one 429 bp in length.

Clone 4 [PNK 2(1)]

Blast n result showed good homology (75%) with *Lycopersicon* clone, 74 per cent with secretory carrier membrane protein of *Arabidopsis* and many other sequences from different organisms. Blastx result showed similar results.

Secretary carrier membrane proteins (SCAMP) constitute a family of putative membrane trafficking proteins (Fernandez *et al*., 2000). Similar results were obtained by earlier workers (Wang *et al*., 2005). One of their sequences showed homology with SCAMP which was induced during water stress in rice.

The sequence had three ORFs with 329 bp being the longest one. The sequence had one conserved domain for secretary carrier membrane protein family, one domain for cystein rich region and eight transmembrane helices.

Clone 5 [PNK1 (2)]

Blastn and Blastx result showed homology of the sequence with fatty acid desaturase gene and proteins respectively in various plants. Plants respond to water stress by modifying aspects of their lipid metabolism. Fatty acid desaturase is required for synthesis of monosaturated fatty acid and for normal distribution of mitochondria. During water stress membrane damage is a common feature and there occurs changes in lipid composition during desiccation (Irina *et al*., 2002). Therefore, this enzyme with others may be involved in protecting membrane by synthesizing fatty acids and maintaining stability of the membrane during water stress.

The sequence had three ORFs with longest one 252 bp in length and five transmembrane helices.

Clone 6 [PNK 10(2)]

Blastn and Blastx result showed (75% to 77%) homology with several gamma thionins in various plants but with low coverage area (35% to 39%). The Interproscan result also indicated the presence of conserved domain for gamma thionin proteins. The sequence had five ORFs with the longest one 246 bp in length and five transmembrane helices.

Gamma thionins also referred to as defensins are small cationic peptides that can inhibit digestive enzymes and acts against pathogens. Hyon *et al*. (2004) found that the accumulation of Pepper (*Capsicum annum*) defensin gene CADEF-1 occurred strongly in response to wounding, high salinity and drought stress. When plants are subjected to stress, they produce various defensive proteins for their defense.

Clone 7 [PNK 4(2)]

Blastn and Blastx result showed homology of the sequence with peptidylprolyl isomerase, FK 506 binding protein in *Arabidopsis, Picea* etc. The sequence had three ORFs with longest one 339 bp in length. The Interproscan result also showed the presence of domains for Peptidylprolyl isomerase, FK 506 binding protein, five transmembrane helices and one conserved domain for Tetratricopeptide repeat domain.

Peptidylprolyl *cis-trans* isomerase also called as rotamases or immunophilins is an enzyme that catalyzes the isomerization of proline residues within proteins. Expression of many FKBP genes is induced by different environmental stresses (Sharma *et al*., 2003).

Clone 8 [PNK 14(1)]

Blastn result showed homology (68% to 88%) of the sequence with *Populus* clone, *Populus* EST from severe drought stressed leaves and with *Brassica napus* seed specific protein. Blastx result also showed homology (60 to 80 bits) of the sequence with unknown protein of *Populus*, seed specific protein of *Brassica* and targeting protein of *Oryza*.

The sequence had five ORFs with the longest one 270 bp in length, indicating that all the ORFs present codes for some proteins. The Interproscan result indicated the presence of conserved domain for targeting protein (TPX_2) and seven transmembrane helices.

Targeting protein represents a conserved region approximately 60 residue long. TPX_2 is a kinesin like protein localized on centrosomes throughout the cell cycle. It is required for centrosome separation and maintenance of spindle bipolarity. Seed specific proteins are induced during water stress and are postulated to form channels that function in metabolite transport (Herman *et al.*, 1992).

Clone 9 [PNK 1(3)]

Blastn result showed homology (80%) of the sequence with *Medicago* clone mte-68q1, *Oryza sativa* clone and *Fagus* putative 3-dehydroquinate synthase mRNA. Similar results were obtained from Blastx. The sequence showed homology (50 to 56 bits) with hypothetical protein of *Oryza*, and putative 3-dehydroquinate synthase protein of *Fagus*.

The sequence had four ORFs with the longest one 158 bp in length, indicating that all the ORFs codes for some proteins. The Interproscan result showed that the sequence had one signal peptide and one transmembrane helix.

Hypothetical protein is a protein whose existence has been predicted, but for which there is no experimental evidence that it is expressed *in vivo.* Dehydroquinate synthase protein is involved in shikimate pathway for the biosynthesis of aromatic amino acids (Brown *et al*., 2003).

Clone 10 [PNK 5(2)]

Blastn result showed homology (76% to 79%) of the sequence with unknown mRNA of *Picea* clone, *Oryza officinalis* clone and *Gymnadenia* expressed DUF 149 protein like mRNA. Blastx result showed homology (145 to 155 bits) of the sequence with unknown protein of *Picea* and hypothetical protein of *Vitis*. The sequence had two ORFs with the longest one 150 bp in length. The Interproscan result indicated the presence of conserved domain for DUF 149, uncharacterized YbaB family and one domain for conserved hypothetical protein. DUF is a group of protein family whose function is unknown. Therefore, it's difficult to assign any function to the sequence as it did not show any homology with the known gene or protein sequence.

Clone 11 [PNK 12(1)]

Blastn result showed homology (74% to 75%) of the sequence with *Solanum* clone, *Populus* EST from mild drought stressed leaves and with *Arabidopsis* NADH-ubiquinone oxidoreductase family. Bastx result showed homology (199 to 213 bits) with unnamed protein product from *Vitis* and *Arabidopsis* NADH-ubiquinone oxidoreductase family protein.

The sequence had nine ORFs with the longest one 342 bp in length, indicating that each ORF codes for some proteins. Although there was no conserved domain found in the sequence, but the Interproscan result indicated presence of domain for NADH-ubiquinone oxidoreductase subunit B17.2 family and nine transmembrane helices.

NADH-ubiquinone oxidoreductase provides the input to the respiratory chain from the NAD linked dehydrogenases of the TCA cycle and is involved in the ATP synthesis. Kohler *et al*. (2003) found that there was abundant NADH-ubiquinone oxidoreductase protein present in water stressed roots of Poplar.

Clone 12 [PNK 3(2)]

Blastn result indicated that the sequence had homology (70% to 80%) with *Vitis vinifera* contig and DSRNA binding protein 2 of Arabidopsis. Blastx result showed homology (40 to 44 bits) with hypothetical protein from *Vitis*, conserved proline rich protein and molecular chaperone from *Mycobacterium.*

The sequence had five ORFs with the longest one 459 bp in length. The Interproscan result showed the presence of three transmembrane helices but did not have any conserved domain.

Since, the score of the sequence was too low with all the other sequences including conserved proline rich protein and molecular chaperon, from *Mycobacterium*, also there were no reports of any domains, it is therefore difficult to assign any function to the sequence. But it was found that proline rich protein is induced during water stress (Silvia *et al*., 2001).

Clone 13 [PNK 4(3)]

Blastn result indicated that the sequence had homology (75% to 93%) with *Vitis* whole genome sequence. Blastx result showed homology (55 to 79 bits) with unnamed protein product of *Vitis,* putative ester/ diacyl glycerol binding protein and PHD finger family protein from *Arabidopsis*. The sequence had six ORFs with the longest one 258 bp in length and one signal peptide, but did not have any

conserved domain. Due to the low identity of the sequence with known sequences, it is difficult to assign any role to it.

Clone 14 [PNK 2(3)]

Blastn result indicated identity (92% to 100%) with low coverage area (5%) of the sequence with *Homo sapiens* clone, *Agaricus* partial mRNA for putative inorganic phosphate and *Vigna* partial mRNA for putative ATP synthase. Blastx result showed homology (34 bits) with conserved hypothetical protein of *Trichomonas*. The sequence had five ORFs with the longest one 180 bp in length and two transmembrane helices with no conserved domain

Clone 15 [PNK 7(2)]

Blastn result indicated identity (80% to 90%) with low coverage area (5%) of the sequence with *Vitis vinifera* contig, Human DNA sequence and *Lotus* genomic DNA chromosome1. Blastx result indicated low homology (40 to 45 bits) of the sequence with hypothetical protein of plasmodium and RNA binding region containing protein from *Dictyostelium*. The sequence had four ORFs with the longest one 273 bp in length with the presence of one transmembrane helix and absence of any conserved domain. Due to low identity it is difficult to assign any role to the sequence.

Clone 16 [PNK 3(3)]

Blastn result showed homology (88%) with low coverage area (13%) of the sequence with *Caenorahabditis* cosmid, complete sequence. Blastx result indicated very low identity of the sequence with polyprotein from human echo virus (33.1 bits). The sequence had three ORFs with the longest one 231 bp in length. Interproscan result indicated the presence of one domain for high potential

iron-sulphur protein, one signal peptide and five transmembrane helices. The sequence did not have any conserved domain.

Clone 17 [PNK 6(1)]

Blastn result indicated homology (85%) with low coverage area (6%) of the sequence with *Pan troglodytes* BAC clone Blastx result indicated homology of the sequence (38.1 bits) with seven TM receptor family protein from *Caenorahabditis* and (35.0 bits) with Glycosyl transferase group 2 family protein from *Pseudomonas*. The sequence was found to have one ORF, 129 bp in length. As the sequence did not show any good homology with the known sequences, therefore it was difficult to assign any role to it.

Clone 18 [PNK 4(1)]

Blastn result indicated homology (90%) with low coverage area (9%) of the sequence with Zebrafish DNA sequence. Blastx result indicated low homology (33.1 bits) with Zinc finger in N recognin family protein from *Tetrahymena*.

The sequence had one ORF, 147 bp in length two transmembrane helices; with no conserved. Zinc finger protein acts as transcriptional repressors that down regulate the transactivation of other transcription factors and are strongly induced by dehydration, high salt, cold stresses and abscisic acid treatment (Hideki *et al*., 2004). As the sequence had low identity even with known gene and protein sequences therefore it is difficult to assign any function to the sequence.

Clone 19 [PNK 2(2)]

Blastn result indicated homology (86%) having low coverage area (11%) of the sequence with *Monosiga* MXI predicted protein. Blastx result indicated

homology (33.1 bits) with zinc finger protein from *Tetrahymena*. The sequence had one ORF, 147 bp in length, five transmembrane helices and without any conserved domain.

Clone 20 [PNK 5(1)]

Blastn result showed homology (75%) having low coverage area (31%) with *Vitis* contig, *Castanea* wound responsive protein mRNA (73%). Blastx result showed homology of the sequence with hypothetical protein from Oryza (48.9 bits), putative Enoyl-COA hydratase from *Oryza* and wound responsive protein from *Castanea*. The sequence had one ORF, 168 bp in length. No hits were reported for Interproscan result and no conserved domain was found.

In conclusion, the results of the present study showed that some of the ESTs with some literature support (Soto *et al*., 1999; Borkird *et al*., 1991; Abe *et al*., 1997; Salekdeh *et al*., 2002; Irina *et al*., 2002; Sharma *et al*., 2003; Kohler *et al*., 2003; Herman *et al*., 1992; Wang *et al*., 2005 and Hyon *et al*., 2004) are potentially involved in drought stress response. While the rest may be considered novel as they showed very low homology with the known sequences. Hence, it was difficult to assign any role to these ESTs at present. But the presence of ORFs in all the ESTs indicates that they code for some proteins that may be involved in providing tolerance to the plant.

5.8. Submission of sequences to dbEST

The sequences were submitted to EST data bank. dbEST provided by NCBI. dbEST is a descriptive catalog of ESTs which was created in 1992 to organize, store and provide access to the great mass of public EST data. There are ESTs for more than 300 organisms and the data can be easily accessed by any one from the website www.ncbi.nlm.nih.gov/dbEST at present there are 30 sequences deposited in the data of which 20 are from the present study.

Future work includes screening of the entire subtracted library so as to identify other genes induced during water stress, full length cloning of cDNAs and characterization and functional analysis of the ESTs that are identified in this study can lead to a more comprehensive understanding of stress tolerance to drought in black pepper. Further, this subtracted library generated in the present study can be used to provide genes for genetic improvement of other black pepper varieties which possess desirable characters but are susceptible to water stress.

Summary

6. SUMMARY

Drought is one of the adverse environmental conditions that limit the plant growth, thus affecting productions in many important crop plants. Water stress induces various physiological and biochemical changes including growth inhibition and to acquire stress tolerance, a number of genes are induced at transcriptional level. Black pepper being a rainfed, perennial spice crop is greatly affected by water stress conditions. The variety Kalluvally is found to be one of the six genotypes that can tolerate water stress at appreciable levels. As a fundamental base for downstream applications of this variety in molecular breeding, an attempt was made to identify the genes that are induced during water stress. This is the first report on the identification of genes that are differentially expressed during water stress conditions in black pepper. The work undertaken and the result obtained in this study are summarized below:

1. As a preliminary step, the soil moisture content of the potted Kalluvally plants was estimated. The soil moisture content varied from 3 to 30 percent on dry weight basis, when the stress period was given up to 96 h during the summer month.
2. The plants under water stress started to show wilting symptoms from the third day onwards when the soil moisture was around 9 percent. Such plants could revive back to their normal condition only when reirrigated within 48 h after induction of wilting.
3. The trizol method adopted could yield good quality RNA from normal and two day stressed plants and the quantity of recovered RNA from normal and stressed plants were 0.253 µg/µl and 0.252 µg/µl respectively. The ratio of OD_{260}/OD_{280} and OD_{260}/OD_{230} for both the samples was approximately 2.0 and greater than 1.0 indicating good quality RNA.
4. Good quality and quantity of mRNA was recovered from total RNA. The quantity of normal and stressed mRNA recovered was 0.017µg μl^{-1} and

0.021µg/µl respectively. The ratio of OD_{260}/OD_{280} and OD_{260}/OD_{230} was greater than 1.9 and 1.0 respectively indicating pure quality mRNA.

5. SSH was carried out using PCR select cDNA subtraction kit as per the manufacturer's guidelines. Control subtraction was first performed and the PCR product of the control subtraction on agarose gel indicated the presence of ϕX174/*Hae*III fragments in the subtracted sample similar to the banding patterns of control subtracted cDNA provided in the kit. Thus the results obtained were satisfactory for carrying out subtraction for the sample.
6. A forward experimental subtraction was carried out using normal mRNA (2 µg) as 'driver' and water stressed mRNA (2 µg) as 'tester'. After synthesis of ds cDNAs the tester and driver cDNAs were hybridized. This resulted in the formation of hybrids between the common ones present in both tester and driver population thus leaving only the differentially expressed ones. The PCR product on agarose gel indicated the presence of smear ranging from 0.2 to 1.5 kb for subtracted sample and greater than 2 kb for unsubtracted; clearly indicating the presence of differentially expressed transcripts in subtracted sample.
7. The cDNA fragments of subtracted sample were eluted and cloned into pGEMT vector and this ligated product was transformed with competent *E.coli* cells. A large number of blue and white colonies were obtained after overnight incubation confirming successful transformation.
8. The presence of insert in white colonies was confirmed through checking the plasmid DNA on agarose gel and PCR analysis.
9. The cloned inserts of twenty selected clones were sequenced using universal primers T7, SP6 and M13.
10. After vector screening and adaptor edition of the sequences, *in silico* analysis of all the twenty sequences using various bioinformatics tools such as Blastn, Blastx, CDD, Interproscan and ORF finder was carried out.
11. All the sequences were numbered as Clone 1 to Clone 20 according to their significance and role in response to water stress. Clone 1 showed

homology with heat shock proteins. Also, it had domains for HSP-17 and 20. These heat shock proteins are found to play an important role during water stress.

12. Clone 2 was found to be similar to MYB transcription factor which acts as transcription activators in the dehydration.
13. Clone 3 was similar with drought induced S like Ribonuclease protein. The sequence did not have any conserved domain.
14. Clone 4 showed good homology with secretory carrier membrane proteins that plays important role in protein trafficking during water stress. Also, it had conserved domain for the same protein.
15. Clone 5 was somewhat similar to fatty acid desaturase enzyme. This protein is found to play role in protection during membrane damage by synthesizing fatty acid during water stress.
16. Clone 6 showed homology with gamma thionins or defensins. These proteins are also found to be induced during water stress. The sequence also had conserved domain for the same protein.
17. Clone 7 showed similarity with peptidylprolyl isomerase. This protein helps in the isomerization of proline residues within the proteins. The sequence had conserved domain for tetratricopeptide repeat domain.
18. Clone 8 showed homology with *Populus* EST from severe drought stressed leaves and targeting protein. The sequence also had a conserved domain for targeting protein.
19. Clone 9 showed identity with different known sequences, 3-dehydroquinate synthase and hypothetical protein. The sequence had one signal peptide and transmembrane helices indicating its role in signal transduction and transportation of molecules across the membrane.
20. Clone 10 and Clone 11 showed homology with DUF protein and NADH ubiquinone oxidoreductase respectively. The role of DUF is unknown while the latter is involved in ATP synthesis and is found to occur in abundance during water stress.

21. Clone 12 to 20 showed very low identity with the known sequences and therefore it was difficult to assign any role to these. But the presence of ORFs in all the sequences signifies that they code for some proteins.
22. The sequences after sequence data analysis were submitted to EST databank referred to as ‘dbEST’.

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Annexures

ANNEXURE I

Composition of Buffers and Dyes used for gel electrophoresis

1. **MOPS Buffer (3-(N-Morpholino)-propanesulfonic acid) 10X (for 1l)**

   0.1M MOPS (pH 7.0)
   80mM Sodium acetate
   5mM EDTA (pH 8.0)

2. **TAE Buffer 50X (for 1l)**

   242g Tris base
   57.1ml glacial acetic acid
   100ml 0.5M EDTA (pH 8.0)

3. **Loading Dye (6X)**

   0.25% bromophenol blue
   0.25% xylene cyanol
   30% glycerol in water

ANNEXURE II

Regents provided in the kit for mRNA isolation

50µl Biotinylated Oligo(dT) Probe (50pmol/µl)
2.8ml 20X SSC Solution (2 × 1.4ml)
9ml Streptavidin MagneSphere® Paramagnetic Particles (15 × 0.6ml)
50ml Nuclease-Free Water (2 × 25ml)
1 each MagneSphere® Magnetic Separation Stand for 1.5ml
microcentrifuge Tubes

The reagents were stored at 4°C. MagneSphere® Magnetic Separation Stand was stored at room temperature.

ANNEXURE III

Reagents provided in the kit for Control and Experimental SSH

1. First-strand synthesis

7µl AMV Reverse Transcriptase (20 units/µl)
10µl cDNA Synthesis Primer (10 µM)
200µl 5X First-Strand Buffer
250mM Tris-HCl (pH 8.5)
40mM MgCl2
150mM KCl
5mM Dithiothreitol

2. Second-strand synthesis

28µl 20X Second-Strand Enzyme Cocktail
DNA polymerase I, 6 units/µl
RNase H, 0.25 units/µl
E. coli DNA ligase, 1.2 units/µl
200µl 5X Second-Strand Buffer
500mM KCl
50mM Ammonium sulfate
25mM MgCl2
0.75mM β-NAD
100mM Tris-HCl (pH 7.5)
0.25mg/ml BSA
14µl T4 DNA Polymerase (3 units/µl)

3. *Rsa*I digestion

300µl 10X Rsa I Restriction Buffer
100mM Bis Tris Propane-HCl (pH 7.0)
100mM MgCl2
1mM DTT
12µl Rsa I (10 units/µl)

4. Adaptor ligation

21µl T4 DNA Ligase (400 units/µl; contains 3 mM ATP)
200µl 5X DNA Ligation Buffer
250mM Tris-HCl (pH 7.8)
50mM MgCl2
10mM DTT
0.25mg/ml BSA
30µl Adaptor 1 (10 µM)
30µl Adaptor 2R (10 µM)

5. Hybridization

200µl 4X Hybridization Buffer
1.4ml Dilution buffer (pH 8.3)
20mM HEPES (pH 6.6)
20mM NaCl
0.2mM EDTA (pH 8.0)

6. PCR amplification

50µl PCR Primer 1 (10 µM)
100µl Nested PCR primer 1 (10 µM)

100µl Nested PCR primer 2R (10 µM)
10µl PCR Control Subtracted cDNA

7. Control reagents

5µl Control Poly A+ RNA (1 µg/µl; from human skeletal
muscle)
5µl Control DNA (3 ng/µl)
(Hae III-digested bacteriophage φX174 DNA)
50µl G3PDH 5' Primer (10 µM)*
50µl G3PDH 3' Primer (10 µM)*

* These primers will amplify human, mouse, and rat species G3PDH genes.

8. General reagents

20µl dNTP Mix (10 mM each dATP, dCTP, dGTP, dTTP)
100µl 20X EDTA/Glycogen Mix (0.2 M EDTA; 1 mg/ml glycogen)
400µl NH4OAc (4 M)
1ml sterile H2O

The following reagents are required but not provided in the kit.

1. *Hae* III digest of bacteriophage fX174
2. 80 & 96 percent Ethanol
3. Phenol:chloroform:isoamyl alcohol (25:24:1)
4. Chloroform:isoamyl alcohol (24:1)
5. 50X Advantage™ 2 Polymerase Mix

ANNEXURE IV

Chemical composition of reagents used for Cloning and Transformation

1. **Composition of SOB medium (pH 7.0)**

20g Tryptone
5g Yeast extraxt
2ml NaCl stock solution (5M)
830µl KCl stock solution (3M)
15 g Agar
1 l Disatilled water

2. **Plasmid isolation solutions**

a) **Solution I (Resuspension buffer)**

50 mM glucose
25 mM Tris
10 mM EDTA

b) **Solution II (Lysis buffer)**

2N NaOH
1 per cent SDS

c) **Solution III (Neutralization buffer)**

5M Potassium acetate (60 ml)
Glacial acetic acid (11.5 ml)
Distilled water (28.5 ml)

ANNEXURE V

Details of Sequence Data obtained from sequencing agencies

Clone 1 [PNK 11(2)]

TTTCGAGCGGCGCCCGGGGCGGTAAGTGGCCCGGGTGGAGAGGAGCA
GCGGCAGGTTCTTGAGGAGGTTTAGGTTGCCGGAGAATGCTAAGGTGG
AGGAGGTGAAGGCGGGGGTGGAGAACGGGGTGCTCACTGTGACTGTG
CCCAAGGTGGAGGTGAAGAAGTCTGAGGTTAAGGCCATTGAAATCTCT
GGAAACTAAGAGTTTCGAAAAAGATAATATTGTGTCAAGGCCATGTGT
GTCACCACGCTTTTCGTGAATGTGTTTGGTCTCGGTTGGAGCTTGTTTT
CTTTATCTTCGTCTTGTAATAAGGTGATGAATGGGTCTATGCGAGGATC
GGTGTTGATGTCAGTTAGTATGAATGGGACATTCAGTTTAAAACTGTT
ATAATTTGATGTCTGATTTTAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAGCTTGTACCTCGGCCGCAACCACGCTAATCACTAGTGCGGCCGC
CTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGC
TTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGGTAATCTGGTCAT
AGCCTGTTCCTGGTGTAAAAATTTGTTAAACCGCTCAACAAATTCCAA
CACAACCATAACGAAGCCGGAAAACCATAAAGGGTTAAAGCCCTGGG
GTGCCCTAATGAATGAACAAACCCA

Clone 2 [PNK1 (1)]

TCTCAGCTATGCATCCACGCGTTGGGAGCTCTCCCATATGGTCGACCTG
CAGGCGGCCGCACTAGTGATTAGCGTGGTCGCGGCCGAGGTACTTCAG
ATGCAGGGGCCTGCTCAGAAGTCCGCTTCTTCAAGGAGGCTCCAACTG
CTTCTGTATCATCAGTAGCAAAATCAAGCCTCATAAGTGCATATTCAT
ACAAAGAACTTGTTGCTCCCGCCAATGATAAAAATGGGCAGTCAAAA
GTTGAAGCAGGAACAAATGAACAGAAGATAGGTTTACTGAATCAACC
GCGGTCAACAGAAGCTGGGGCGACTGGCCTTTCAAGCGCTTTTTACCA
GAGAAGTTCTGCCACAAAGCTTCTCTCCACCTCATGACGATGCAAGAA
GCAAGGTGCCCACCAAGAGCATAGCAGAGGACGAGCATACGAGGCAA
GCTTCAAGGCAAGTTGAAGGAGTGAGCAAGCTCAAGCTGGATTTGGA
GCTTGGCTGCAGGATGCAACCATCTCCCTCTCATGCGGTTCCTGCCCGA

Clone 3 [PNK 3(1)]

AACTCAGCTATGCATCCACGCGTTGGGAGCTCTCCCATATGGTCGACC
TGCAGGCGGCCGCACTAGTGATTAGCGTGGTCGCGGCCGAGGTACAA
ACCATTATTCGCTTCATATCATGAAACAAATAAAGAGGAAATTTATTC
TTCTTATTCTAATTCCACCTTACTAACATTATTAGCTAAGGCACCAAAT
ATGGTCATATCCATGATCACACTTGTGACATAATATTAAAGCTATCCAT
TTGATGGCTAGCATCGATCACTGGGCAACCACCATTTCGATGGGGTTG
TCCTCCACCTCCAAAGTGTCGGTGCTATTTGGCAGCACATCTAGCTTGA
CAGACGCCGGACAGGTCGAGCTCCGCGGCGTCGGGCAGGTGATGAAT
TTGGTGGCGGTGATGTCCACACACAACCTCACATTCATGATCCTCTCCA
ACATGACCGAGCCGCAGTGAAGCACCGCCGTGTTCACTCCCAATCCTT
GGTTGATAGCGTTCACCACATCGACGGTCAGGTATTTGTTGGTGTGAG
ATGGCCTGATACCTCTTCTAGAGAGGAGATAGAGGAGGTTCGCCTTAT
CGCGAAGTGCAAGTGCTCTGGAGAAGTAGTCGCTCACCGTCAACCAGG
TGCAGGGGCCGTTGTCTTTCCAAGTCTGCTCCCACGCCACCATGTTGTT
GTCGTTGAGCATGCAGCCGAGCTCGGCCAATTCTGGTTGAGACTTCTA
TGATATTTTGGAGAGCCTTGGGATCGAAGGAATTTAGAGCATTGATGC
TGCGATCAAATTCCTTGCCAAAACCGGACGACAAGTCGTCTTTTTGGT
CCAGGTGGAGGGAGCAGCATCGCCTTGCTGGGGGCAGA

Clone 4 [PNK 2(1)]

CACTCAAGCTATGCATCCAACGCGTTGGGAGCTCTCCCATATGGTCGA
CCTGCAGGCGGCCGCACTAGTGATTAGCGGCCGCCCGGGCAGGTAAC
ACGAGCTGGTGTGCCAGTCAATGACAAGAATTGGCCCCCTTCTTTCCT
GTTATCCATCATGACATAACCAATGAGATACCAGCCCATGTTCAGAGG
TTGCAGTATTTGGCATTTGCAAGTTGGTTGGGTATTGTCTTTTGCCTTG
TGTTTAACATTGTTGCGATCATTGTCTATTGGATAAGAGGAGGAGGTG
TTAGGATCTTTCTCCTTGCAGTTATCTATGCATTATCTGGATGTCCTCTT
TCATATGTTCTGTGGTATAGGCCTCTTTATCGGGCTATGAGGACTGACA
GTGCTCTGAAGTTCAGCTGGTTTTTCCTGTTTTATCTGCTTCACATAGG
ATTTTGCATTTTTGCAACAATCGCCCCTCCAGTAGTTTTTCGTGGGAAA
TCATTGACAGGCATACTTGCTGCAATTGATGTGATCTCAGACAGCACG
TTGGCTGGGATATTTTACTTCGTTGGGGCTGGATTGTTTTGTCTTGAAG
TACCTCGGCCGCGACCACGCTAATCCCGCGGCCATGGCGGCCGGGAGC
ATGCGACGTCGGGCCCAATTCGCCCTATAGTGAGTCGTATTACATTCA
CTGGCCGTCGTTTTACACGTCGTGACTGGGAAAACCCTGCGTTACCCA
ACTTAATCGCCTTGCAGCACATCCCCTTTCGCCAGCTGCGTAATAGCG
AAAAGCCCGCACCGATCGCCCTTCCCACAGTTGCGCAGCCTGAATGGC
GAATGAAGCGCCTGTAGCGGCGCATTA

Clone 5 [PNK 1(2)]

ACGATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCCGCGGGAT
TTTCGAGCGGCCGCCCGGGCAGGTACTACCGTTTCGATGGCACCCCTG
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CCCGACGAGGGGTCGCCGGAGAAGGGCGTCTACTGGTACGGACACAA
GTTTTGAGACAAGATCTTGCGTCGGGAATCGATGGTTGGGAAGTGGGT
GGTCGATTATCTTGCCGTTCTTGTCTTTGTTATAATTTAGTATTGTTATG
GTTATTTAGTCCGTCATTGCCGGCGACATGGTCGTAATTTAATTTAATT
GAGGGATACTATTGTTGGAAGCCGCCCATCTTTTTTGGGTTTATAGTCG
GTATCTGTACCTCGGCCGCGACCACGCTAATCACTAGTGCGGCCGCCT
GCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTT
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GCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATA
CGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAG
CTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGA
AACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGA
GGCGGTTTGCGTATTGGGCGCTCTTCGCTTCTCGCTCACTGACTCGCTG
CGCTCGTCGTCGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTA
ATACGTTATCCACAGAATCAGGGATAACGCAGAAGAAACATGTGAGC
CAAAG

Clone 6 [PNK 10(2)]

TTGACGTGGTCGCCCGCGGGAGGTCAGGTTGCCGGCGATCTTTCTTGG
GTGGTGAGAGGTAGTATTCTTGAGCCATGATCTCCAAATAGAGCTGAT
CCTTGAGCTTCCTAGGATGGTGAAAGCTCGCAGAGACCCAGAGAGGC
AGAAGAAGAAGAAGAAGATCAGACAGCTTATTGCCTAGAATGGCTAT
TCTTACTCAAGCCAAAAGAGTTTCAATTTCTCTCCCACAGCTTAGCATC
GTGCTCAGCACGTCTTCACACAAAAGCAGCGACGGCGGAAACCACGG
CAGTGCCCACCGGGGAAGCCCTCTGTCTGGCAGACACTGGCGCAGTTG
CTGTCCCTCACACACGGACCTTTGAACCTGTGGCTCTGTGACTCGCAG
ATCCTTGCCTCGGCAAAGCTCGGCCCAACATTTCCAAGTGGACATGAT
CAGCAAAACCATGATAAAAATGGCTGGGAACAGACGTGATCACCATT
AGTCCTGAAAGCTAGAATCAGAAGCCGAGGATTTGAAGACCTGCCCG
GGGG

Clone 7 [PNK 4(2)]

AAGAACTTTTGTATACGACTCCTATAGGGCGAATTGGGCTCGACGTCG
CATAAGCTCCCGGCCGCCATGGCCGCGGGATTAGCGTGGTCGCGGCCG
AGGTACTTTCAATCAGGAGCAGAAAAAGATGGGTAACTTTTTCCGAGT
GACCTGCATTTTGAATAATGCAGCTTGCAAACTGAAACTGAATGAATT
CCGGGAAGCTGCGAAGCTCTGTGCAA

AGGCTTTGCAATTAGAGCCTGACAACGTGAAGGCTCTCTACCGTAGAG
CTCAGTCTTACATTGAGACAAGTGATCTGGACTTGGCAGAAATAGACT
TATTGGCTGCAAAGGAAATTGAACCAGACAATGAGCCATTAAATCAAC
TACTGGAGAGAGTGAAAAAACTAAAGACACGAGCCAGTAAAATGGAT
ATGAAATTTTACGCAAACATGCTGGCAAAGGTTAACATTGCACACTGT
AAATAACTCAGAAATATCTTTATACTGCTCATTTTTGTTGAGTTAAAAC
AGATCTGATTAGGGTTGGAAAAATAAGCATTGAAGAGATTGTTTGCCT
CAGCGGCTCTGTATCATTCTATAGGCGAGAGCTTTTAATTTCTTTTGTG
ATTAGTGATAAAAGAAGGGACGAGATAAATAATTTGGTGGGCATATG
TTTGGAATGTATTCTGCATGATGAGCTGTTGTTGATAAATGCTTGAAAG
GGATGATGATATGTATAATAATACACAGGCTGCTGGGAGTATATATAT
GTAACTTGTACCTGCCCGGGCGGCGCTCAATCAAAAGGGGCC

Clone 8 [PNK 14(1)]

CCCAACCATTTACTTTAACAGCAGAAAAATGTGGATTTCAAGAACTTG
GAGCTGAAACTACTACTGGTGGGACCAAAATTGTGGGCACTAATGTGC
AGTCTCCAACAGTAAAGAAGGCTCAGCCAATGGAACCTTCACAACCA
GATAGCACCAAGCATGGTGATGAAGAGGATGCTTGCTCTGTTGCTTCT
TCATAAATTCATGCTACCTTTTTGCAGCCAATGAAACCTTCACAACCAG
ATAACACCAAGCATGGTGATGAAGAGGATGCTTACTCTGTTGCTTCTT
TCACTGCTGCATCAATTCAAGCAATCAAATCAAGGATTATTGCCGCAA
GTGCTCGAGTATTTAGGGTCACTGAACGTGCAGAGAAGCGGAAAGAA
TTTTACCGAAAGCTAGAAGAGAAATACCAAGCTCTAGAAGCTGAAAA
AAACCAGTCTGAAGCAACGACCAAGATTTTTGATTTTTTCAGATTTGTT
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TTGGGCGTATCTGGTCATTAGCTTGTTTCCTGGTGTTGAAAATTTGGTT
ATTCCGGTCAACAATTCCACCAACAACTTACGAGCCGGAAGATAAAGT
GGTAAGGCCTGGGGTGCCTAATGGAAGTGATTAATCCAAATTTATTGG
NTTGGGCNCCATGGCGGTTTCAGTGGGGAAACTGTCGGGCCTTGCTNA
GGAATCGGCA

Clone 9 [PNK 1(3)]

AACAAAGACATTAAAATACGACTCCTATAGGGCGAATTGGGCTCCGAC
GTCGCATGCTCCCGGCCGCCATGGCCGCGGGATTAGCGTGGTCGCGGC
CGAGGTATGGCTGTCGACAAGAAAGTAGCTGATGGATTGCTGAGACTT
ATACTACTGAAAGGGCCTCTTGGTAGCTGCGTTTTTACCGGTGATTATG
ACAGAAAAGCTCTTGATGAAACTCTTCATGCATTCTGCAGTAGATGAT
ACTGGTTCTACAGTCAGATTTGTGTGATTCTG

GAAGCATTTCTTGCAATCATGCTTATTTCGCAGCCTTGTCCATAGATAT
GAGGGTCAAAGATTTTACAAGACTTACTGTTTTACTAAAACCATCTGT
GTACCTGCCCGGGCGGCCGCTCGAAATCACTAGTGCGGCCGCCTGCAG
GTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGT
ATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGT
TTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGC
CGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAAC
TCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCT
GTCGTGCCAGCTGCATT
AATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGC
TCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGC
GCGAGCGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAT
CAGGGATACGCAGGAAAGAACATGGTGAGCAAAAGC

Clone 10 [PNK 5(2)]

AACCGACAATAGTATACGACTCACTATAGGGCGAATTGGGCTCCGACG
TCGCATAGCTCCCGGCCGCCATGGCCGCGGGATTTCGAGCGGCCGCCC
GGGCAGGTACAGAAAGAGCTTGCTGAGACTGAATTTGATGGCTACTGT
GAAGGTGAACTAGTAAAGGTCACACTTTCTGGGAATCAACAGCCCGTT
AGGACTGAAATTACTGAGGCTGCCATGGAACTAGGCGCTGATAAGCTC
TCTCTGTTGATTACTGAAGCATATAAGGATGCACACCAGAAGAGTGTG
CAGGCCATGAAGGAGAGAATGAGTAATCTAGCACAGAGTTTGGGAAT
GCCTCCTGGTTTGAGCGATGGTTTGAAGTAGTGCTAGAAAAAAAAATG
CAAATGTACCTCGGCCGCGACCACGCTAATCACTAGTGCGGCCGCCTG
CAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTG
AGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAG
CTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATAC
GAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGC
TAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAA
ACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAG
GCGGTTTGCGTATTGGGCGCTCTTCGCTTCCTCGCTCACTGACTCGCTG
CGCTCGGTCGTTCGGCTGCGCGAGCGTATCAGCTCACTCAAGGCGGTA
TACGTTATCCACAGATCAGGGATACGCGGAAGAC

Clone 11 [PNK 12(1)]

GCGTGGTCGCGGGCGAGGCCAGAGTGAAATTTGTTACAAGTAAAACTT
CACAACATAGGTGCAAGACTTGTTGGTGTCGACCAATTTGGGAACAAA
TATTATGAGAAACTTGAAGGCGTGATGTATGGGAGACACAGATGGGT
GGAATATGCAAAAAAGGATCGCCACAATGCTTCTCAAGTGCCTCCTGA
GTGGCATGGCTGGCTGCATAACATTACTGATCCTACTGGTGATGAATT
GTTGATGCTCAGACCAAAGAGATATGGGGTGGC

GCACAGAGAGAACTTTTCAGGAGAGGGAGAAGAATACATCCACCATT
CAAAGGGTCACGCACTCAATCCAGATCAGAAAGACTGGACAAGATAT
CAGCCGTGGCAGCCTGCAGAGAAGTCTTAGGAAATCAAAAGAAAATG
TGGATGGGCGTAAGCTTTGTTGTTTGTTTCTTAAATTTATTCGTACCTG
CCCGGGGCGGCCGTCTAATCACTTGTGGCGGGCCGCCTGCCACGGTCG
ACCATTATGGGAAGAAGCTCCCACCGCGTTGGGATGCATAACTTGCAG
TATTTCTAATAGTGTCACCTTTATAGCCATTGGGCTTAATTCATGATTA
TTTATGTATTCCGGTGTAAAAGGGATACGTTACCATAAACCAAAACAT
ATAGCAAGAACGAGAAATAAAAGTAAAG

Clone 12 [PNK 3(2)]

GACGATGTATACGACTCACTATAGGGCGAATTGGGCCCGACGTCGCAT
GCTCCCGGCCGCCATGGCCGCGGGATTTCGAGCGGCCGCCCGGGCAGG
TACTTACATCACAACAGTTTGCCACTACAGAAAACCGCGGATGTAGAT
CTCAGAAGTTTCCTACTGCAGTAGCGGCACCCTACGTTCCTGTGCGGC
ACTACCAGAATGTTTATCATGGGATGGCCCCACCTGTAACTGTAAGAA
CGGCAGTTCCTGTGTTTTCAGCACCTCCTCATCCACCAACACCTGGCTG
CTGTGCTCCTCCAGGAATGGGGCCGGGAGTGCGAATGGCTCCAGCTGT
GAATATAAGATCTGTTGTGCCAGTATTTGCAGCACCTCCATCCGTGGTC
AGAATTGAGGACCCCCCATCAGTTTTTGCTGCTCCTACAACACTGGCA
AGGTCATCCATAAAGATCGAGGAGAAGGGTAATCCCACTTCATCAACA
GCAGTTATAGCTCCACCTCCGCCCACACAGACTTCAGTGAAGACGGAT
GACATTACCAATTTGCCAGGGCCTTCCGTTTCCGCAGCTCCTCACTCTG
TCCAGCCATCGGTTATAGTAGAAGAAACTAGCATCCCTGCTCCAAAGA
ACATGCAAAACCCAGTCATACAGAACCTACAACAGCTAAAGATATGA
TGAGGAGGTTGTAAGTTGGAAAATGATTCTCTTTCTCCGTGCTGTTCCT
TAACGTATTTGTTTGGTTTTTCCGATGTATGTCGTAAATCATCCTGCGT
AGTTAGCTTATGGTAGTACTCGGCCGCGACCACGCTAATCACTAGTGC
GGCCGCCTGCAGGTCGACCATATGGA

Clone 13 [PNK 4(3)]

TCGAGCGGCCGCCCGGGCAGGTACAGGAGAAAAAGTGATAGAATTTT
ATCTTCTGGAAAGCAACTCCTTCATGGGCTTACTTCTGTTCACTCTAAA
AATGCTGGGGTGGACGTTGAAAAGAAGTCCAAGGCTAGAAAGGACAA
AGCTGGAATTTTGTGTCATGCATCTTATGCTGCTATAAAGCAATATGGT
TCACTCCCGTCTACTTGAATTCTTCACATTGAGACTTTGCAGAAAGAAA
TGATTATGACATCCGATCAGGCTTCTAACCAAAATCAATGGTTACCTA
AAGGTTTTGCGTATGTTCCAATAGGTTGCTTATCGAAGGAGAAGCAAG
AACCTGATGGATAATGTCTCCCCTTCAATGGGCGACTTGTTTGCACTTT
CATCATGACTCCCAATTTTGCTCAAGCATATTGTTTGCCGCAATGGTGG
AGCT

TCAATGCTGTGGTAACTGTTTGATTAAAAGCAAAAAAAAAAAAAAACC
AGATCAATTGGTCAGCTGAGTTTTTGATGGAGCTCCTCAAGCTCCTGTT
ACTGCAGAGAAATGACAAGGCCAGGCTATCCTGCAGGATTTCTGGGG
GTGGCTTTGCAAAATGTTCATTGCCAGCGGAAAGAGAGACCAGGGTG
GTGTCATGATTTTTAACAGAACGTTAGTTGAATAGCTATGTTTGGCCTG
TCCAATGGAGATGAGATTTTACTCTGATACTTGTATATTAGGAAAGCT
GGTGGCTTTCTTTTCTGTAGAATCTGTAAATTAGACCGTTGTATTGTGT
CG

Clone 14 [PNK 2(3)]

AACGATTGGGCCGACGTCGCATGCTCCCGGCCGCCATGGCCGCGGGAT
TAGCGTGGTCGCGGCCGAGGTACAAATTCAGCACCGACATTCCCCAAT
ATATGAAATTGTAACAGAAGCACCATCTTGCGTAGAGCAGGAGGATG
AGCTCAGTTCCACTGGAAAACCAGTCCACATCTGCTATATCCTTGAAC
ATCTCTGAATCTATACACTTCGTTGCAGAATTGCGCAACAAACTGATT
GCGAGAAAGGCTATCCACCCCTTGCGAAAGATAAGGAAAACATATCT
AAAAAGACTCCTATCAACCGATTCTTAAGCATATTGCAATCAACCACT
CTATGCTAAACCCTGTCAGAATCAAAAAGAAGTTTCACCTTACAGTTC
TGCAGTCCACTAATACCCCACAACAGAAATTCAATAGTCCATATGATA
CTTGTCATAAAAAGGAAGCCTAGTAAAACTCTTGCCCTCTTAAGCCAG
TTCCTAGATGAGATTCCATATGTTGATCATGATCAAACTTCATTGTCCG
TCTGTTACCACTAAAAAGTGCACACAAGACTTGGAGGTAACTGCGTGT
ACCTGCCCG

Clone 15 [PNK 7(2)]

TTGCAGTGGTCGCCGCGGGAGGGATGATATCCGAATCATACACTAAGG
TGTTTGATTCTGAGAGAGATGCCAAGAGAAGCCAACAGGATGATTCGA
GATCATATGGTAAACGAGATGATTCCGAGAGAGATGCTAAGAGGAGC
GGACAGGATGATTCAAGATCATACGGTAAGCGTGACAACTATCGTAA
ATTCGATACCGAGAGCGCCAGGCGATAGGCCTTGTGAGAGAGACCAA
CGATATGCCTACTCAGTTATATTGTGCTCTCTGTTTAAGACTATCTGGA
ATGTGCATTTAATATGCTTGATTGAATGTTGCATAATGTGGTTACAGTG
GTAGCCTTGGGGCAAGTAGCTGTAATCTAAACTTCGTAGTATTTCCTGT
AAGATGTGTTGCTACTCGAGCTTGATTTTCAAGTTTTCTAGTGCTACTA
CAATGTACCTGCCCGGGCGGCCGCTCGAAATCACTAGTGCGGCCGCCT
GCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTT
GAGTATTCTATAGTGTCACCTAAATACTTGGCGTAGTCATTGGTCATAC
TGTTACCTGAGTAGCAGAATAAGATTATCCGATCACAAACCACACCAG
NCNTAAGACCCAGAAAAGTTAAAAAAAAACCNGAGGTGACAATGAAT
AGAAAAAAACCAAAAAGGCTANTCNACAATCAATAAAC

Clone 16 [PNK 3(3)]

ACGATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCCGCGGGAT
TTCGAGCGGCCGCCCGGGCAGGTCGTTGTGTTACTTAGGTTAAGCATT
GATGGCTTCCACAAATAAAACAAAAACAGCCGAGCAGCATATATTTTG
GATATTGGAAATTTTGGAAGCTTGCTGTGGCTTATATGGAATGGTTGTT
TTTTTGTGCTTTATGTTCCAACGGGGAGTCAGGTGTGGAGTGAAGGGC
CATGTTAGCCATTGTAATGTTAATTTGTGAATATTGGCAAAAATGGAT
ATTGTAATTTTTGGGATACATTCAACCATTTTTTGTGAATTGTACCTCG
GCCGCGACCACGCTAATCACTAGTGCGGCCGCCTGCAGGTCGACCATA
TGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGT
GTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTG
AAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCAT
AAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAAT
TGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCA
GCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTAT
TGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTT
CGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGCGGTAATACGGTTAT
CCACAAATCAGGAAACGCAGAAAGAACATGTGAGCCAAAGGCAGCAA
AGGCAGGAACCG

Clone 17 [PNK 6(1)]

ACGATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCCGCGGGAT
TTCGAGCGGCCGCCCGGGCAGGTAATGAGGGGAACTAGCGTAGTGTT
GTTATAGGCGAACCTTTTATGACTTTGATCTATCATATGGAGGACTGTA
TGATTTTTATCTGCTCTTGATCGAGTTTGACATAAATGCGCTCTTTAAT
AACTTCGATTACCTAATATTACGTCTTTTATTCCTTGGATACATTGTTTT
GGCTTTTGATGTTCTGTATTATTGCGTGCTGCACTGTTTCTATGGTTTGC
TTCCAATGAAGAAGTGCAGGCCATTGCACCTCTGAAGATATTGTCTAT
TGCGGGTATTGTTAAAGGGAGGACTATATTCTTGTAATATTTAGTTGCA
CATTTTTTATCAAATAGTTAATTTTAGTATATATTGCTTTACTTTTATTC
CGATTGACATGGTATTTTGAAGTGCTTGCTATTTTGCAGGATCAGAGGT
TGTTTTTCATTGAAGCATCTGAAACTATTCTATGGATTATTTAACTCGA
AGTTCATGTGTCTGAAAGCTGATTTTATCGTTATGCAATTTGCAGGCAA
AAACTAGTGGTCGAATCCTCTTGGAGCTGCTAAGACGAGCTGCCGCAG
AAATTTCCTAATGGGTGCTAATTTCCAGTTTGAGACGAGGTTGACAAT
TGAAGTACCTCGGCCGCGACCACGCTAATCACTAGTGCGGCCGCCTGC
AGTCGACCATATGGGAGAGCTCCACGCGTTGGATGCATAGCTTGAGTA
TTCTATAGTGTCACCTAAATAGCTTGGCGTATCATGGTCATAGCTGTTT
CCTGTGTGAAA

Clone 18 [PNK 4(1)]

CAATAAAGCTATGCATCCACGCGTTGGGAGCTCCTCCCATATGGTCGA
CCTGCAGGCGGCCGCACTAGTGATTTCGAGCGGCCGCCCGGGCAGGTA
CTGCTCTATTATTCCAAATAAAGAAGTATTCACATTATACAAATTTATC
CGAAAAGTTGTGATCAACTAAAACGAATAATGGACTTAACCTACCTGC
AAAAAACAAAGACTACCAAACCGCGCAAATGCCTTTGTCATGACAGCT
TAACTCATGCAAATTACGACTACTAAACAATAATTAGTAATAACTTCA
CTTTGTTAAACTAACATAACGCAACAGATTTGGAGCAGAGCAGGCATC
CTTCTTTCCATTAGCACCGCTAATTCCTTCCACATGCAGTCAAACAGCT
GCTTGCACAGGACCATCTGATCTTCCCCGCCCGCCGCGTCCTCGGCCTC
GGCCATGACCTCGGCCGCGACCACGCTAATCCCGCGGCCATGGCGGCC
GGGAGCATGCGACGTCGGGCCCAATTCGCCCTATAGTGAGTCGTATTA
CAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGG
CGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGG
CGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGC
AGCCTGAATGGCGAATGGACGCGCCCTGTAGCGGCGCATTAAGCGCG
GCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCC
CTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCCGTTCGCC
GGCTTTCCCGTCAAGCTCTAATCGGGGCTCCTTTAAGGTTCCGATTTAT
GCTTTACGGCACCTCAACCC

Clone 19 [PNK 2(2)]

AAACGACTCAGTATACGACTCACTATAGGGCGAATTGGGTCCGACGTC
GCATGCTCCCGGCCGCCATGGCCGCGGGATTAGCGTGGTCGCGGCCGA
GGTCATGGCCGAGGCCGAGGACGCGGCGGGCGGGGAAGATCAGCTGG
TCCTGTGCAAGCAGCTGTTTGACTGCATGTGGAAGGAATTAGTGGTGC
TAATGGAAAGAAGGATGCCTGCTCTGCTCCAAATCTGTTGCGTTATGT
TAGTTTAACAAAGTGAAGTTATTACTAATTATTGTTTAGTAGTCGTAAT
TTGCATGAGTTAAGCTGTCATGACAAAGGCATTTGCGCGGTTTGGTAG
TCTTTGTTTTTTGCAGGTAGGTTAAGTCCATTATTCGTTTTAGTTGATCA
CAACTTTTCGGATAAATTTGTATAATGTGAATACTTCTTTATTTGGAAT
AATAGAGCAGTACCTGCCCGGGCGGCCGCTCGAAATCACTAGTGCGGC
CGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCAT
AGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGT
CATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAA
CATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAG
TGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTC
GGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGG
GAGAGGCGGTTTGCGTATTGGGCGCTCTTCGCTTCCTCGCTCACTGACT

CCCTGCGCTCGGTCGTCGCTGCGCGAGCGTATCAGCTCACTCAAAGCG
TATACGTTATCCCGATCAGGGAAACGCAGAAGACTGTGAC

Clone 20 [PNK 5(1)]

CGACTCAGCTATGCATCCACGCGTTGGGAGCTCCTCCCATATGGTCAG
ACCTGCAGGCGGCCGCACTAGTGATTTCGAGCGGCCGCCCGGGCAGGT
ACTAATCTGTGTAGAAATCAGCAAACAGCATTTCCACAAAAACTTCCT
GAACATGACGATTTACAAACAAAATCAAACTCCTGCAACCAATCATCT
TCAAGAACAATCTGCTGTAAAATCCCAGTTCTGGTAAACCTAACCGCT
TGCCGTCCAAGGCCCCCCTGCTGATGGCGTTATGTTTCTTTGCTTTCTG
AACAATAAATTAGCATCGTGAACTGCTGATTCTGCATTCAGATGAAAG
CTTCTCTCACTGCAGTAGGTAACTTTAATTCAGGCTCAAATACACCAA
GTGGGCCAAAATAACTATCAACCATGTCCTTTGGTACCTCGGCCGCGA
CCACGCTAATCCCGCGGCCATGGCGGCCGGGAGCATGCGACGTCGGG
CCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTT
TACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCC
TTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCC
GCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGA
CGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCG
CAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCT
TTCTTCCCTTCCTTTCTCGCCACGTTCGCGCTTTCCCGTCAGCTCTAAAT
CGGGGCTCCCTTTAGGTTCCGATTAGTGCTTTACGCACCTCGACCCAAA
ACTTGATA

DEVELOPMENT AND ANALYSIS OF ESTs (EXPRESSED SEQUENCE TAGS) IN BLACK PEPPER (*Piper nigrum* L.)

By

RENU KUSHWAH

ABSTRACT OF THE THESIS

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Centre for Plant Biotechnology and Molecular Biology

COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR - 680 656

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ABSTRACT

Soil moisture is one of the major factors that influence plant growth and productivity. The stress is the second most important factor that affects crop production in black pepper. Various genotypes of black pepper are reported to vary in their response to water stress and the variety Kalluvally has been identified as a drought tolerant one among the cultivated genotypes.

Plants respond to stress by adaptation of the biochemical and physiological processes. These biochemical and physiological reactions are regulated by several genes that are induced during drought conditions. Thus the gene products directly or indirectly provide tolerance to the plants so that they can survive under water stressed conditions.

In the present study, an attempt was made to identify such water stress induced genes in variety Kalluvally using the molecular technique called Suppression Subtractive Hybridization (SSH) and finally to develop and analyze Expressed Sequence Tags (ESTs). Total RNA and mRNA were isolated from normal and water stressed plants and were used respectively as 'driver' and 'tester' in SSH reaction. The reactions were performed utilizing the PCR select cDNA subtraction kit provided by CLONTECH, USA.

Control subtraction was carried out first using PCR select™ cDNA subtraction kit, which gave satisfactory and expected results. For experimental subtraction, double stranded cDNAs were synthesized from 2µg mRNA from normal 'driver' and water stressed 'tester'. Two tester populations were created and each ligated to two different adaptors. This was followed by two hybridization reactions and finally a selective PCR amplification. Only differentially expressed cDNAs were amplified exponentially. This was confirmed by analyzing the PCR products on agarose gel, which showed a smear ranging from 0.2 to1.5kb in the subtracted sample and was different from smear pattern of unsubtracted ones. The cDNA fragments from subtracted sample were

cloned in pGEMT vector and sequenced. Total twenty clones were sequenced and analysed after vector and adaptor editing.

In silico analysis using bioinformatics tools revealed that some of the cloned sequences showed good homology with known sequences which play important role during water stress conditions directly or indirectly. These included Heat Shock Proteins (HSP-17 & 20), Secretory Carrier Membrane Protein (SCAMP), gamma thionins, MYB transcription factor, Ribonuclease enzyme, fatty acid desaturase, peptidylprolyl isomerase and NADH-ubiquinone oxidoreductase family protein. Also, these sequences had conserved domains for the above mentioned proteins. The rest of the clones did not show any good homology and therefore it was difficult to assign any reported role to these. In addition to this, all the sequences possessed Open Reading Frames (ORFs) many had transmembrane helices and some were found to have signal peptide.

The sequences were submitted to dbEST. For further exploitation of these sequences it is necessary to clone full length cDNA. ESTs thus generated in the present study will be of great use in future for further downstream applications.