STUDY ON

THE EXPRESSION OF VACUOLAR PROTON PUMP ATPase SUBUNIT F THAT CONTRIBUTES IN ABIOTIC STRESS TOLERANCE OF JUTE (*Corchorus olitorious)*

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Certificate

*This is to certify that the research work embodying the results reported in this thesis entitled "**Study on the expression of vacuolar proton pump ATPase subunit F that contributes in abiotic stress tolerance of jute (Corchorus olitorious)**" submitted by **Md. Ahasanur Rahman**, has been carried out under our supervision in the Molecular Biology Laboratory, Department of Biochemistry and Molecular Biology, University of Dhaka and MNS department, BRAC University. It is further certified that the research work presented here is original and suitable for submission for the partial fulfillment of the degree of Master of Science in Biotechnology, BRAC University, Dhaka.*

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DEDICATED

TO

THE MEMORY OF MY FATHER

WHO I MISS EACH & EVERY MOMENT

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Abstract

Jute (*Corchorus spp.*) is the second most important fibre producing plant in the world. It is a very season specific crop and requires certain temperature and moisture conditions for growth. To imporve the quality of this agronomicaly improtant fibre yielding crop, conventional breeding between species (*C. capsularis* and *C. olitorius*) was attempted and failed due to sexual incompatibility of the species. Hence, study of jute at the molecular level is of great importance.

To make jute tolerant to environmental stresses, the study of genes and transcriptional factors involved in stress-tolerance mechanism cannot be overemphasized. In this study, vacuolar proton pump ATPase subunit F, which functions as central stalk of a multi-subuint complex protein vacuolar ATPase, was found to be responsive in dehydration stress in Jute (*Corchorus olitorius*). Expression study reveals that the expression of V-ATPase subunit F gene is induced by salt, low temperature and drought stress and variation of expression is observed in time dependent manner. This finding will enable us to attempt the production of jute under dehydrated condition and resurrect the golden age of our jute fibre.

Keywords: Jute, Salt, Low temperature, Drought, Stress tolerance, Dehydration response, Vacuolar Proton Pump ATPase subunit F.

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LIST OF ABBREVIATIONS

bp	Base pair
BJRI	Bangladesh Jute Research Institute
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary DNA
Conc.	Concentration
dd H_2O	Distilled de-ionized water
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothritol
dNTPs	Deoxyribonucleotide triphosphates
dATPs	Deoxyadenosine triphosphates
dTTPs	Deoxythymidine triphosphates
dCTPs	Deoxycytidine triphosphates
dGTPs	Deoxyguanosine triphosphates
dUTPs	Deoxyuridine triphosphates
DNase	Deoxyribonuclease
EDTA	Ethylene diamine tetra acetic acid
e.g.	For example
Et. Br.	Ethidium bromide
et al.	With others
etc.	Etceteras
g	Gram
HCl	Hydrochloric acid
hrs.	Hours
kb	Kilobase

L	Liter
M	Molar
mg	Milligram
min.	Minute
ml	Milliliter
mM	Millimolar
2-ME	2-Mercaptoethanol
N	Normal
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
ng	Nanogram
NaOH	Sodium hydroxide
No./no.	Number
O.D.	Optical density
PCR	Polymerase chain reaction
p^H	Negative logarithm of hydrogen ion
RACE	Rapid amplification cDNA end
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolution per minute
RT	Reverse transcription
sec.	Second
SDS	Sodium dodecyl sulphate
SOPMA	Self Optimized Prediction Method from Alignment
TAE	Tris acetate EDTA
TE	Tris EDTA
TEMED	N,N,N',N'- tetramethyl ethylene diamine
TMHMM	TransMembrane prediction using Hidden Markov Models

UV	Ultra violet
Var	Variety
V-H+ATPase	Vacuolar Proton Pump ATPase
vol.	Volume
µl	Micro liter
µg	Micro gram

[Introduction]

[Chapter 1]

Pages 01-19

1.9 Necessity of the Present Study:

Plants are exposed to various adverse environmental conditions such as drought, high salt and high/low temperature etc. during their life cycles. These environmental stimuli, commonly known as "environmental or abiotic stresses" severely limits plant growth and productivity. One of the plant's responses to these stresses is the induced expression of a large number of genes, whose products are known or believed to be involved in various adaptive functions under stress conditions. Due to its agronomic importance, identification of the stress-induced gene products has been a subject of intense study. As a result, numerous stress-inducible proteins have been identified and their corresponding genes have been isolated. In parallel, numerous molecular biological studies have been performed in order to decipher regulatory elements of these stress-modulated genes.

Unlike their mobile animal counterparts, sessile plants have to adjust their gene expression pattern as well as metabolite profile to cope with altered local environmental conditions (Kulheim et al. 2002). A number of genes are involved in these pathways which can either be up-regulated (Holappa and Walker-Simmons 1995; Hannah et al. 2005; Yan et al. 2005) or down-regulated (Hannah et al. 2005; Yan et al. 2005) based on their mode of function. Identification of these genes may lead to the development of commercial crops which are better suited to grow in a particular unfavorable condition. Due to cross-species transferability of gene function, this improvement can be achieved in non-source plant species as well.

flood or cold, fungal diseases, photoperiod sensitivity, poor fiber quality etc. Nevertheless, the production quality and yield of this economically important crop is affected by several biotic e.g. fungi, pest, insect, nematode, virus, mite (Ghosh 1983; Keka et al. 2008) and abiotic factors e.g. salinity, submergence and low temperature (Hossain et al. 2003; Samira et al. 2010) . Addressing these challenges through traditional plant breeding program has limitations due to lack of genetic diversity among cultivated jute varieties. Therefore, for developing superior jute varieties with traits such as biotic and abiotic stress tolerance, genetic modification with foreign gene(s) may provide a suitable alternative.

In spite of its enormous commercial value, only a few genes or part of genes from jute have been identified or sequenced (Islam et al. 2005; Wazni et al. 2007; Ahmed et al. 2009; Alam et al. 2010; Samira et al. 2010). Here we report a transcript from *Corchorus olitorus* var 9897 responsive to abiotic stress.

1.10 Objective of the study:

Since the V- H+ ATPase gene was found to be of significant importance in numerous stress response pathways in different species, and no previous study has been found in the case of jute, current study aimed to functional characterize the gene from *Corchorus* spp. With these goals in mind, the objectives of the current study were set as follows:

- Study the expression level of vacuolar proton pump ATPase subunit F under abiotic stresses (salt, low temperature and osmotic stress using mannitol) and non-stress condition by semi-quantitative RT-PCR in the *Corchorus olitorious* var 9897 to identify both constitutive and induced changes in gene expression which may assist in the elucidation of molecular basis of abiotic stress resistance in wild jute species.
- Comparing the expression of the transcript in different time intervals.
- Bioinformatics analysis of the transcript.

[Introduction]

[Chapter 1]

Pages 01-19

1.1 General Overview of Jute:

Jute is one of the most affordable natural fibers and is second only to cotton in amount produced and variety of uses of vegetable fibers. Jute has various inherent characteristics like, high tensile strength, low extensibility, long durability, fire and heat resistance, silkiness, luster and long staple length. Jute fiber has a lustrous and shiny golden color, hence it is called ***"The Golden Fiber"***. Because of its high tensile strength and low extensive nature, jute fiber has been the best substitute for any other vegetable fibers and the best natural substitute for polypropylene.

Jute is comprised of two cultivated species, *Corchorus capsularis* L. and *C. olitorius* L. *C. capsularis* is called White Jute and *C. olitorius* is called Tossa Jute. The two species are distinct in their growth habitat, branching habit, and characteristics relating to leaf, flower, fruit, seed, bast fiber, and photosensitivity (Basu et al. 2004). *C. olitorius* is supposed to have originated in Africa, while the Indo-Burma region is believed to be the centre of origin of *C. capsularis (*Roy et al. 2006*)*.

Figure1.1: A typical jute plant (*Corchorus spp.)*

1.1.1 Origin of Jute and its producers:

The cultivars of *C. olitorus* (Tossa) are thought to have originated in many African countries for example, Mozambique, Tanzania, Zimbabwe, Zaire, Ethiopia, Somalia, Kenya, Uganda and Sudan. Evidence suggests it grows in Anatolia (Turkey), Socotra (Yemen) and Iraq. In the Middle East *C. olitorius* leaves have been in use as potherbs since Biblical times. Cultivation in commercial scale is restricted to Bangladesh, India, Nepal, Taiwan and Brazil.

The cultivars of *Corchorus capsularis* (white jute) occur in southern China, northern Burma including the Shan States, Malaysia, the Indonesian islands of Halmahera, Celebes and Timur, the northeastern hills of Meghalay, Mizoram, Nagaland and Tripura in India. Recently types have been collected in India from M.P., Mirzapur, A.P. and Eastern Ghats .*C. capsularis* types have also been found in Sri Lanka, Philippines and Pakistan.

1.1.2 Taxonomical classification:

It is classified in the Magnoliophyta division, Magnoliopsida class, Malvales order and Malvaceae family (Subfamily: Grewioideae). It is produced from plants in the genus Corchorus. Over 170 Corchorus species names are given in the Kewensis

Fig 1.2: Different vegetative part of Jute

Scientific classification

Domain:	Eukaryota
Kingdom:	Plantae (plants)
Division:	Magnoliophyta (flowering)
Class:	Magnoliopsida (dicotyledons)
Order:	Malvales
Family:	*Malvaceae*
Genus:	*Corchorus*

(Source: NCBI Taxonomy Browser)

1.1.3 Physical characteristic:

Jute is a long, soft, shiny vegetable fiber that can be spun into coarse, strong threads (Hossain et al. 2003). Jute plants are tall, usually annual herbs, reaching a height of 2-4 m, unbranched or with only a few side branches. The leaves are alternate, simple, elliptic-lanceolate, apically acute or acuminate, 5-15 cm long with an acuminate tip and a finely serrated or lobed margin. At the harvest stage varieties of *C. capsularis* attain a height of about 5-12 feet and those of *C. olitorius* 5-15 feet or more. The stems of both are cylindrical. Flowers of both the species look yellow, are small in size (2-3 cm diameter) and occur in condensed cymes. Flowers occur in the axel of leaves and are composed of 4-5 sepals and petals, 5 to numerous stamens. Sepals are about 3 mm long, oblong, apiculate. Ovary is 2-6 locular. Seeds are small and numerous. Seeds of *C. olitorius* are smaller of the two species. *C. capsularis* seed is coppery in colour and weigh about 500-600 seed per 1 gram. *C. olitorius* seed is greyish in colour and weigh about 1000 seeds per gram. Both the species are mostly self-pollinating (Wikipedia).

1.1.4 Chemical characteristics:

Jute fibers are composed primarily of the plant materials cellulose (major component of plant fiber) and lignin (major components wood fiber). It is thus a ligno-cellulosic fiber that is partially a textile fiber and partially wood (Wikipedia).1.4.1

Constituents	Percentage (%)
Cellulose	59-61
Pentosan	15-17
Lignin	12.5-13.5
Polyuronide	4.8-5.2
Acetyl value	2.8-3.5
Fat and Wax	0.9-1.4
Nitrogenous matter	1.56-1.87
Mineral Substance	0.5-0.79

Table 1.1: Chemical composition of jute fibre

1.2 Environmental Factors for Jute Cultivation in Bangladesh:

Jute cultivation requires specific climate and land. It requires early rains in March, May and June and intermittent rain and sunlight thereafter till August, temperature between 28°C and 35°C and humidity between 70% and 90%. This type of climate is available in areas between 30° Latitude North and South of Bangladesh. Most of the jute species are acclimatized to warm temperature during summer and are adversely affected by low temperature.

Photoperiod (length of light and the dark period in a day) has direct effect on the growth of the plant and formation of flowers and fruits. Jute is grown from March to July in the transition from short to long days. As jute is grown for its fibre, the plants are allowed to attain maximum vegetative growth during the long days and after this when the day length starts to shorten then flowering appears and vegetative growth slows down or ceases.

Jute grows well where the annual rainfall is 1500 mm or more, with at least 250 mm during each of the months of March, April and May. In the months of November to April, the water balance is negative which means that transpiration exceeds water gain. Since this period of the year has deficit of water, normal germination, growth and development of most of the varieties of jute crop suffer. To make jute available throughout the year as raw material for jute based industries home and abroad, novel variety of jute is required that can withstand any environmental stress without compromising fibre quality.

1.3 Jute, the Cash Crop of Bangladesh:

Two species of jute, *C. capsularis* and *C. olitorius* are widely cultivated in Bangladesh. It is grown in almost all the districts of the country, Faridpur, Tangail, Jessore, Dhaka, Sirajganj, Bogra, and Jamalpur are considered the better growing areas. It is our principal cash crop. On an average Bangladesh produces 0.794 million tons of raw jute on about 0.392 million hectares of land i.e. national average production is 2.03 tons per hectare (BBS, 2004). This average production is encouraging because the fertile lands are occupied by food crops and

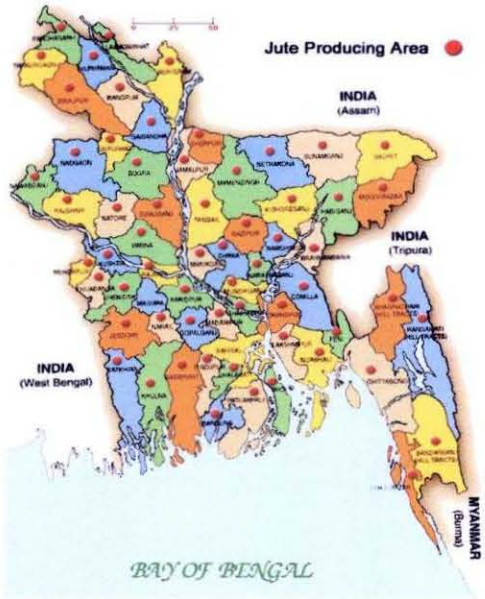


Fig 1.3: Major jute producing area of Bangladesh.

jute is being pushed to the marginal land. It contributes to about 6% of the total export earning. About 30 million people are directly or indirectly dependent on the jute sector as 3.5 million farmers are engaged in growing jute, 0.2 million people are working in jute factories, 0.1 million are engaged in jute trade, and a large number of people provide other services. Jute offers cash earnings to 5 million farm families (IJSG & The Daily News Today, 13 April, 2011) and total average export earning of Bangladesh from jute & jute goods is about US$ 600 million per year (IJSG 2007/08-2009/10) which is one of largest export earners of Bangladesh.

1.4 Use of Jute and Jute Products:

1.4.1 Nutritional and Medicinal Utility

1.4.1.1 Edible Uses

Jute is rich in calcium, phosphorus, iron and potassium. It has also been determined that 100 grams of jute leaf contains a good amount of vitamin A, thiamine, riboflavin and ascorbic acid. In Japan dry jute leaf is used as a substitute of coffee and tea. In Europe, jute leaves are used for preparing soup.

1.4.1.2 As a Tonic and Diuretic

The seeds of jute are used as a purgative and the leaves as a tonic and diuretic (Chopra 1956). The leaves are also used as appetizer, carminative, demulcent and laxative. An infusion is used in the treatment of fevers, dyspepsia and liver disorders. A decoction of the roots and unripe fruits is used in the treatment of dysentery and constipation. The seeds contain a substance that has a similar action to Digitalin on the heart (from *Digitalis* spp.), though less intense in its action (Khalique 1954).

Jute leaf powder and its water-soluble viscous solution are found to decrease total serum and liver cholesterol concentrations and increase the faecal excretion of bile acids, total neutral sterols and cholesterol (Inammi 1995).

1.4.1.3 As an Anti-tumour Agent

Jute leaves are used as health-food in Japan. Jute leaves contain anti- tumour agents as phytol and monogalactosyl-diacylglycerol which may reduce risk of cancer (Wang et al. 2002).

1.4.1.4 As an Anti-Oxidant

Six phenolic antioxidative compounds have been identified in the leaves of *C. olitorius*. Their antioxidant activities were measured and this showed that 5-caffeoylquinic acid was a predominant phenolic antioxidant in jute leaves (Azuma et al. 1999).

1.4.2 Industrial Uses

Jute is used for the production of different conventional jute products which include:

Jute yarn - of various counts

Jute twine - made by winding two or more yarn strings

Jute cloth - of different variety and for different applications like

- General packaging purpose
- Converting into sacks for carrying food grain, vegetable, sugar, fertilizer, cement etc.

Another area where jute has been put to use of late is the diversified sector where a wide range of technologies has been developed. Jute has an infinite range of applications.

Fig 1.4 : Jute sacks.

Fine quality yarn- used in weaving finer quality of fabric. Blended fabric where even better quality of fabric is woven blending jute yarn with other superior yarn.

Jute fabric- could be chemically processed, dyed and printed to produce colorful fabric for manufacturing shopping bags, designer bags, brief cases and suitcases.

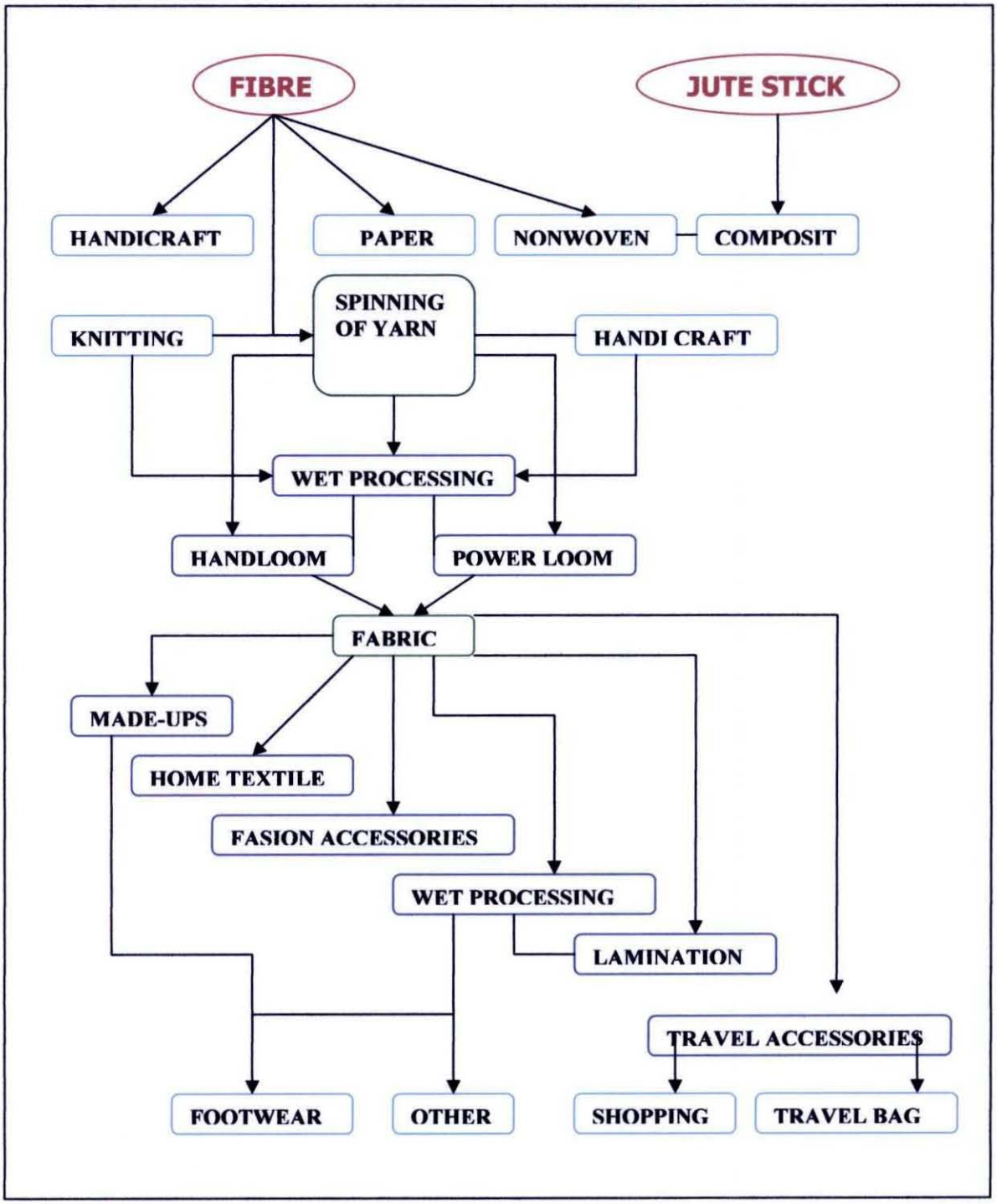
Jute pulp- for applications like making paper moulds etc.

Jute particles boards -made of jute sticks. This in now being used in house hold interior applications and knockdown furniture.

Jute geo-textile- applications in holding soil erosion, making rural roads, embankment building, landslide prevention, landscaping etc.

1.4.2.1 Possible Jute Diversified Industries:

Here is a summary of possible jute diversified industries.



1.5 On-going Research on Molecular Biology of Jute:

In spite of being one of the most important and useful fiber crops, it is surprising that research on this important cash crop is limited worldwide and very few literatures are available.

1.5.1 Analyzing genetic diversity based on molecular markers:

Analysis of jute genetic diversity has been reported by (Hossain et al. 2002) and Qi et al. (2003) using RAPD; (Basu et al. 2004) using SSR and (Roy et al. 2006) using STMS, ISSR and RAPD markers. (Hossain et al. 2003) characterized cold-tolerant and cold-sensitive jute germplasms and Qi *et al.* (2003) classified wild jute species using Inter Simple Sequence Repeat (ISSR) marker. Recently (Mir et al. 2007; Islam et al. 2008) reported the utility of studying genetic variability for different traits in jute genotypes using jute specific SSR markers.

1.5.2 Tissue culture independent transformation:

Since two main jute varieties (*Corchorus capsularis* and *Corchorus olitorius*) are self-pollinating, attempts to cross different jute species to combine useful characteristics were unsuccessful (Finlow 1917, 1921, 1923; Datta et al.1960; Patel and Datta 1960; Islam and Rashid 1961; Chaudhuri and Mia 1961; Islam 1964; Hoque et al. 1988). Whole plant regeneration following tissue culture and transformation was also not very successful with jute (Sarker et al. 2007).

A successful transformation protocol for *C. olitorius* using a tissue culture independent technique was reported by (Islam et al. 2008). The method involved *Agrobacterium* mediated transgene introduction to apical meristems of the plant that are subsequently allowed to grow into plants and produce seeds. Inoculation of naked DNA into ovaries has also been reported to produce transformed progeny.

1.5.3 Somatic Hybridization

Somatic hybridization was attempted between *C. capsularis* and *C. olitorius* as sexual hybridization between the two cultivated species could not be achieved. For this purpose, protoplast was isolated and callus production and somatic embryogenesis was induced from protoplasts. The (putative) hybrid showed green pigmentation and vigorous growth which was absent in the parents. In isoenzyme profiles (acid phosphatase), hybrid cell lines showed prominent hybrid bands and included both parental bands (Khatun 2007).

1.5.4 Gene Annotation, Characterization and Genome Sequencing

Islam and co-workers (Islam et al. 2005) reported sequences of 15 jute genomic and cDNA clones which had significant similarity to *Arabidopsis* genes. Wazni and his group (Wazni et al. 2007) reported 16 ESTs showing significant similarity to *Arabidopsis* or other higher plant genes. Both the studies took the approach of homology based gene prediction and revealed genes which might be involved in a number of metabolic and stress related pathways.

From the structurally annotated genes researchers align the sequences of these genes, design degenerate primers, conduct PCR, clone them in vector, sequence the genes, design gene specific primers and then with cDNA end amplification process finds the full-length cDNA and conduct expression analysis (Alam et al. 2010). Computational approach for developing jute ESTs from genomic clones (Ahmed et al. 2009) and gene identification and confirmation from sequences of SSR library (Samira et al. 2010) has also been reported.

In this backdrop of the extent of jute genomics study, the Prime Minister of Bangladesh has recently declared the completion of Jute Genome Sequencing by a group of Bangladeshi biologists and computer scientists (The Daily Star, June17, 2010) but the sequence is not yet available in the public domain.

1.6 Necessity for the Study of Jute at Molecular Level:

Bangladesh holds the largest gene bank of jute and allied fiber (JAF) crops. Bangladesh Jute Research Institute (BJRI) premises preserve 5936 accessions of jute and allied fiber germplasm including 15 species of *Corchorus*, 22 species of *Hibiscus* and 15 of allied genera yet to be characterized. Although a number of varieties have been developed experimentally from time to time from inter-varietal crosses using conventional breeding techniques but none has been stable enough to be commercially released (A. S. Islam 1992).

Jute cultivars are currently distinguished by morpho-physiological characters such as pigmentation pattern in plant, leaf shape, stipule, seed coat color of mature field grown plants (Anon, 1997). This method is slow and unreliable (Cooke, 1999) and phenotypic identification based on morphological traits is subject to environmental variation (Nielson, 1985). As jute is a self-fertilized crop, its natural genetic variability is very narrow, that makes the plant breeder helpless in the development of this crop. Cultivars that are closely related or have low genetic variability cannot be readily distinguished by morphological indices (Deng *et al.* 1994 & Degani *et al.* 1998). But the use of molecular technique can be a better alternative to this. Jute is a new crop in the field of molecular biology. So any kind of molecular level information would be helpful for its improvement.

1.7 Jute and Environmental stresses:

Plants can be subjected to two different types of stresses viz. biotic stress and abiotic stress. Fungal infection is the major biotic stress for jute (Keka et al. 2008). Abiotic stresses can be cold, dehydration, salt and so on (Hossain et al. 2003; Sarker et al. 2008). Many of these environmental stresses affect jute production significantly (Hossain et al. 2003; Sarker et al. 2008).

Jute cultivation requires specific climate and land. It requires early rains in March, May and June and intermittent rain and sunlight thereafter till August, temperature between 28°C and 35°C and humidity between 70% and 90% .

This stringent climatic requirement makes jute less lucrative to farmers for cultivation. Using modern molecular biological techniques, it is possible to develop jute varieties which can overcome these requirements. But, to do that, the knowledge of stress responsive pathways is a prerequisite. A number of proteins are known to function in these pathways.

1.8 Vacuolar Proton Pump ATPase (V- H+ ATPase):

The vacuolar proton pump ATPase (V- H+ ATPase) is a transmembrane protein complex of about 800 k Da found in all eukaryotes. The V-ATPase is localized in the endomembrane system, where it acidifies diverse cell compartments and generates a proton motive force (PMF) used to energize secondary transport (Sze et al. 1999, Kluge et al.2003). The ability of the V-ATPase to maintain the cytosolic pH homeostasis and to acidify the endomembrane compartments is important during essential processes such as growth and cell elongation (Smart et al.1998, Viereck etal. 1996). In plants the V-ATPase is composed of 13 V- H+ ATPase subunits (VHA) distributed among the cytosolic V1-domain and the membrane bound V0-domain (Sze et al. 2002).

The V1-domain is dominated by the hexameric head assembled from three copies each of VHA- A and-B. Subunits VHA–D and –F function as central stalk and transducer conformational changes in VHA- A established by ATP-hydrolysis into rotation of the proteolipid ring. The remaining V1-subunits (VHA-E to –H) constitute at least one or possibly upto three peripheral stalks which immobilize the catalytic head during rotation of the rotor (Domgall et al. 2002, Wilkens et al. 2004). VHA-E and – G form a tetramer containing two copies of each subunit interacting with single copies of VHA-C and-H (Fethiere et al.2004, Seidel et al. 2005)

1.8.1 Vacuolar H+ ATPase and Environmental Stress:

The vacuolar H+ATPase is of prime importance for plant cell expansion and stress adaptation. This may partly be attributed to the presence of the large central vacuole as one of the peculiar structures of plant cells that may occupy as much as 99% of the symplastic cell space, for example in parenchymatic tissues of succulent plants. The vacuole functions as intermediate and terminal storage compartment for salts, metabolites, carbohydrates like fructans, amino acids, and conjugates. All these functions are intimately connected to transport processes (Martinoia et al. 2000).

Many environmental parameters affect V-H+ ATPase expression and activity. For that reason Luttge et al. (2001) denominated the V H+ATPase as the "eco-enzyme" of higher plants. In addition to its function as house-keeping enzyme, V- H+ ATPase undergoes specific modifications in structure and activity that are crucial for environmental adaptation.

1.8.2 Literature Review:

Under stress conditions such as salinity, cold, drought, acid stress or excess heavy metals in the soil, survival of the cells strongly depends on maintaining or adjusting the activity of V-H+ ATPase. Several reports suggested the pivotal role of V-H+ATPase when plants are challenged with environmental stresses, with salinity being the best studied example followed by chilling and other stresses.

1.8.2.1 Salinity:

In plants central vacuoles plays a crucial role for the regulation of cytoplasmic ion homeostasis. Efficient exclusion of excess sodium from the cytoplasmic compartment and the vacuolar sodium accumulation are the main mechanism for adaptation of plants to excess sodium chloride concentrations (Dietz et al. 2001). The vacuolar sodium sequestration is mediated by a secondary active Na+/ H+ antiport at the tonoplast (Barkla et al. 1995; Apse et al. 1999) and it is energized by a proton motive force that is driven by the vacuolar proton pump ATPase (Dietz et al. 2001).

Detailed analysis of the regulation of the V-H+ ATPase and of the transcription and translation of V-H+ ATPase have been carried out for the facultative halophyte *Mesembryanthemum crystallinum* (common ice plant). Salt-induced transcriptional activation of V-H+ ATPase subunits A, B, E, G and c have been reported in *M. crystallinum* (Dietz 1996; Low et al. 1996). In *M. crystallinum,* Dietz et al. (2001) showed that the expression of subunits A, B, E, F and c increased upon salt stress in leaves, but not in roots of 5-week-old plants treated with 400mm NaCl for 72h. However, a decline in the expression of subunit E was found in root cortex cells and the root vascular cylinder whereas in leaves an increase was found. Interestingly, seedlings of *M. crystallinum* that are not salt tolerant do not show transcriptional changes of the V-H+ ATPase subunits A, B, E, F and c under salt stress (Golldack 2001). A Coordinated salt-induced increase of transcript amounts of V- H+ ATPase subunits has been reported for other halotolerant plants as well. In the halotolerant sugar beet, transcripts of the V-H+ ATPase subunits A and c were found in root and leaf tissue and NaCl treatment caused an increase of the transcript levels in leaves, but not in the roots (Kirsch et al. 1996; Lehr et al. 1999). In barley plants exposed to 300mM NaCl subunit E proteins showed a slight increase in the root tissue but no changes in leaves (Dietz et al. 1995).

LI et al. (2004) showed that the expression of V-H+ ATPase B subunit from leaves of *S. salsa* was significantly up-regulated and was coordinated with subunit c at transcription and translation level under NaCl stress. While for glycophytes, salt-reduced protein amount of V- H+ATPase subunit B was reported in wheat (Wang et al. 2000) and the protein amount of subunit B in pea was not changed by sodium chloride exposure (Yu H-F et al. 2001). These data suggested that salt stress affected V-H+ATPase subunit B expression differently in glycophytes and halophytes.

Increases of the transcript levels of the subunit A of the V- H+ATPase are also known from salt stressed and salt adapted cell suspension cultures of tobaccao (Narasimhan et al. 1991) .

Studies on salt sensitive species including *Arabidopsis thaliana*, suggesting that increased V-H+ ATPase level and/or activity may be required to drive Na+ sequestration under salt stress (Kluge et al. 2003). In *Arabidopsis*, majority of subunits from both V1 and V0 sectors are upregulated in response to salt, particularly in the later stages (24-96 hr) of the treatment (Maathuis et al. 2003). However, it has been reported that in *Arabidopsis Thaliana*, the expression of subunit D of the vacuolar ATPase was not modified by NaCl treatment (Kluge et al. 1999). Recently Dietz et al. (2007) showed that in Arabidopsis under heat and drought conditions though the pollen specific isoforms VHA-E2 and-G3 as well as VHA- a2 were up-regulated, however, salinity did not affect the expression level of VHA-a, VHA-E and VHA-G subunits.

1.8.2.2 Low Temperature:

Growth of plants at low temperatures seems to have an impact on V-H+ ATPase activity. Many tropical and subtropical plant species are damaged by low temperatures ranging from +10 to -1^{0}C. Plant sensitivity towards low temperature and development of chilling and frost hardiness implicate three vacuolar events related to V-H+ ATPase activity (Yoshida et al. 1999): (1) one of the primary events of chilling injury appears to be an inhibition of V- H+ATPase activity. (2) as a consequence, the formation of pH gradients is inhibited and probably compartmentation of solutes distributed. (3) the fluidity of membranes has to be adjusted to low temperatures by an increase of the unsaturated fatty acids in cell membrane.

When chilling-sensitive *V. radiata* seedlings were grown at temperatures below 10^{0}C, H^+ transport activity of the V-H+ATPase decreased to a very low level. After incubation of *V .radiata* hypocotyls for 3 days at 0^{0}C the amount of several V1-subunits were decreased while the amount of subunit c was not altered (Matsuura et al. 1990; Yoshida et al. 1991). However, This effect was not observed by low

temperature stress of chilling-insensitive *Pisumsativum* seedlings (Yoshida et al. 1991).

Yoshida et al. (1992) investigated in seedlings of mung bean (*Vigna radiate*) chilling at 0^0C for 3 days and they found that the amounts of the eight subunits of ATPase, but not the 16-kD subunit, decreased during chilling in vivo. They demonstrated that the 16-kD subunit is an integral membrane protein, and the interaction between the subunit and membrane lipids was not broken. They concluded that the V-H+ ATPase of mung bean dissociates easily and is sensitive target of cold stress. A clear correlation was established between chilling sensitivity and cytoplasmic acidosis (Yoshida 1994). Perhaps acidification of the cytosol as a consequence of decreased V-H+ ATPase activity accounts for chilling sensitivity, since pH homeostasis within narrow pH range is a central element of functional cell metabolism, and directly depends on V-H+ ATPase activity (Dietz et al. 2001). Interestingly mRNA levels of subunit c and subunit E were not modified in *M. crystallinum* plants that were osmotically stressed with mannitol (Dietz et al. 2001).

1.8.2.3 Dehydration:

Little attention has been paid to the function of the V- H+ ATPase under osmotic stress. Since maintenance of vacuolar compartmentation is of fundamental importance particularly under conditions of water and turgor loss and increasing ion and metabolite concentration, there is a need to investigate in more detail the expression and activity of V-H+ATPase under osmotic stress.

Studies with *Mesembryanthemum crystallinum* suggested that the application of osmotic stress using mannitol resulted in a different expression pattern. The level of all vacuolar H+ ATPase transcripts decreased in leaves and roots with quantitative differences. The decrease was less pronounced in leaves. The most pronounced decrease was seen for subunit B and C, whereas only a small effect was measured for G (Dietz, Kluge et al. 2003).

1.9 Necessity of the Present Study:

Plants are exposed to various adverse environmental conditions such as drought, high salt and high/low temperature etc. during their life cycles. These environmental stimuli, commonly known as "environmental or abiotic stresses" severely limits plant growth and productivity. One of the plant's responses to these stresses is the induced expression of a large number of genes, whose products are known or believed to be involved in various adaptive functions under stress conditions. Due to its agronomic importance, identification of the stress-induced gene products has been a subject of intense study. As a result, numerous stress-inducible proteins have been identified and their corresponding genes have been isolated. In parallel, numerous molecular biological studies have been performed in order to decipher regulatory elements of these stress-modulated genes.

Unlike their mobile animal counterparts, sessile plants have to adjust their gene expression pattern as well as metabolite profile to cope with altered local environmental conditions (Kulheim et al. 2002). A number of genes are involved in these pathways which can either be up-regulated (Holappa and Walker-Simmons 1995; Hannah et al. 2005; Yan et al. 2005) or down-regulated (Hannah et al. 2005; Yan et al. 2005) based on their mode of function. Identification of these genes may lead to the development of commercial crops which are better suited to grow in a particular unfavorable condition. Due to cross-species transferability of gene function, this improvement can be achieved in non-source plant species as well.

Jute (*Corchorus olitorius*) is the most important fibre producing plant (Kundu 1956). It is the major fibre producing plant of Bangladesh (Hossain et al. 2003) and one of the major fibre crops of the Indian subcontinent (Basu et al. 2004).

Though there is strong demand for jute in both local and international market, there are problems in increasing the productivity and profitability of jute. Some of the major challenges include low yield under unfavorable growth conditions such as salinity, drought,

flood or cold, fungal diseases, photoperiod sensitivity, poor fiber quality etc. Nevertheless, the production quality and yield of this economically important crop is affected by several biotic e.g. fungi, pest, insect, nematode, virus, mite (Ghosh 1983; Keka et al. 2008) and abiotic factors e.g. salinity, submergence and low temperature (Hossain et al. 2003; Samira et al. 2010) . Addressing these challenges through traditional plant breeding program has limitations due to lack of genetic diversity among cultivated jute varieties. Therefore, for developing superior jute varieties with traits such as biotic and abiotic stress tolerance, genetic modification with foreign gene(s) may provide a suitable alternative.

In spite of its enormous commercial value, only a few genes or part of genes from jute have been identified or sequenced (Islam et al. 2005; Wazni et al. 2007; Ahmed et al. 2009; Alam et al. 2010; Samira et al. 2010). Here we report a transcript from *Corchorus olitorus* var 9897 responsive to abiotic stress.

1.10 Objective of the study:

Since the V- H+ ATPase gene was found to be of significant importance in numerous stress response pathways in different species, and no previous study has been found in the case of jute, current study aimed to functional characterize the gene from *Corchorus* spp. With these goals in mind, the objectives of the current study were set as follows:

- Study the expression level of vacuolar proton pump ATPase subunit F under abiotic stresses (salt, low temperature and osmotic stress using mannitol) and non-stress condition by semi-quantitative RT-PCR in the *Corchorus olitorious* var 9897 to identify both constitutive and induced changes in gene expression which may assist in the elucidation of molecular basis of abiotic stress resistance in wild jute species.
- Comparing the expression of the transcript in different time intervals.
- Bioinformatics analysis of the transcript.

[Materials and Methods]

[Chapter 2]

Pages 20-37

2.1 Expression Study

For the study of expression pattern of the verified genes under abiotic stresses, seeds were first germinated and treated with respective stress condition.

2.1.1 Seed Germination

The seeds of *Corchorus olitorius* var O-9897 were grown on the moist tissue paper at different stressed and non stressed conditions. Initially the seeds were incubated at room temperature in the absence of light for four days in Petri dishes in presence of water only. Germinated seedlings were subjected to different stress conditions from the day 4 of their germination.

2.1.2 Stress treatment

Jute seedlings were subjected to abiotic (salt, dehydration, Low temperature) stress.

2.1.2.1 Salt Stress

On day 4, the seedlings were treated with 200 mM NaCl and stressed seedlings were collected in 6, 12 and 24 hours time intervals respectively.

2.1.2.2 Dehydration Stress

On day 4, the seedlings were treated with 100 mM Manitol to create an environment similar to water deficit condition. Then seedlings were collected in 6, 12 and 24 hours time intervals respectively.

2.1.2.3 Low temperature Stress

For cold stress, the seedlings were transferred to an incubator at a temperature of 10^0C on 4^{th} day. The incubation was carried on in the absence of light and stressed seedlings were collected in 6, 12 and 24 hours time intervals respectively.

2.2 Isolation of RNA

The main goal of the isolation process is to recover the maximum yield of high quality total RNA from plant tissues, especially those rich in polyphenolics or starch. TRIZOL Reagent (GibcoBRL) was used for RNA isolation.

2.2.1 Materials

- Jute seedlings
- Liquid nitrogen
- Mortar and pestle
- RNase-free microcentrifuge tubes
- TRIZOL reagent®
- Chloroform
- Isopropanol
- 75 %(v/v) Ethanol
- DEPC-treated water

2.2.2 Methods

The following steps were followed for successful RNA isolation from jute seedlings:

- 1g of frozen seedlings (each of the four samples, 3 stressed and a control) was ground to powder in liquid nitrogen using mortar and pestle.
- 1 mL TRIZOL reagent was added to 1.0g of the grounded tissue, homogenized thoroughly and incubated at room temperature for 10 minutes.
- The sample was centrifuged at 12,000x g for 10 minutes at 4°C.
    - Supernatant (aqueous phase) was transferred to a new tube and 0.2 ml chloroform was added and shaken vigorously by hand for 15 seconds and was incubated at room temperature for 3 minutes.

- The sample was again centrifuged at 12,000x g for 15 minutes at 4°C and supernatant was transferred (aqueous phase) to a new tube. 0.25 ml of iso-propanol and 0.25ml of 2M NaCl was be added to this supernatant.
- This mixture was mixed by inversion and incubated for 10 minutes at room temperature.
- This was centrifuged at 12,000xg for 10 minutes at 4°C and the supernatant (aqueous phase) was removed and the pellet was washed with 75% (v/v) ethanol (2 times).
- The pellet was air-dried and re-suspended in DEPC-treated water.
- Isolated RNA was stored at -80°C.
- The concentration of total RNA was adjusted to 1 µg of total RNA per µl of solution.

2.2.3 Precautions for isolating total RNA

The precautions listed below were strictly maintained to avoid contamination with RNases and subsequent damage of RNA:

- Disposable gloves were used and changed frequently during isolation process.
- Non-disposable items were treated with RNase AWAY™ to remove RNase contamination.
- DEPC treated doubly autoclaved water was used for all solution preparations and dilutions.

- RNA was isolated under a laminar flow hood. The air conditioner was switched off before the start of the work to prevent in-flow of air into the laminar air flow hood.
- RNAses are also found in living cells, so when working with samples it was important to inactivate RNAses before they have a chance to degrade the RNA in the sample. To inactivate cellular RNAses, TRIZOL was added rapidly after grounding of the plant tissue.

2.2.4 Preparation of 0.1% DEPC treated water

0.1% (v/v) solution of Diethyl Pyrocarbonate (DEPC) in distilled water was prepared, shaken vigorously, and allowed to stand one night at room temperature. The solution was autoclaved twice for removing DEPC activity completely.

2.3 Quality of isolated RNA

Quality of purified RNA was determined by electrophoresis in 1.3 % agarose gel.

2.3.1 Materials

- Ultra pure agarose
- MOPS buffer
- RNA gel loading dye
- Gel electrophoresis kit

2.3.1.1 Preparation of Stock Solution (1 liter) of 10X MOPS Buffer

The following reagents were mixed in the given amounts:

MOPS (MW=209.27)	41.86g
Na-Acetate	4.1g
0.5M EDTA (pH 8.0)	20.0 ml

- The pH was adjusted to 7.0 using 1N NaOH prepared with DEPC treated water.
- This volume was made up to 1000 ml with DEPC treated water.

2.3.1.2 Preparation of 1.3% Agarose Gel (50mL)

2.3.1.2.1 Materials

- 1×MOPS (Electrophoresis buffer)
- Formaldehyde
- Agarose
- DEPC-treated water

2.3.1.2.2 Methods

- To prepare 50 ml of 1.3% gel, 0.65g of agarose powder was mixed with 5.0 ml of 10x MOPS (3-Morpholinopropane sulfonic acid) buffer and 42.50 ml of DEPC treated water in a conical flask.
- The agarose was melted in a micro-oven at 60^0C for 2 minutes.

- As the mixture was cooled to 40-45ºC temperature, 2.5 ml formaldehyde was added and mixed by gentle swirling.
- The molten solution was poured on a gel-case to solidify the gel.

2.3.1.3 Sample preparation and gel electrophoresis

2.3.1.3.1 Materials

- Thermal cycler
- RNA-loading buffer (dye)
- RNA sample

2.3.1.3.1.2 Composition of RNA loading buffer

10×MOPS	40µL
Formamide	200µL
37% Formaldehyde	70µL
Glycerol	20µL
DEPC-treated Water	20µL
0.5 M EDTA	0.5µL
10 mg/mL Ethidium bromide	2.0µL
A few drops of Bromophenol Blue	

2.3.1.3.1.3 Methods

- 1.0 µL of RNA sample was added to 3.0 µL of dye and the samples were denatured in a thermal cycler at 65^{0}C for 10 min.
- Denatured samples were quickly chilled on ice for at least 1 minute.
- The gel was assembled in the electrophoresis system and samples were applied into the wells and electrophoresis was carried out at 45 voltage for 4 hours.
- Photograph of the gel was taken on a trans-illuminator under UV light.

2.4 Quantitation of RNA

Quantitation of RNA was carried out using nanodrop (ND-1000). Following steps were followed for RNA quantitation:

1. Blank was set using the spectophotometric reading of DEPC treated H_2O.

2. 1 µL of RNA sample was used to quantitate RNA content.

2.5 Understanding the RNA quality:

Intact total RNA run on a denaturing gel will have sharp 28S and 18S rRNA bands (eukaryotic samples). The 28S rRNA band should be approximately twice as intense as the 18S rRNA band (Figure 2.1, lane 3). This 2:1 ratio (28S:18S) is a good indication that the RNA is intact. Partially degraded RNA will have a smeared appearance, will lack the sharp rRNA bands, or will not exhibit a 2:1 ratio. Completely degraded RNA will appear as a very low molecular weight smear (Figure2.1, lane 2). Inclusion of RNA size markers on the gel will allow the size of

any bands or smears to be determined and will also serve as a good control to ensure the gel was run properly (Figure 2.1, lane 1).

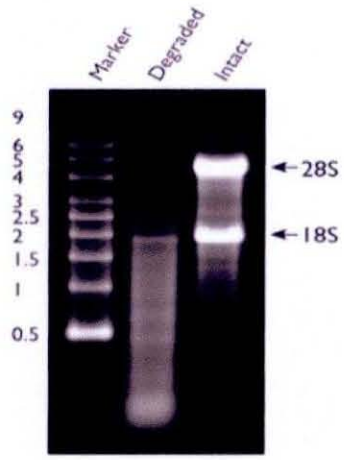


Figure 2.1: Intact vs. Degraded RNA. The 18S and 28S ribosomal RNA bands are clearly visible in the intact RNA sample. The degraded RNA appears as a lower molecular weight smear.

2.6 First Strand cDNA Synthesis

First strand cDNA were synthesized as stated before for all the samples with T_{18} arbitrary primer and PCR was conducted with gene specific primers.

Following steps were followed for cDNA preparation.

1. 4µg of RNA was taken in 0.2 µL PCR tube.

2. 1 µL of 10 mM dNTP was added.

3. 1 µL of primer was added.

4. DEPC treated H_2O was added to make the final volume 13 µL.

5. The mix was heated to 65^0C for 5 minutes and incubated on ice

6. Following reagents were added afterwards:

a.	5x 1st strand buffer	4 µL
b.	0.1 M DTT	1 µL
c.	RNase In	1 µL
d.	RT Enzyme	0.75 µL

7. The mix was subjected to following thermal profiling:

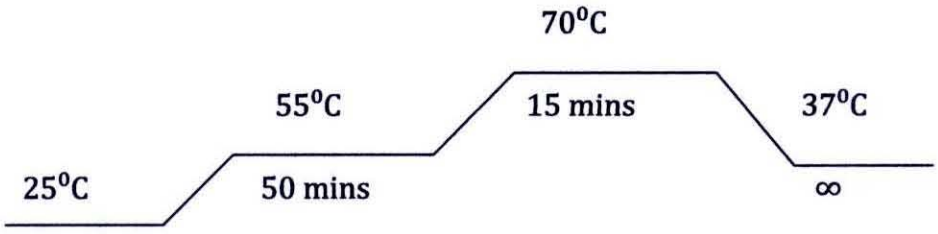


Fig 2.2 Thermal Cycle for cDNA synthesis

8. 0.2 µL of RNase H was added in the mixture and heated at 37°C for 20 min.

2.7 Polymerase Chain Reaction (PCR) with Gene Specific and Actin Primers

2.7.1 Designing of Primers

Following steps were followed to design primers:

1. Web browser was pointed to Primer3Plus URL

(http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).

2. Selected contig was pasted to input box.

3. The program was run.

4. Primer pairs which were from different reads and had similar melting temperature was selected.

When choosing two PCR amplification primers, the following guidelines should be considered, Primer length should be 18-30 bases, The optimal melting temperatures for primers in the range of 52-58°C, Primers should have GC content between 45 and 60 percent, It is well established that the 3' terminal position in PCR primers is essential for the control of mis-priming. Primers should not contain complementary (palindromes) within themselves and they must have unique sequence, Primers need to be designed with absolutely no intra-primer homology beyond 3 base pairs.

2.7.1.1 List of primers for RT-PCR (Semi-quantitative analysis)

All primers were designed by using primer3 tool & analyzed by oligo analyzer

2.7.1.1.1 Gene Specific Primers

Table –2.1: List of primers for semi-quantitative PCR and their properties

					Primer	
Primer I.D	Oligo Name	Length	Desired Band Size (bp)	^ Ta (°C)	5´-Sequence -3´	TARGET GENE
v-H_F1	Forward primer	20	220	56.3	TCG TTG ATT CGA AAACCACA	V- H+ ATPase
v-H_R1	Reverse primer-1	20	220	60.4	CGG ATT CAG TCG AAA AGAGG	V- H+ ATPase
v-H_R2	Reverse primer-2	20	248	60.4	CAG ATG CAA CGG ATTCAGTC	V- H+ ATPase

2.7.1.1.2 Actin primers

Table – 2.2: List of actin primers as control for semi-quantitative analysis.

Name of the Primers	Sequence of the primers 5′-Sequence -3′	Length	T_m(°C)			GC Content (%)
Actin deg Forward	TGGCATCAYACWTTC TACAATGA	23	MIN 59.8	MEAN 60.9	MAX 62.2	37
Actin deg Reverse	GNRYATTGAAVGTCT CAAACAT	23	56.8	61.2	65.7	39.9

2.7.2 Dilution of Primer

The working concentrations of gene specific primers were 10ng/μL. Therefore, the stock primer solutions were diluted using TE buffer to the desired concentration

2.7.3 Preparation of dNTPs Mixtures

20 μL of each of dATP, dGTP, dCTP, dTTP (their concentrations being 100 mM each) were mixed in fresh, autoclaved eppendorf tube and the final volume was made 200 μL by adding PCR water and stored at -20^0C. The concentration of each of the nucleotide in the above mixture was 10 mM.

2.7.4 Preparing Master Mix for PCR

The following reaction mixture was added to a 0.2 or 0.5 ml thin-wall PCR tube sitting on ice for each sample.

Reagent	Volume (µL)
10x PCR Buffer	1.5
25 mM MgCl2	0.8
10 mM dNTP	1.0
PCR H_2O	8.5
Taq Polymerase	0.2
cDNA	2.0
Forward Primer (10x)	0.5
Reverse Primer (10x)	0.5
Total	15

The thermal cycling profiles that were programmed to amplify the gene by Polymerase Chain Reaction (PCR) for 25 cycles are as follows:

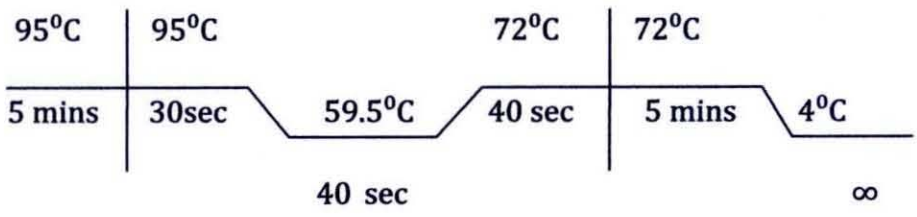


Fig 2.3: Thermal Cycling Profile Used in PCR

2.7.5 Analysis of RT-PCR product

PCR products were analyzed by agarose gel electrophoresis.

2.7.5.1 Agarose gel electrophoresis of DNA

The standard method was used to separate and identify DNA fragments through agarose gel electrophoresis.

2.7.5.1.1 Materials

- Ultra pure agarose,
- TAE buffer,
- Gel loading dye and
- Gel electrophoresis kit.

2.7.5.1.1.1 Preparation of stock solution (1 liter) of 50X TAE buffer

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

This solution was made up to 1000 ml with ddH_2O.

2.7.5.1.1.2 Preparation of 1.50 % agarose gel (50 mL)

- To prepare 50 ml of 1.5% agarose gel, 0.75g of agarose powder was weighed in conical flask.

- 1mL of 50X TAE was taken in a measuring cylinder and the volume was made up to 50 ml with dd water.
- The mixture of 50X TAE and H_20 was poured into flask containing agarose and then melted in micro-oven at 60^0C for 2-3 minutes.

2.7.5.1.1.3 Composition of DNA dye

i.	Xylene cyanol	:	0.25%
ii.	Bromophenol blue	:	0.25%
iii.	Glycerol	:	30%

2.7.5.1.1.4 Preparation of 1X TAE buffer used in gel electrophoresis

For 250 ml of TAE

50X TAE	5 ml
dd H_2O	245 ml

The gel was loaded and analyzed by following procedure:

2.7.5.1.1.5 Materials

- Electrophoresis buffer (1X TAE buffer).
- Ladder.
- Loading dye.
- PCR products.

2.7.5.1.1.6 Method

- Before sample application, the electrophoresis apparatus and gels were assembled. Then 500 ml of electrophoresis buffer was added such that the top of gel was well covered.
- Samples (2 µL of PCR products + 3 µL of dye) and ladder (3µL) were applied to wells.
- Electrophoresis was carried out for 1 hour at 100 V.
- The gel was stained with ethidium bromide.
- Band was visualized by UV.

2.8 Bioinformatics: Sequence analysis

Table 2.3: Bioinformatics tools used for analysis the jute v-H+ATPase subunit F gene

Source	URL	Service
BLAST (blastn, blastx and blastp)	www.ncbi.nih.gov/blast	Database homology search
CDD	http://www.ncbi.nlm.nih.gov/cdd/	Conserved Domain Identification
Translate Tool	http://www.expasy.ch/tools/dna.html	Translate sequence

Source	URL	Service
ClustalW (1.83)	http://www.ebi.ac.uk/tools/clustalw	Multiple sequence alignment and phylogenetic tree construction
SOPMA	http://npsapbil.ibcp.fr/cgibin/npsa_automat.pl?page=npsa_sopma.html	Secondary structure analysis
Kyte and Doolittle scale	http://www.expasy.ch/cgi-bin/protscale.pl	Search hydrophobic membrane spanning segments
TMHMM	http://www.cbs.dtu.dk/services/TMHMM/	Prediction of transmembrane helices in proteins
PSI-PRED	http://bioinf.cs.ucl.ac.uk/psipred	Secondary structure analysis
Swiss Model	http://swissmodel.expasy.org/	Protein structure homology-modeling analysis
Mega4	http://www.megasoftware.net/	Phylogenetic tree analysis

2.8.1 Procedure:

- The browser was pointed to www.ncbi.nlm.nih.gov/BLASTClicked the "Nucleotide query- Translated database" link.
- Pasted the nucleotide sequence into the BLAST search window in FASTA format.
- The BLAST button was clicked.
- The Results page appeared and maximum perfect matches were selected choosing higher score and lowest E-value.

In the same manner all other analysis were carried out.

[Results]

[Chapter 3]

Pages 38-59

3.1 RNA Estimation:

For the purpose of RNA isolation seeds of *C. olitorius* var 9897 were germinated on petri dishes. They showed better germination at 30 to 35°C than that of lower temperature at 25°C.

3.1.1 Qualitative estimation of RNA:

Although the ratio of the isolated RNA was good, it could sometimes be misleading, as RNA is very sensitive and is prone to damage and even get degraded due to improper handling. Gel electrophoresis was used to check for genomic DNA contamination and RNA decay. Optical density was used to assay the RNA yield and to check for contamination by salt, solvent, protein, *etc.* So the isolated RNA was checked by agarose gel electrophoresis (Fig 3.1).

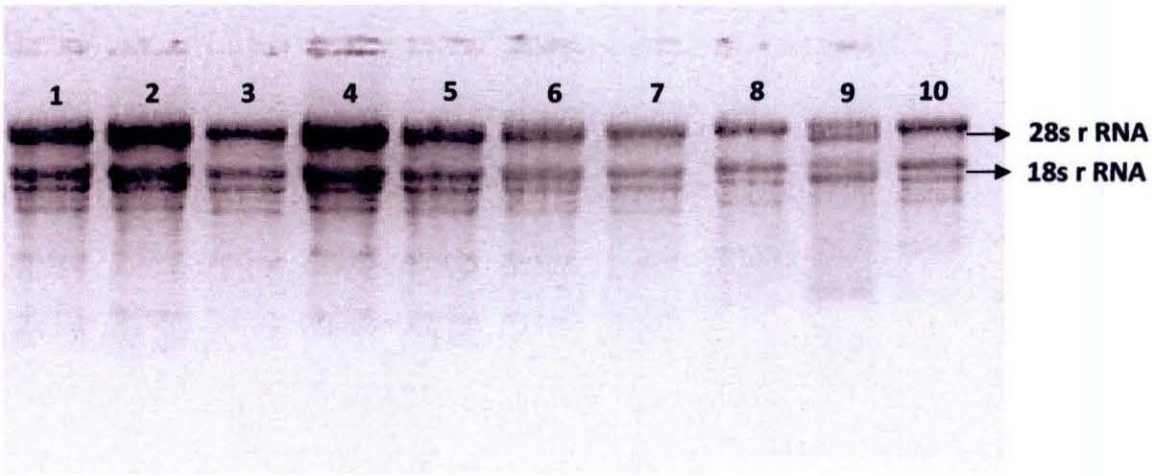


Fig 3.1: Agarose gel electrophoresis of isolated total RNA from Normal and Stressed seedlings of *C. olitorius* var 9897.

Isolated total RNA showing (Fig 3.1) the ribosomal bands in each lane as follows; from Left to right;

Lane 1: *C. olitorius,* var 9897 (Normal)

Lane 2-4*: C. olitorius,* var 9897 (6 hr stressed Low temperature, Salt, Mannitol)

Lane 5-7: *C. olitorius,* var 9897 (12 hr stressed Low temperature, Salt, Mannitol)

Lane 8-10: C.olitorius, var 9897 (24 hr stressed Low temperature, Salt, Mannitol)

3.1.2 Quantitative estimation of RNA:

Concentration and quality of the isolated RNA were determined by spectroscopic analysis using a nanodrop (Table 3.1).

Table 3.1: Spectrophotometric analysis of isolated RNA

Jute Genotype	Condition	Time Interval	$OD_{260/280}$	$OD_{260/230}$	Concentration (ng/µL)
O – 9897	Normal	0 hr	1.96	2.05	3308.6
	Salt	6 hr	2.11	2.11	1245.3
	Low Temp.	6 hr	2.11	2.13	2289.9
	Osmotic	6hr	1.91	1.96	1836.9
O – 9897	Salt	12 hr	2.10	1.60	1412.5
	Low Temp.	12 hr	2.06	1.75	2377.5
	Osmotic	12 hr	1.89	0.92	2475.7
O – 9897	Salt	24 hr	2.09	1.35	1189.4
	Low Temp.	24 hr	2.09	0.88	1626.7
	Osmotic	24 hr	2.04	1.66	1542

3.2 Expression Analysis of Vacuolar H⁺ -ATPase subunit F in *C. olitorius,* var 9897

To perform semi-quantitaive RT-PCR for detrmining the expression pattern of V-H+ATPase sybunit F in *C. Oloitorius* O-9897 varieties, cDNA was synthesized with T_{18} primer. Same cDNA was used for normalization using Actin primers shown in figure 3.2.

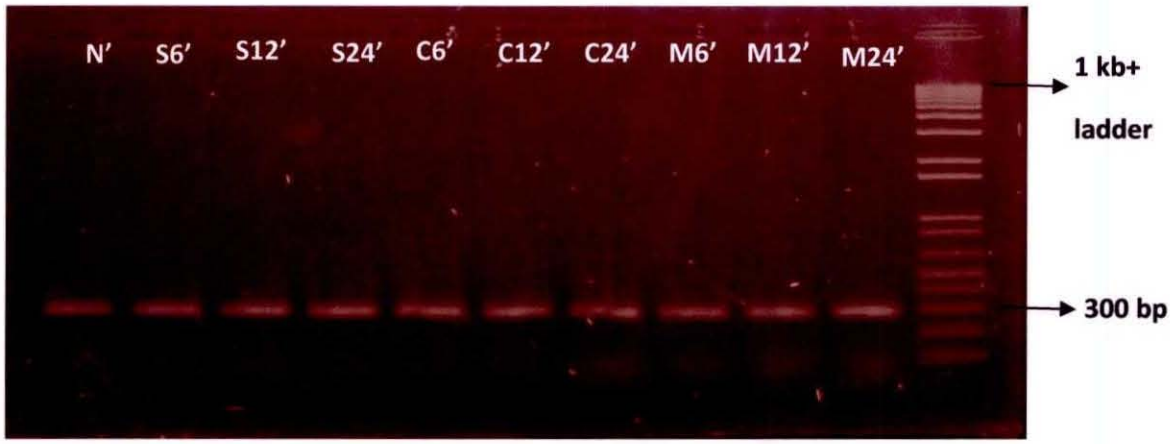


Fig 3.2: RT-PCR was carried out with cDNA using Actin primers (25 cycles). cDNAs used were of O-9897-Normal, Salt 6,12 and 24hrs, Low temperature 6, 12 and 24 hrs and Drought 3, 6 and 24 hrs stress conditions.

In normalization, all the bands for RT-PCR products were of same intensity. RT-PCR was conducted with gene-specific primers of V-H+ATPase subunit F using cDNAs from normal and different stressed conditions. All the amplified products were run on 1.5% agarose gel and the following figure (Fig: 3.3) show the results obtained.

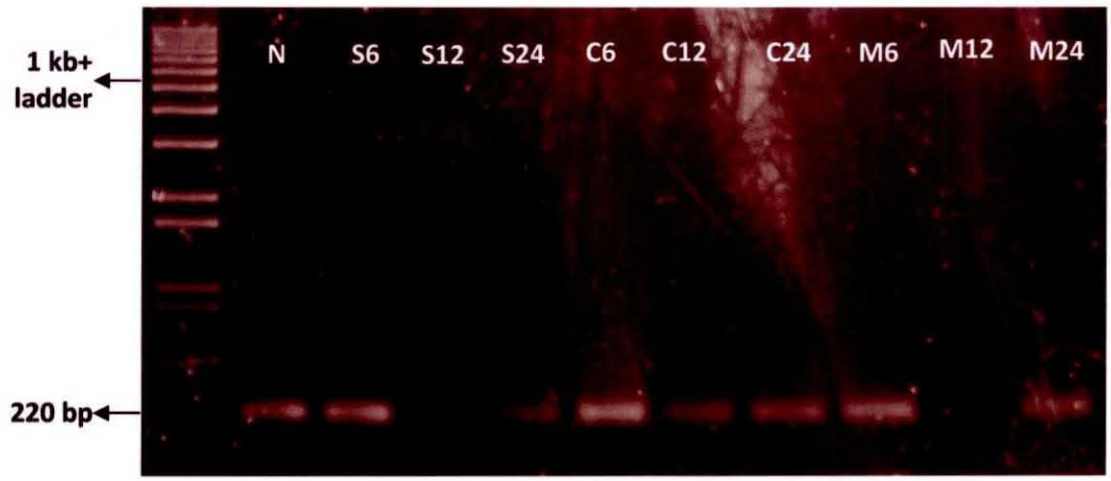


Fig 3.3: RT-PCR analysis of the F subunit of Vacuolar H+ ATPase under different stress conditions. RT-PCR was carried out with cDNA and v-H+ATPase subunit F primers (25 cycles). cDNAs used were of O-9897-Normal, Salt- 6,12 and 24hrs, Low temperature- 6, 12 and 24 hrs and Drought- 6,12 and 24 hrs stress conditions.

Agarose gel electrophoresis of PCR products showed (Fig: 3.3) the band of desired size (220bp). Each of the lanes showed the expected band of *C. olitorius* var 9897 as follows; from left to right:

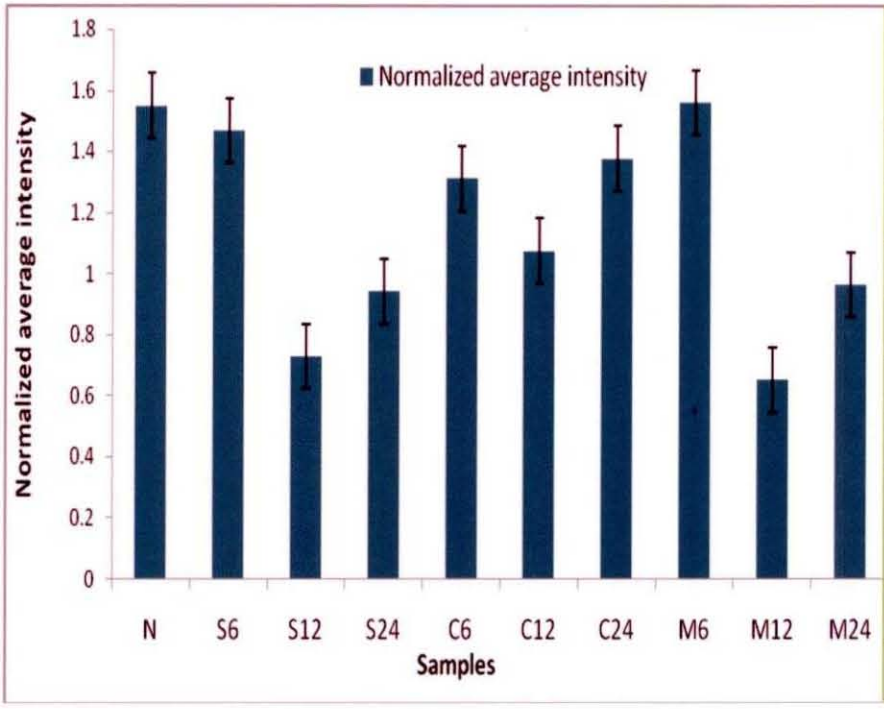
Lane N: RT-PCR product of ***C. olitorius***, var 9897-normal

Lane S6, S12, S24: RT-PCR product of ***C. olitorius***, var 9897- Salt (200 mM NaCl) stress for 6 hrs, 12 hrs and 24 hrs respectively.

Lane C6, C12, C24: RT-PCR product of ***C. olitorius***, var 9897-Low Temperature (10^0C) stress for 6 hrs, 12 hrs and 24 hrs respectively.

Lane M6, M12, M24: RT-PCR product of ***C. olitorius***, var 9897 –Drought (100 mM Mannitol) stress for 6 hrs, 12 hrs and 24 hrs.

The gel images were analyzed using GelScan version 6 software available freely in web (http://www.bioscitec.de/download_gelscan.htm). Based on the band intensity variation observed in the gel, this software gave numerical value for all the bands as well as bar diagrams (Fig: 3.4). For authenticity, RT-PCRs were performed in duplications and average intensity values were taken to plot the bar diagram.



Sample	Normalizes Average Intensity
N	1.549741231
S6	1.467864372
S12	0.729556729
S24	0.940529961
C6	1.310150376
C12	1.074461207
C24	1.377076049
M6	1.560683325
M12	0.651579444
M24	0.964650767

Fig: 3.4 Band Intensity of V-H+-ATPase F-subunit gene in *C. Olitorious* var 9897 **at normal condition and when seedlings treated with NaCl (200 mM), Mannitol (100 mM) and low temperature (10^{0}C) for 6, 12 and 24 hrs respectively.**

In the fig 3.4, average intensity of the bands of RT-PCR (done in duplicates) products of the samples O-9897- Normal, Salt (200 mM NaCl), Low temperature (at 10^{0}C) and drought (100 mM Mannitol) stress for 6, 12 and 24hrs respectively.

Band intensity values are produced by the software and shown in the adjacent bar diagram where variations in expression under different stressed conditions in comparison to without stress were observed.

Expression pattern of V-ATPase subunit F also shown in time dependent manner (Fig: 3.5). Based on the values the software produced the adjacent data are plotted along a time (0, 6, 12 and 24 hrs) dimension where variation in expression under different stressed conditions in comparison to without stress was observed.

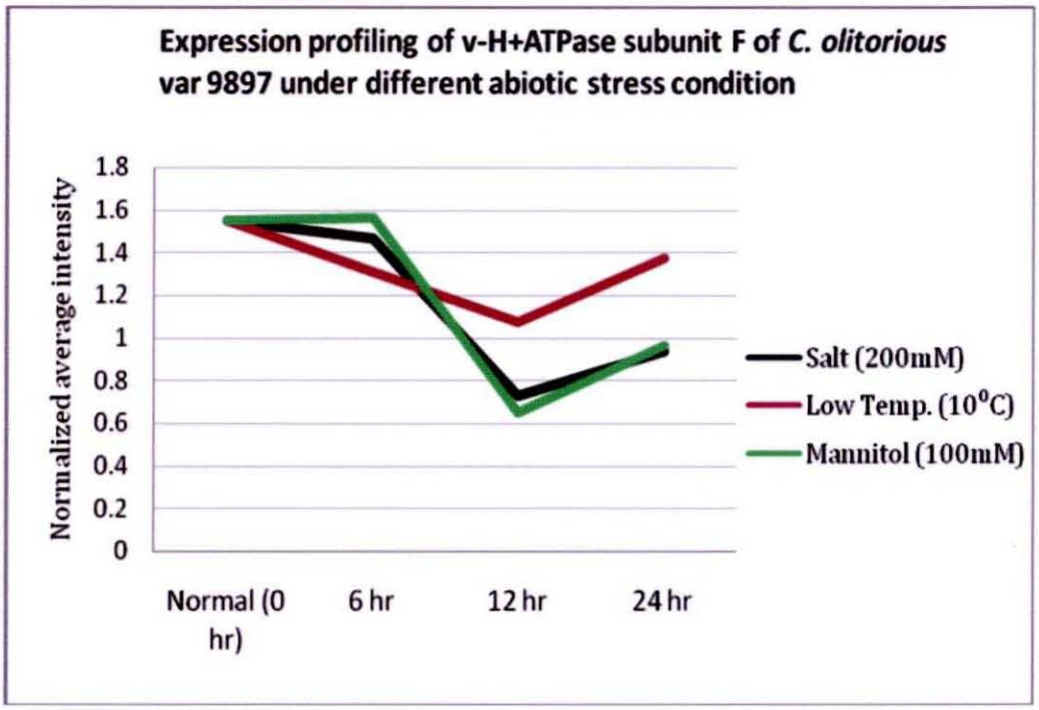


Fig 3.5 Time series graph based on band intensity shows the expression pattern of V-H+-ATPase F-subunit gene in *C. olitorious* var 9897 at Normal unstressed condition and when seedlings were treated with NaCl (200 mM), Mannitol (100 mM) and low temperature (10⁰C) for 6, 12 and 24 hrs .

3.5 Bioinformatics analysis:

Bioinformatics analysis was performed by using different bioinformatics tools like Primer3, ExPASY, BLAST, SOPMA, CLUSTALW etc.

3.5.1 Sequence Identification

The sequence of *Corchorus olitorius* vacuolar proton pump subunit F mRNA, complete cds was first identified and submitted to NCBI (ACCESSION EU024516) by Taliafero et al. (09-JUL-2007). From NCBI database the sequence was obtained (Fig: 3.6).

Corchorus olitorius vacuolar proton pump subunit F mRNA, complete cds

GenBank: EU024516.1

FASTA Graphics

Go to:

```
LOCUS       EU024516                393 bp    mRNA    linear   PLN 05-AUG-2007
DEFINITION  Corchorus olitorius vacuolar proton pump subunit F mRNA, complete
            cds.
ACCESSION   EU024516
VERSION     EU024516.1  GI:154269265
KEYWORDS    .
SOURCE      Corchorus olitorius
  ORGANISM  Corchorus olitorius
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
            rosids; malvids; Malvales; Malvaceae; Grewioideae; Corchorus.
REFERENCE   1  (bases 1 to 393)
  AUTHORS   Taliaferro,J.M., Islam,A.S., Wazni,M.W. and Sathasivan,K.
  TITLE     Expressed Sequence Tags (ESTs) from a Jute (Corchorus olitorius)
            cDNA Library
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 393)
  AUTHORS   Taliaferro,J.M., Islam,A.S., Wazni,M.W. and Sathasivan,K.
  TITLE     Direct Submission
  JOURNAL   Submitted (09-JUL-2007) Molecular, Cell & Developmental Biology,
            University of Texas at Austin, 2400 Speedway, ESB 1B, Austin, TX
            78712, USA
FEATURES             Location/Qualifiers
     source          1..393
                     /organism="Corchorus olitorius"
                     /mol_type="mRNA"
                     /db_xref="taxon:93759"
     CDS             1..393
                     /note="ATPase"
                     /codon_start=1
                     /product="vacuolar proton pump subunit F"
                     /protein_id="ABS72193.1"
                     /db_xref="GI:154269266"
                     /translation="MAGRAQIKTSSSALIAMIADEDTVTGFLMAGVGNVDLRRKTNYL
                     IVDSKTTVKAIEDAFKEFTTREDIAIILISQYVANMIRFLVDSYNNPIPAILEIPSKD
                     HPYDPAHDSVLSRVKYLFSTESVASGRY"
ORIGIN
        1 atggcgggaa gagctcaaat taagacaagc agctcagcac taattgctat gattgctgat
       61 gaggatacag taactggatt tttgatggct ggagttggaa atgtggattt aaggagaaaa
      121 accaactatc tgatcgttga ttcgaaaacc acagtgaagg ctattgaaga cgcattcaaa
      181 gagtttacca caagggaaga cattgcaatt attttgatca gtcaatatgt cgcaaacatg
      241 ataaggtttc tagttgatag ctacaataat ccaattccag caatcttgga aatcccttcc
      301 aaagatcatc cttatgatcc tgctcatgat tctgttctct cacgtgtgaa gtacctcttt
      361 tcgactgaat ccgttgcatc tggaaggtat tga
//
```

Fig: 3.6 Submitted Sequence of *Corchorus olitorius* vacuolar proton pump subunit F mRNA.

3.5.2 Primer Characterization:

3.5.2.1 Gene specific primer:

For studying the expression level, gene specific primers (v-H-F1, v-H_R1, v-H_R2) were designed from the 393 bp sequence. Primers were designed using free software "Primer3".

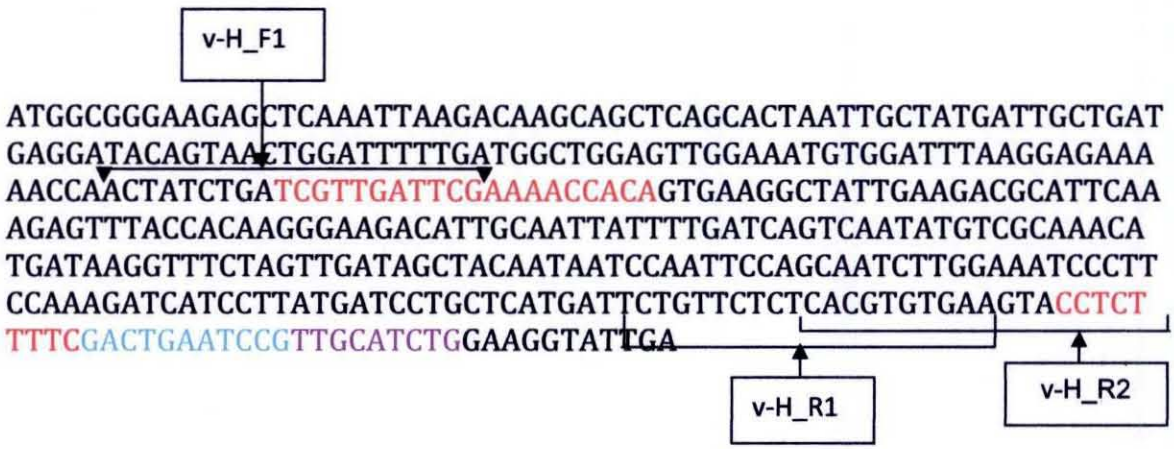


Figure 3.7: Sequence showing the positions of the primers (v-H_F1, v-H_R1 and v-H_R2)

3.5.3 Translation of the sequence into protein:

The nucleotide sequence was translated into protein by using ExPASY Translate tool.

XXXXXXXXXXXXXXXXXXXXMAGRAQIKTSSSALIAMIADEDTVTGFLMAG
VGNVDLRRKTNYLIVDSKTTVKAIEDAFKEFTTREDIAIILISQYVANMIRFLV
DSYNNPIPAILEIPSKDHPYDPAHDSVLSRVKYLFSTESVASGRY**Stop**

Fig 3.8: Translated protein sequence of vacuolar proton pump ATPase subunit F (*C. olitorious* var 9897).

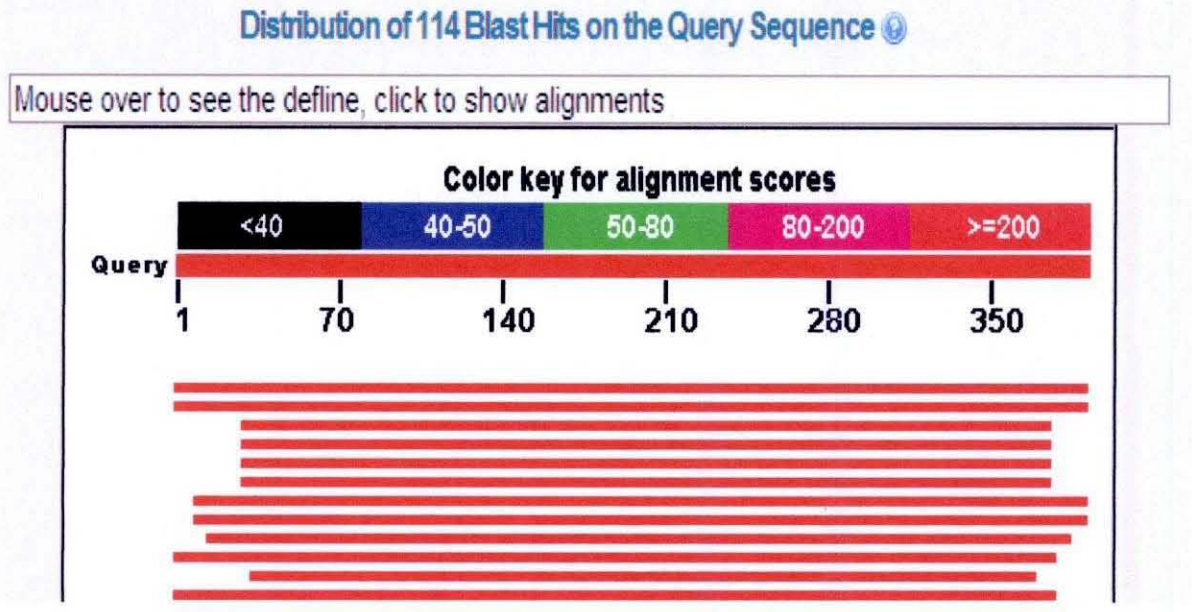
3.5.4 BLAST analysis:

BLAST (Basic Local Alignment Search Tool) was used to compare the sequence with sequences stored in NCBI sequence database (NCBI). Top hits of the search result are enlisted in the Table 3.5.2.1, 3.5.2.2 and 3.5.2.3

3.5.4.1 BLASTn output:

BLAST search (blastn) was carried out with complete nucleotide sequence. It was searched in nucleotide database using nucleotide sequence.

GRAPHICAL SUMMARY



Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
EU024516.1	Corchorus olitorius vacuolar proton pump subunit F mRNA, complete c	710	710	100%	0.0	100%	
XM_002268025.1	PREDICTED: Vitis vinifera hypothetical protein LOC100267411 (LOC10	376	376	100%	7e-101	81%	UGM
AK324363.1	Solanum lycopersicum cDNA, clone: LEFL1076DH07, HTC in leaf	349	349	88%	9e-93	82%	U
AK246221.1	Solanum lycopersicum cDNA, clone: FC04BA06, HTC in fruit	349	349	88%	9e-93	82%	U
BT012798.1	Lycopersicon esculentum clone 113812R, mRNA sequence	349	349	88%	9e-93	82%	U
AK328948.1	Solanum lycopersicum cDNA, clone: LEFL3074J17, HTC in root	343	343	88%	4e-91	81%	U
XM_002306777.1	Populus trichocarpa predicted protein, mRNA	336	336	97%	6e-89	79%	UG
EF144930.1	Populus trichocarpa clone WS0111_A02 unknown mRNA	336	336	97%	6e-89	79%	UG
BT089174.1	Soybean clone JCVI-FLGm-1E8 unknown mRNA	333	333	94%	7e-88	79%	UG
AK224280.1	Oryza punctata cDNA, clone: BBS21A10, expressed in shoot apical m	307	307	96%	3e-80	77%	
BT102094.1	Picea glauca clone GQ0166_D16 mRNA sequence	304	304	86%	3e-79	79%	
AK224312.1	Oryza officinalis cDNA, clone: CCP01D08, expressed in panicle of CC	298	298	96%	1e-77	77%	
DQ317309.1	Musa acuminata putative vacuolar ATP synthase subunit F mRNA, pa	298	298	89%	1e-77	78%	

Fig: 3.9 Graphical summary of BLASTn output for vacuolar proton pump ATPase subunit F gene in *C. olitorious* var 9897.

GENE ID	Protein ID	Organism	Score	E-value	Similarity
gb|EU024516.1|	vacuolar proton pump subunit F mRNA	*Corchorus olitorius*	710	0.0	100%
ref|XM_002268025.1|	hypothetical protein	*Vitis vinifera*	376	7e-101	81%
ref|XM_002306777.1|	predicted protein	*Populus trichocarpa*	336	6e-89	79%
ref|NM_116496.3|	V-type proton ATPase subunit F	*Arabidopsis thaliana*	282	1e-72	78%
gb|DQ198167.1|	Vacuolar ATP-related protein	*Nicotiana tabacum cultivar Samsun NN*	214	5e-52	85%

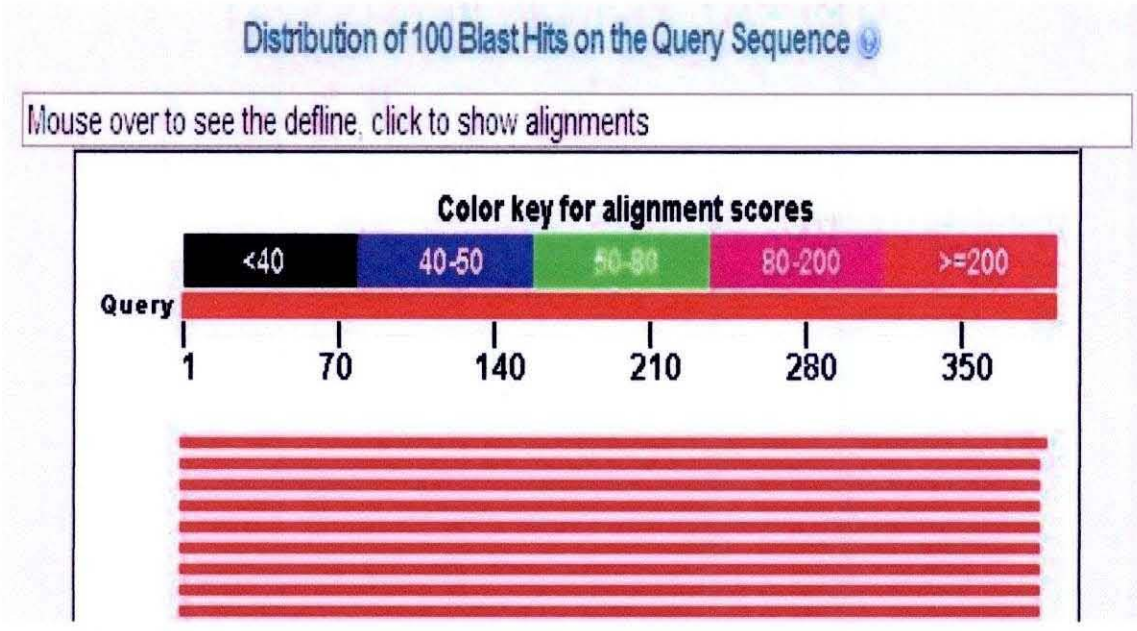
Table 3.2: BLASTn output for vacuolar proton pump ATPase subunit F gene in *C. olitorious* var 9897.

The BLASTn result revealed that the sequence identified from *C. olitorious* var 9897 has similarity with hypothetical protein of *Vitis vinifera* (81%) and with V-type proton ATPase subunit F of *Arabidopsis thaliana* (78%).

3.5.4.2 BLASTx output:

BLAST search (blastx) was carried out with nucleotide sequence. It was searched in protein database using a nucleotide sequence.

GRAPHICAL SUMMARY



Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E – value	Max ident	Links
ABS72193.1	vacuolar proton pump subunit F [Corchorus olitorius]	253	253	99%	5e-86	100%	
XP_002268061.1	PREDICTED: hypothetical protein [Vitis vinifera] >emb|CAN63147.1| h	237	237	98%	8e-80	93%	UG
ACU13254.1	unknown [Glycine max]	231	231	98%	2e-77	89%	G
XP_002461176.1	hypothetical protein SORBIDRAFT_02g042380 [Sorghum bicolor] >gb|	229	229	98%	1e-76	88%	UG
NP_001149476.1	vacuolar ATP synthase subunit F [Zea mays] >gb|ACG35548.1| vacu	229	229	98%	1e-76	88%	UG
NP_001048577.1	Os02g0824700 [Oryza sativa Japonica Group] >dbj|BAD22867.1| puta	228	228	98%	3e-76	89%	UG
NP_001149843.1	vacuolar ATP synthase subunit F [Zea mays] >gb|ACG26498.1| vacu	228	228	98%	5e-76	87%	UG
XP_002453078.1	hypothetical protein SORBIDRAFT_04g037920 [Sorghum bicolor] >gb|	226	226	98%	3e-75	87%	UG
ACF82913.1	unknown [Zea mays] >gb|ACG26199.1| vacuolar ATP synthase subui	226	226	98%	3e-75	86%	G
XP_002306813.1	predicted protein [Populus trichocarpa] >gb|ABK93132.1| unknown [I	226	226	98%	3e-75	88%	UG
XP_002522069.1	vacuolar ATP synthase subunit f, putative [Ricinus communis] >gb|EE	222	222	98%	7e-74	85%	G
ABF29867.1	vacuolar ATPase subunit F [Triticum aestivum] >dbj|BAJ94730.1| pre	220	220	98%	6e-73	85%	G

Fig: 3.10 Graphical summary of BLASTx output for vacuolar proton pump ATPase subunit F gene in *C. olitorious* var 9897.

GENE ID	Protein ID	Organism	Score	E-value	Similarity
gb|ABS72193.1|	vacuolar proton pump subunit F	*Corchorus olitorius*	253	4e-86	100%
ref|XP_002268061.1|	hypothetical protein	*Vitis vinifera*	237	8e-80	93%
ref|NP_001149476.1|	vacuolar ATP synthase subunit F	*Zea mays*	229	1e-76	88%
dbj|BAD22867.1|	putative Vacuolar ATP synthase subunit F	*Oryza sativa Japonica*	228	3e-76	89%
ref|XP_002306813.1|	predicted protein	*Populus trichocarpa*	226	3e-75	88%
ref|NP_192171.1|	V-type proton ATPase subunit F	*Arabidopsis thaliana*	218	5e-72	86%

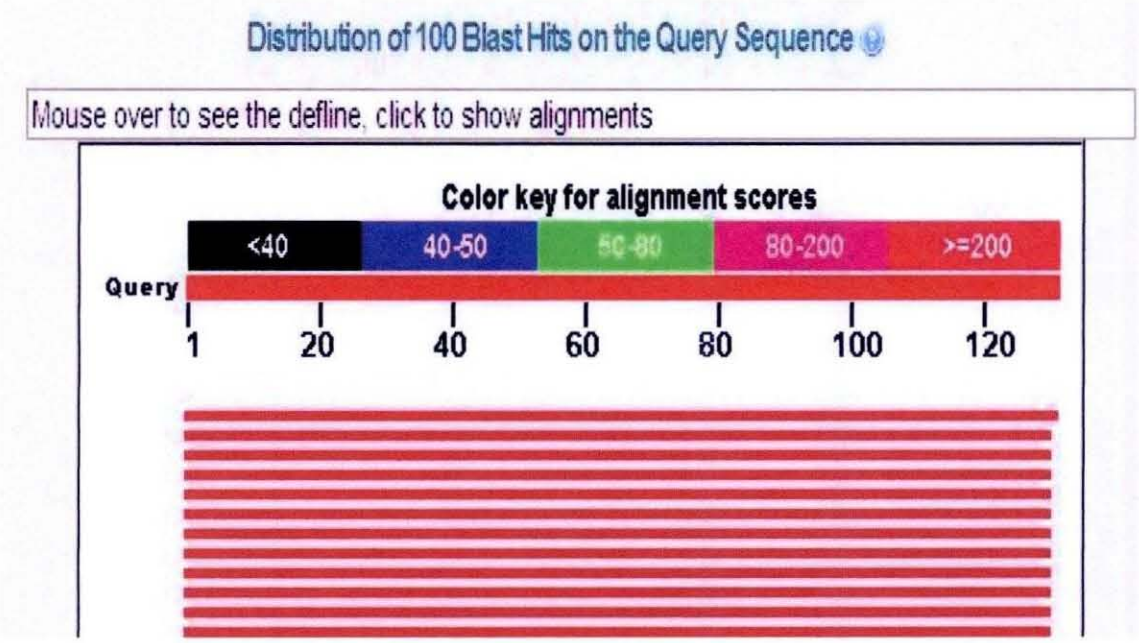
Table 3.3: BLASTx output for vacuolar proton pump ATPase subunit F gene in *C. olitorious* var 9897.

The BLASTx result revealed that the sequence identified from *C. olitorious* var 9897 has significant similarity with hypothetical protein of *Vitis vinifera* (93%) & V-type proton ATPase subunit F of *Arabidopsis thaliana* (86%).

3.5.4.3 BLASTp output:

Protein-BLAST search (blastp) was carried out with translated nucleotide sequence. It searched the entire protein database at NCBI using a translated protein sequence.

GRAPHICAL SUMMARY



Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Links
ABS72193.1	vacuolar proton pump subunit F [Corchorus olitorius]	265	265	100%	7e-91	
XP_002268061.1	PREDICTED: hypothetical protein [Vitis vinifera] >emb|CAN63147.1| hy	248	248	99%	3e-84	UG
ACU13254.1	unknown [Glycine max]	241	241	99%	2e-81	G
NP_001149476.1	vacuolar ATP synthase subunit F [Zea mays] >gb|ACG35548.1| vacuol	238	238	99%	3e-80	UG
XP_002461176.1	hypothetical protein SORBIDRAFT_02g042380 [Sorghum bicolor] >gb|EE	238	238	99%	3e-80	UG
XP_002306813.1	predicted protein [Populus trichocarpa] >gb|ABK93132.1| unknown [Po	237	237	99%	6e-80	UG
NP_001048577.1	Os02g0824700 [Oryza sativa Japonica Group] >dbj|BAD22867.1| putati	237	237	99%	8e-80	UG
NP_001149843.1	vacuolar ATP synthase subunit F [Zea mays] >gb|ACG26498.1| vacuol	236	236	99%	1e-79	UG
ACF82913.1	unknown [Zea mays] >gb|ACG26199.1| vacuolar ATP synthase subunit	234	234	99%	8e-79	G
XP_002453078.1	hypothetical protein SORBIDRAFT_04g037920 [Sorghum bicolor] >gb|EE	234	234	99%	8e-79	UG
XP_002522069.1	vacuolar ATP synthase subunit f, putative [Ricinus communis] >gb|EEF	233	233	99%	3e-78	G

Fig: 3.11 Graphical summary of BLASTp output for vacuolar proton pump ATPase subunit F gene in *C. olitorious* var 9897.

GENE ID	Protein ID	Organism	Score (total)	E-value	Similarity
gb|ABS72193.1|	vacuolar proton pump subunit F	*Corchorus olitorius*	265	7e-91	100%
emb|CAN63147.1|	hypothetical protein	*Vitis vinifera*	248	3e-84	99%
gb|ACG35548.1|	vacuolar ATP synthase subunit F	*Zea mays*	238	3e-80	99%
dbj|BAD22867.1|	putative Vacuolar ATP synthase subunit F	*Oryza sativa Japonica*	237	8e-80	89%
ref|NP_192171.1|	V-type proton ATPase subunit F	*Arabidopsis thaliana*	227	8e-76	85%

Table3.4: BLASTp output for the vacuolar proton pump ATPase subunit F gene in *C. olitorious* var 9897.

The BLASTp result revealed that the sequence identified from *C. olitorious* var 9897 has significant similarity with hypothetical protein of *Vitis vinifera* (99%), vacuolar ATP synthase subunit F of *Zea mays* (99%) and V-type proton ATPase subunit F of *Arabidopsis thaliana* (85%).

3.5.5 Determination of Conserved Domain:

Conserved domain is identified using NCBI conserved domain tools.

Conserved domain analysis showed that ATP-synthase (F/14-kDa) subunit domain is present in this subunit. This family includes 14-kDa subunit from V-ATPase, which is in the peripheral catalytic part of the complex (Fig: 3.12).

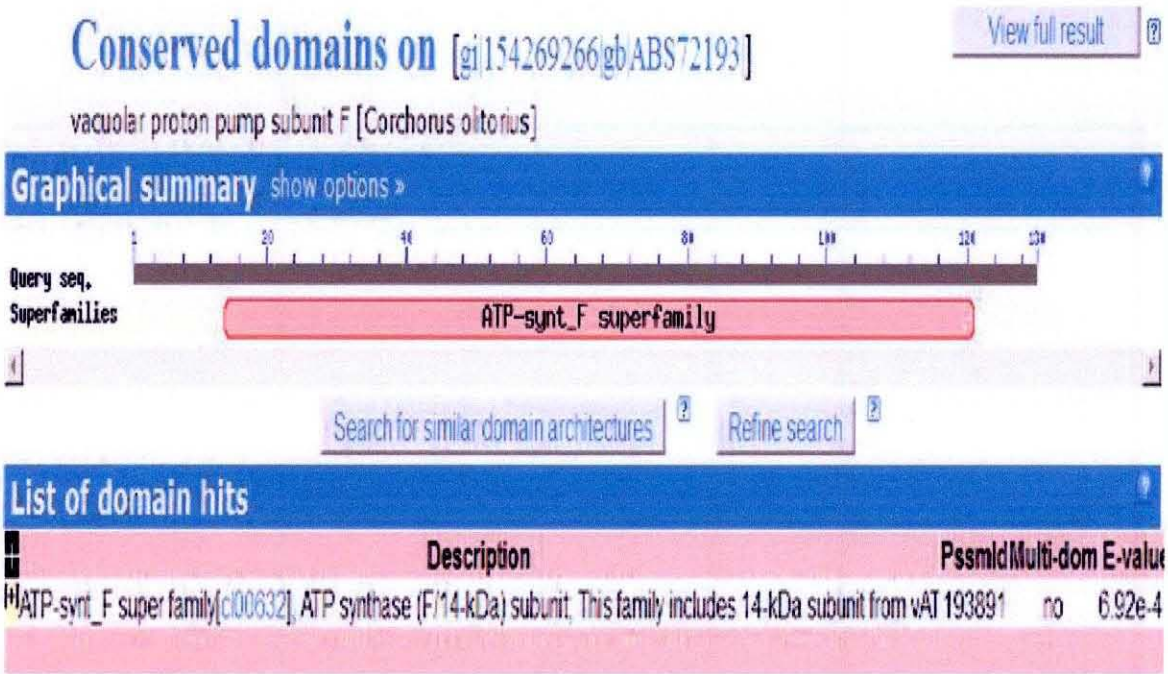


Fig 3.12: Conserved domain of vacuolar proton pump ATPase subunit F in *C. olitorious* var 9897.

3.5.6 Transmembrane Segment Prediction:

To identify whether the protein is transmembrane or not, the sequence was analyzed in Kyte-Doolittle and TMHMM scale. Two prominent peaks (above 1.6 score) were found indicating a high probability that the sequence contains two transmembrane segments (Fig: 3.13 and Fig: 3.14).

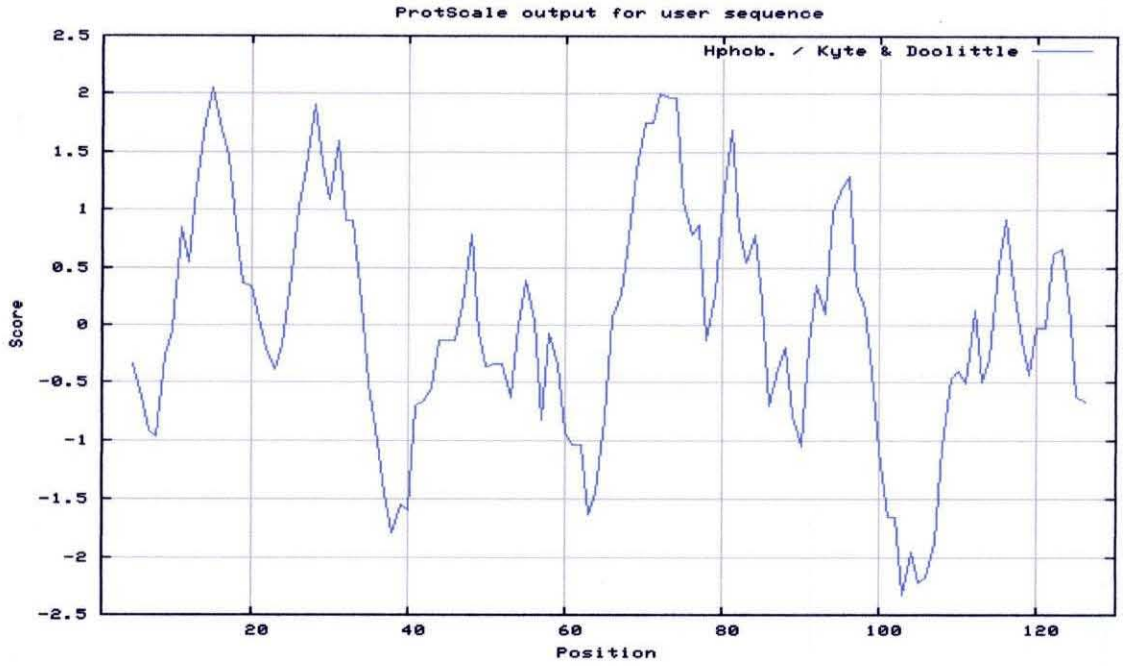


Fig 3.13: Kyte-Doolittle scale shows the transmembrane segments of vacuolar proton pump ATPase subunit F *C. olitorious* var 9897.

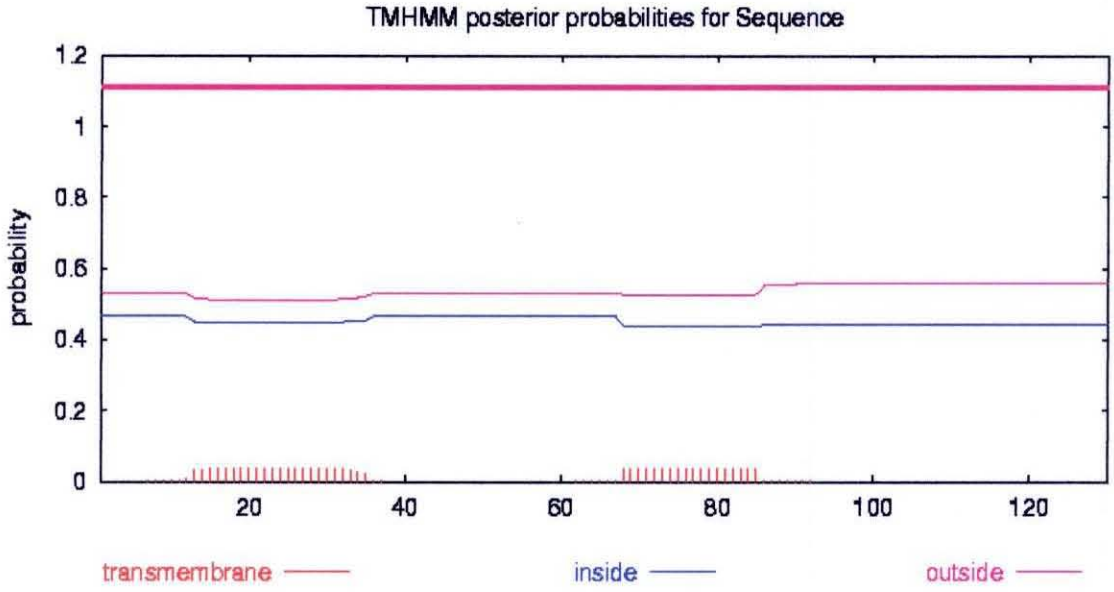


Fig 3.14: TMHMM scale for transmembrane segments of vacuolar proton pump ATPase subunit F *C. olitorious* var 9897.

3.5.7 Secondary Structure Analysis:

The function of a protein is firmly related with its structure. To understand the structure-function relationship, the secondary structure of translated jute sequence was predicted using SOPMA and PSIPRED (Fig: 3.12- a,b), which showed a good number of alpha helices (36.15%), random coils (41.54%) and extended strands (20%) with absolute absence of β sheets (0.0%).

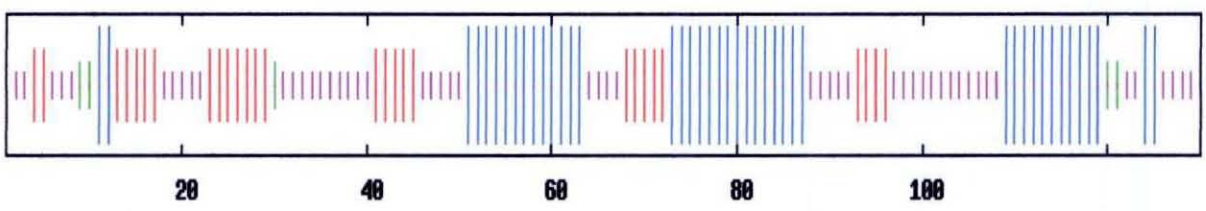
a.

```
       10         20         30         40         50         60
70
        |          |          |          |          |          |
|
MAGRAQIKTSSSALIAMIADEDTVTGFLMAGVGNVDLRRKTNYLIVDSKTTVKAIEDAFKEFTTR
EDIAI
hcccehcccchheeeeccccchheeeeetcccccccccceeeeecccchhhhhhhhhhhhhc
cccee
ILISQYVANMIRFLVDSYNNPIPAILEIPSKDHPYDPAHDSVLSRVKYLFSTESVASGRY
eeehhhhhhhhhhhhhhccccceeeecccccccccccchhhhhhhhhhhttcchhcccc

Sequence length:   130
```

SOPMA:

Alpha helix	**(Hh)**	**:**	**47 is**	**36.15%**
3_{10} helix	**(Gg)**	**:**	**0 is**	**0.00%**
Pi helix	**(Ii)**	**:**	**0 is**	**0.00%**
Beta bridge	**(Bb)**	**:**	**0 is**	**0.00%**
Extended strand	**(Ee)**	**:**	**26 is**	**20.00%**
Beta turn	**(Tt)**	**:**	**3 is**	**2.31%**
Bend region	**(Ss)**	**:**	**0 is**	**0.00%**
Random coil	**(Cc)**	**:**	**54 is**	**41.54%**
Ambigous states	**(?)**	**:**	**0 is**	**0.00%**
Other states		**:**	**0 is**	**0.00%**



b.

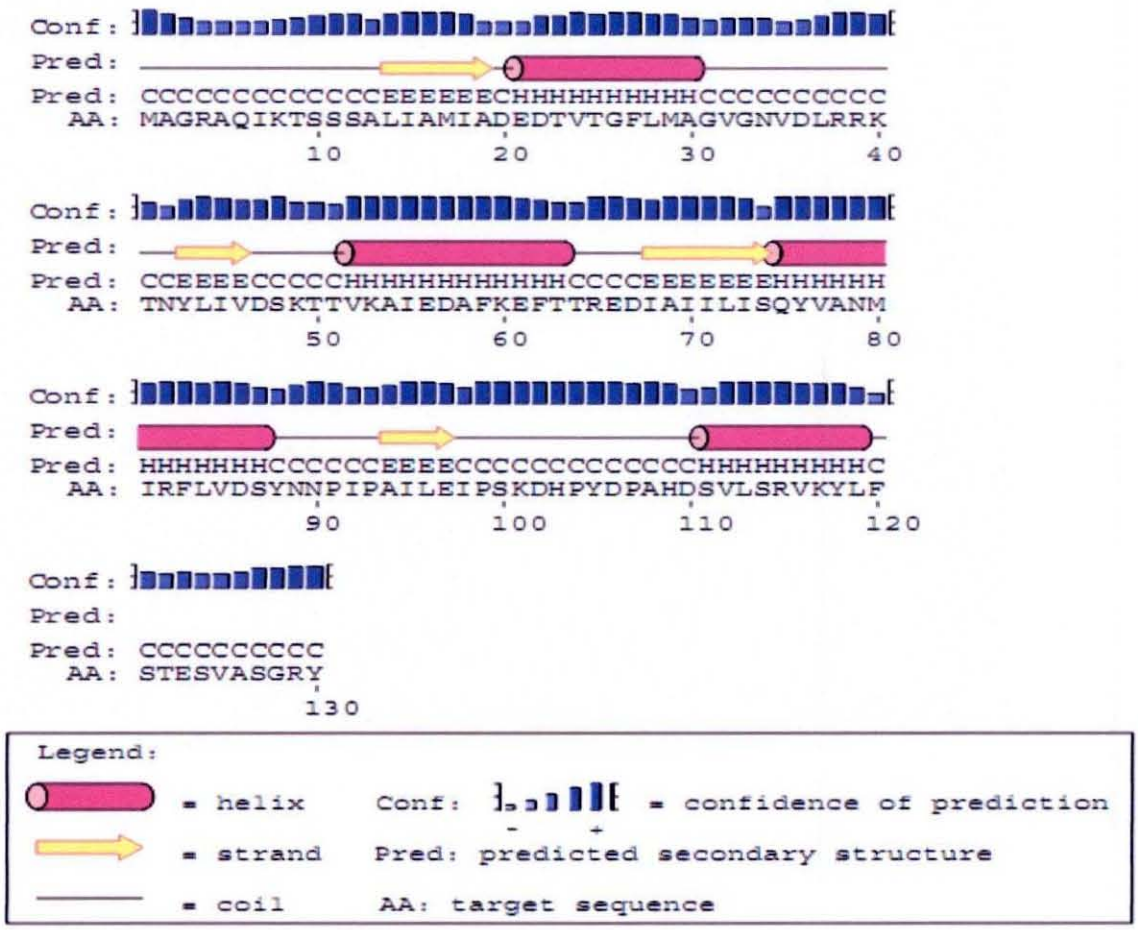


Fig 3.15: Secondary structure analysis of translated sequence of vacuolar proton pump ATPase subunit F gene using (a) SOPMA and (b) PSIPRED software.

3.5.8 Multiple sequence alignment:

In order to construct a phylogenetic tree multiple sequence alignment was done by ClustalW. All of these protein sequences were aligned with the jute sequence.

A multiple alignment of sequences of different plant species namely *Vitis vinifera, Zea mays, Sorghum bicolor, Populus trichocarpa, Ricinus communis, Oryza sativa* and

Arabidopsis thaliana along with jute was done using ClustalW (Fig: 3.16) and the conserved domains were found as follows:

```
CLUSTAL 2.1 multiple sequence alignment


Vitis_vinifera              MAGRAQIPTKSSALIAMIADEDTITGFLLAGVGNVDLRRKTNYLIVDSKT 50
Corchorus_olitorius         MAGRAQIKTSSSALIAMIADEDTVTGFLMAGVGNVDLRRKTNYLIVDSKT 50
Zea_mays                    MAGRANIPTNNSALIAIIADEDTVTGFLMAGVGNVDLRKKTNYLLVDNKT 50
Sorghum_bicolor             MAGRASIPTNNSALIAIIADEDTVTGFLMAGVGNVDLRKKTNYLLVDNKT 50
Oryza_sativa_Japonica       MAGRPSIPTNSSALIAIIADEDTVTGFLLAGVGNVDLRKKTNYLIVDNKT 50
Populus_trichocarpa         MANRAQIATNNSALIAMIADEDTIVGLLMAGVGNVDLRRKTNYLIVDSKT 50
Ricinus_communis            MANRNQIRTNNSALIAMIADEDTVVGFLLAGVGNVDLRRKTNYLLVDSKT 50
Arabidopsis_thaliana        MAGRATIPARNSALIAMIADEDTVVGFLMAGVGNVDIRRKTNYLIVDSKT 50
Arabidopsis_lyrata          MAGRAPIPARNSALIAMIADEDTVVGFLMAGVGNVDIRRKTNYLIVDSKT 50
                            **.*  * : .*****:******:.*:*:*******:*:*****:**.**

Vitis_vinifera              TVKQIEDAFKEFTTKEDIAIILISQYVANMIRFLVDSYNKPVPAILEIPS 100
Corchorus_olitorius         TVKAIEDAFKEFTTREDIAIILISQYVANMIRFLVDSYNNPIPAILEIPS 100
Zea_mays                    TVKQIEDAFKEFTTREDIAIVLISQYIANMIRFLVDSYNKPVPAILEIPS 100
Sorghum_bicolor             TVKQIEDAFKEFTTREDIAIVLISQYIANMIRFLVDSYNKPVPAILEIPS 100
Oryza_sativa_Japonica       TVKQIEDAFKEFTTREDIAIVLISQYVANMIRFLVDSYNRPVPAILEIPS 100
Populus_trichocarpa         TVKQIEDAFKEFTTREDIAIVLISQFVANMIRFLVDSYNKPVPAILEIPS 100
Ricinus_communis            TVKQIEDAFKDFTTREDIAIVMISQYVANMIRFVVDSYNKPVPAILEIPS 100
Arabidopsis_thaliana        TVRQIEDAFKEFSARDDIAIILLSQYIANMIRFLVDSYNKPVPAILEIPS 100
Arabidopsis_lyrata          TVRQIEDAFKEFSARDDIAIILLSQYIANMIRFLVDSYNKPVPAILEIPS 100
                            **: ******:*:::****:::**::******:*****.*:********

Vitis_vinifera              KDHPYDPAHDSVLSRVKYLFSAESVASGRR 130
Corchorus_olitorius         KDHPYDPAHDSVLSRVKYLFSTESVASGRY 130
Zea_mays                    KDHPYDPAHDSVLSRVKYLFSAESVASDRR 130
Sorghum_bicolor             KDHPYDPAHDSVLSRVKYLFSAESVASDRR 130
Oryza_sativa_Japonica       KDHPYDPAHDSVLSRVKYLFSAESVASDRR 130
Populus_trichocarpa         KDHPYDPTQDSVLSRVKYLFSAESVASGRR 130
Ricinus_communis            KDHPYDPSQDSVLSRVKHLFSAESVASGRR 130
Arabidopsis_thaliana        KDHPYDPAHDSVLSRVKYLFSAESVSQR-- 128
Arabidopsis_lyrata          KDHPYDPAHDSVLSRVKYLFSAESVSQR-- 128
                            *******::********:***:***:.
```

Fig 3.16: Multiple sequence alignment of jute and other species

* = this column of the alignment contains identical amino acid residues in all sequences.

: = this column of the alignment contains different but highly conserved (very similar) amino acids.

. = this column of the alignment contains different amino acids that are somewhat similar.

blank = this column of the alignment contains dissimilar amino acids or gaps.

3.5.9 3-D Model of vacuolar proton pump ATPase subunit F

The 3D structure of the protein was predicted by SWISS MODEL server (http://swissmodel.expasy.org/) which is a protein homology based modelling server.

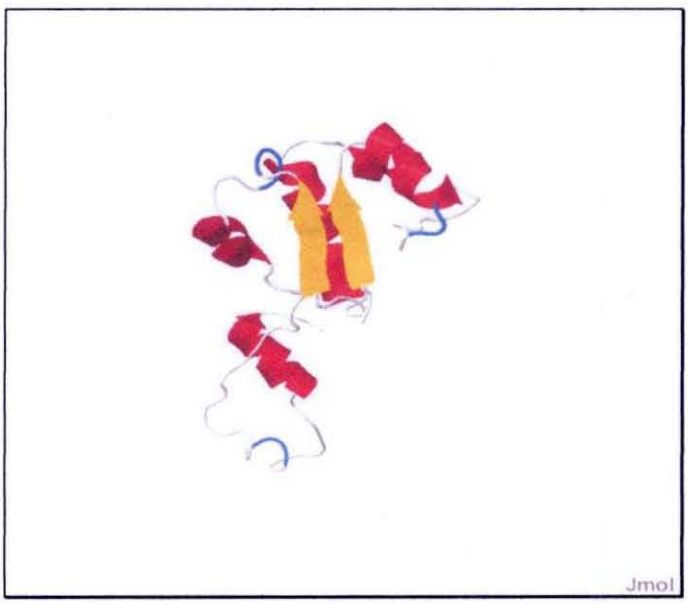


Fig 3.17: 3-D model prediction of vacuolar proton pump ATPase subunit F

Predicted protein (Fig: 3.17) was viewed by Jmol viewer which is an open-source Java viewer for chemical structures in 3D (http://www.jmol.org/). In this predicted model the structures in yellow are the two parallel β-sheets and the red helical structure is α-helix characteristic of V-H+ATPase Subunit F.

3.5.10 Phylogenetic Tree (Neighbour Joining Method):

A Phylogenetic analysis of vacuolar proton pump ATPase subunit F was performed. The tree was based on an alignment of the following protein sequences deposited in GenBank

Phylogenetic tree (Fig: 3.18) showed that the protein resides in the same clade with *Vitis vinifera*. It also revealed that *C. olitorious* var 9897, *P. trichocarpa* and *R. communis* were in the same group.

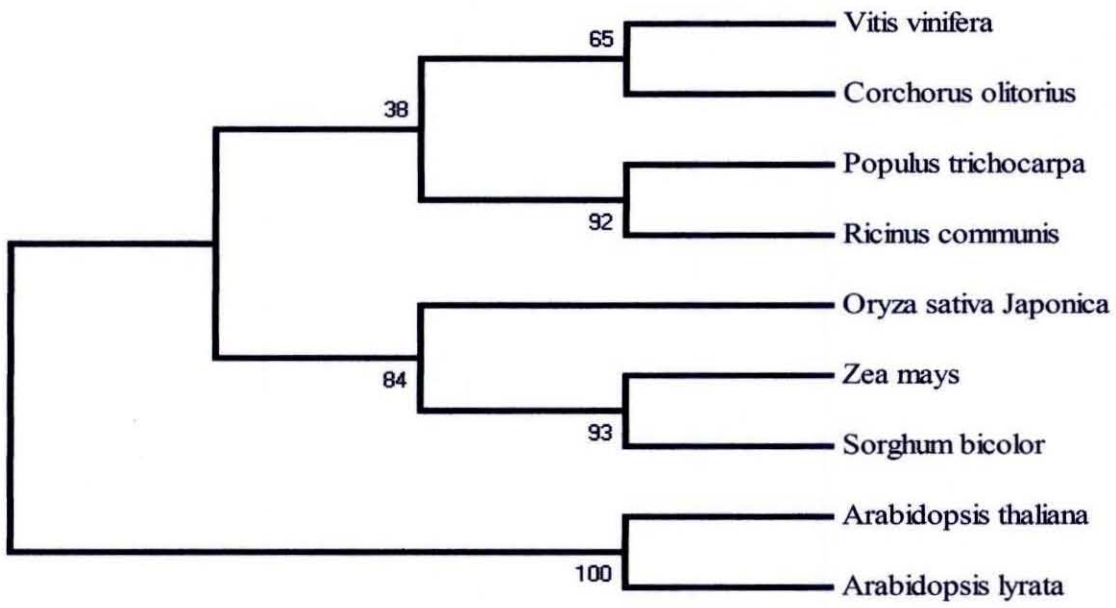


Fig 3.18: Phylogenetic tree of jute and other species using Neighbour Joining Method.

[Discussion and Conclusion]

[Chapter 4]

Pages 60-69

Jute is one of the most important fibre crops, and stands second only to cotton, in providing environment-friendly, biodegradable and renewable ligno-cellulose fibre. But in the field of molecular biology jute is a new crop and only a few research references are available at the molecular level on DNA markers, tissue culture and genetic transformation. The whole genome sequence of jute has been unveiled and it will unravel the huge opportunity for the development of the jute fiber.

Abiotic stress is the primary cause of crop loss world wide, reducing average yields for most major crop plants by more than 50% (Boyer 1982; Bray et al. 2000). Drought and salinity are becoming particularly widespread in many regions, and may cause serious salinization of more than 50% of all arable lands by the year 2050 (Wang et al. 2003). Abiotic stress leads to a series of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and productivity (Wang et al. 2001). Drought, salinity, low/extreme temperatures and oxidative stress are often interconnected, and may induce similar cellular damage. For example, drought and/or salinization are manifested primarily as osmotic stress, resulting in the disruption of homeostasis and ion distribution in the cell (Zhu 2001). Oxidative stress, which frequently accompanies high temperature, salinity, or drought stress, may cause denaturation of functional and structural proteins (Wang et al. 2003). Plants have adapted to respond to these stresses at the molecular and cellular levels as well as at the physiological and biochemical levels, thus enabling them to survive.

Expression of a variety of genes is induced by these stresses in various plants (Ingram and Bartels 1996; Thomashow 1999; Shinozaki and Yamaguchi-Shinozaki 2000). The products of these genes function not only in stress tolerance but also in the regulation of gene expression and signal transduction in stress responses (Bartels et al. 2005; Shinozaki et al. 2003; Xiong et al. 2002). Environmental conditions are highly variable, stress sensing and response mechanisms are expected to change rapidly and require constant innovation. Under stress conditions

such as salinity and drought the survival of plant cells largely relies on the maintenance of V-ATPase activity, indicating that V-ATPase plays an important role in plant adaptation to stress (Dietz et al. 2001). Several reports suggested that V-ATPase subunit expression and protein synthesis is altered in response to environmental stress. Thus, it is likely that V-ATPase genes are highly regulated under different stress conditions.

As per literature documentation this is the first attempt to study the V-H+ ATPase subunit F gene expression patterns under different abiotic stresses in jute. In a previous study (Wazni et al. 2007) first described the identification of vacuolar proton pump ATPase subunit F mRNA as a new expressed sequence tag (ESTs) from a cDNA library of *Corchorus olitorius* L. var. O-4 (GenBank Accession EU024516).

The present study has two distinct parts:

1) Expression profiling of vacuolar proton pump ATPase subunit F gene at abiotic stress conditions in jute *Corchorus olitorius* var O-9897 and

2) Bioinformatics analysis of this subunit.

4.1 Expression Pattern of Vacuolar Proton Pump ATPase Subunit F at Different Stress Conditions:

To characterize the V-H+ATPase gene functionally, the expression of the gene under normal and abiotic stress conditions was monitored. For this, very high quality total RNA was isolated from non-stressed and stressed (Salt with 200mM NaCl, Low temperature at 10^0C, Drought with 100mM mannitol) seedlings of O-9897 variety. Due to high mucilage content with other polysaccharide 4 days old seedlings were preferred for molecular analysis in this study. The quantity of RNA measured by spectrophotometer showed high value with very good 260/280 and 260/230 ratios (Table 3.1) which eliminated the presence of protein or DNA contaminants. Gel electrophoresis confirmed the integrity of the RNA molecule as distinct bands of ribosomal RNA could be seen (Fig: 3.1)

For cDNA synthesis 4µg of RNA was taken and commercially available RT-PCR kit was used for the same purpose. Normalization with actin primers was done for all the cDNAs and then semi-quantitative RT-PCR was conducted with V-H+ATPase subunit F specific primers.

The current understanding indicates that V-H+ATPase subunit F is positively regulated in response to abiotic stress. Interestingly, we got a similar profile for transcript expression of V-H+ATPase subunit F in jute when treated with different abiotic stresses for 24 hrs. This type of expression pattern is similar to the expression patterns observed in all other plants specially in common ice plant *Mesembryanthemum crystallinum* (Dietz et al. 2001). However, we observed a different scenario when seedlings of jute were exposed with stress for short duration i.e. 6 hours.

From the semi-quantitative RT-PCR, it was found that the expression level of the vacuolar proton pump ATPase subunit F gene had remained almost unchanged in first six hour under 200 mM NaCl, low temperature at 10^0C and 100 mM mannitol stress when compared to the normal condition. However, it showed a decreased expression pattern when treated with salt, low temperature and mannitol for 12 hour (Fig 3.3). Interestingly current study revealed the subsequent enhanced expression level of the V-H+ ATPase subunit F gene in 24 hours compared to the 12 hour.

Although the V-H+ ATPase subunit F gene appears to be involved in a general stress response to physiological stress that plant cells experience as a result of dehydration, high salt and low temperature. Dehydration causes osmotic stress by a direct loss of water (Hong et al. 1997). High salt causes osmotic stress by reducing water potential (Hong et al. 1997). Low temperature stress is also known to cause osmotic stress by reducing the water supply from the roots to green tissues (Yamaguchi-Shinozaki and Shinozaki 1993). Thus, it is possible that induction of the V-H+ ATPase subunit F gene is due to a common osmotic stress and that the gene is involved in an osmotic rather than in a general stress response.

During the first six hour stress, the gene did not show significant altered expression compared to the no stress condition. Initial period of the given stress conditions used in this study was not enough to elicit sufficient osmotic response within a very short period of time.

However, the expression of V-H+ ATPase subunit F is significantly altered after 12 hours in all given stresses compared to normal one (Fig 3.2); this could possibly be due to the fact that the failure of the plant to establish an adaptive response or since micro RNA mediated gene regulation is a common phenomena in plants, the decreased expression level can be due to the micro RNA(Carrington and Ambros 2003; Dugas and Bartel 2004; Zhang et al. 2006). However, it has not yet been

determined whether this decline in V-H+-ATPase subunit F mRNA is the result of selective transcriptional down-regulation and/or post transcriptional events.

Most important finding of this study is the subsequent upregulation of the transcript when treated with 200 mM NaCl, 100 mM mannitol and low temperature at 10^0C for 24 hours. This may be due to the reestablishment of cellular homeostasis. As a result, the level of subunit F mRNA in seedlings reached the level normally found in unstressed control seedlings of jute. Jute was able to retain the activity of V-H+-ATPase during the period of 24 hour stress exposure. The results also suggest that the adaptation to high salt concentrations, osmotic stress and low temperature depend on an increase in the amount of V-H+ATPase transcripts. Similar findings were reported by Dietz et al. (2001). The increased expression of the gene for 24 hr stress exposure may be due to the accumulation of ABA as indicated by Tsiantis et al. (1996) for subunit c in *M. crystallinum* and by Kasai et al. (1993) in barley or by accumulation of gibberelin as reported by Cooley and coworkers (1999) for subunit A and B in tomato at the infection site. However, another study suggested G proteins involvement in the signal transduction pathways affecting V-ATPase activity (Golldack and Dietz 2001). As of date a little is known about the regulation of this gene in plant species at molecular level and it opens a field for future investigation.

As per literature survey, the findings of this study concur with that of previous experiments where short-term stress treatments were investigated for a small subset of V-ATPase genes in other species and other subunits as well (Dietz and Arbinger 1996; Low et al. 1996; Dietz et al. 2001; Dietz et al. 2003; Gao et al. 2011; Wang et al. 2011)

Since jute is an important cash crop, a functional study of V-H+ ATPase subunit F by biochemical and transgenic approach should give valuable information on the signal transduction pathway activated by osmotic stress and/or by the stress hormone. These findings would help in understanding a jute plant better with regard to its mechanism for withstanding various stresses it has to face in the inhospitable terrains where it is now grown.

4.2 Bioinformatics analysis:

In BLAST search, **Blastn** was carried out with nucleotide sequence V-ATPase subunit F mRNA (*C. olitorious* var 9897) to search the nucleotide database for related nucleotide sequences. **Blastx** was carried out to search the protein databases for related protein sequences. **Blastp** was carried out with translated protein sequence (*C. oitorious* var.9897) to search the protein databases for related protein sequences. Results obtained were significant.

The sequence identified from V-ATPase subunit F mRNA (*C. olitorious* var.9897) has significant similarity with a vacuolar proton pump subunit F mRNA of *Vitis vinifera* (81%) and with a predicted protein of *Populus trichocarpa* (79%) and with a V-type proton ATPase subunit F of *Arabidopsis thaliana* (78%) in blastn (Table 3.2).

In blastx, 93% similarity was found with hypothetical protein of *Vitis vinifera* and 86% similarity was found with V-type proton pump ATPase subunit F of *A. thaliana* (score : 218 , E-value: 5e-72) as shown in table 3.3.

From the analysis by blastp we can see that the vacuolar-ATPase subunit F of *C. olitorious* var.9897 has 99% similarity with that of hypothetical protein of *Vitis vinifera* (score: 248, E-value: 3e-84) and with vacuolar ATP synthase subunit F of *Zea mays* and 89% similarity with a putative vacuolar ATP synthase subunit F of *Oryza sativa* and also 85% similarity with the V-type proton ATPase subunit F of *A. thaliana* (Table 3.3).

The score of *C. olitorious* of vacuolar-ATPase subunit F (blastn, blastx, blastp) was highest as the query sequence and e – value of other species were low indicating a strong homology with this protein. The high score and low e – value indicates a strong homology with the *Vitis vinifera, Populus* and *Arabidopsis* protein with the *C.olitorious* var 9897 sequence.

It was also revealed from the blastp analysis that the vacuolar-ATPase subunit F has a single conserved domain, ATP-synthase (F/14-kDa) subunit domain. This family includes a 14-kDa subunit from V-ATPase, which is in the peripheral catalytic part of the complex (Fig 3.12).

Protein sequences were analyzed by **Kyte-Doolittle** scale and **TMHMM** scale. Few peaks were found with two having values more than 1.6 (Fig 3.13 and Fig: 3.14). Probably two transmembrane segments are present in the vacuolar-ATPase subunit F gene of *C. olitorious* var 9897. This analysis was used in determining the transmembrane location of the protein.

In **Secondary Structure Analysis** the protein showed (Fig 3.15) a good number of alpha helices (36.15%), random coils (41.54%) and extended strands (20%), with absolute absence of beta sheets (0.0%). α-helices are generally considered slightly weaker than β-sheets, and are readily attacked by the ambient water molecules. α helices have particular significance in DNA binding motifs, including helix-turn-helix motifs, leucine zipper motifs and zinc finger motifs(Kohn, Mant et al. 1997). Coiled-coil α helices are highly stable forms in which two or more helices wrap around each other in a "supercoil" structure (Mason and Arndt 2004). They indicate sites for potential protein-protein interaction (Mason and Arndt 2004).

The online server (SWISS MODEL server) predicted a 3-D structure (Fig: 3.17) of vacuolar-ATPase subunit F based on homology modeling which showed the presence of two parallel β sheets and alpha helices. The model was automated based on a deposited similar structure of subunit F of V-type ATPase of *Thermus thermophilus* (RCSB PDB ID: 2D00).

The **Multiple Sequence Alignment** of the translated jute sequence with other proteins showed highly conserved regions among the sequences, indicating strong

homology in both structure and function with the vacuolar-ATPase subunit F of other species (Fig:3.16).

Phylogenetic tree showed that the protein resides in the same clade with *Vitis vinifera*. It also revealed that *C. olitorious* var 9897, *P. trichocarpa* and *R. communis* were in the same group. Since the genes placed in the same clade of phylogenetic tree are expected to share similar biochemical or biological functions (Eisen and Wu 2002), it is likely that homologues of vacuolar proton pump ATPase subunit F from *V. vinifera*, *P. trichocarpa* and *R. communis* might have similar function in stress response pathway. However, a detail study is needed to find out any correlation between expression and phylogenetic placement of the genes of V-H+ ATPase family.

4.3 Conclusion

The current study is a significant step towards understanding the expression pattern of vacuolar proton pump ATPase subunit F gene in jute species. The findings may be summarized as-

- The expression is slightly modified within the first six hours and decreases in the next 12 hours but eventually increase with delayed exposure (24 hours) under different abiotic stress conditions which indicate that this gene is actively responsive to abiotic stresses.
- The current study indicates a more transient expression profile of V-H+ATPase F subunit.

4.4 Future Perspectives:

Despite the significant progress made in elucidating the structure of the V-ATPase, identification of regulatory mechanisms and cloning subunit genes, there remain many open questions which needs to be addressed in the near future: (1) The complete understanding of the process of V-ATPase assembly, including the differentiation of self assembly processes and catalyzed reactions, (2) the role of subunit isoforms in the structural, enzymatic and regulatory context of V-ATPase function, (3) the expressional regulation of subunits in distinct tissues, developmental stages and under environmental stress.

Based on this study the future research can be aimed to

1. Identify the full length sequence of the F subunit of V-H+ATPse through the RACE strategy (Frohman et al. 1988).
2. Real time study for expression profiling under different stress conditions.
3. Investigation of the interaction of V-H+ATPase F subunit with other subunits to determine whether there is a coordinate or non coordinate expressional regulation at stress conditions.
4. Mutant analysis of this subunit b might reveal the detailed role of this enzyme for giving rise to different physiological and morphological characteristics of jute.
5. Over expression of this subunit might be useful to develop a dehydration tolerant trait of jute.

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APPENDIX

COMMON LABORATORY APPARATUS USED IN THE BIOCHEMICAL RESEARCH

NAME	DESCRIPTION	SOURCE
Eppendorf tubes	Size 1.5 ml, colorless	Eppndorf, Germany
Micropipette tips	Vol. range 0.5-200µl and 200-1000µl	Labsystems, Finland.
PCR tubes	Size0.2 ml, 0.5 ml	Perkin Elmer Cetus,USA.
Gloves	Disposable	Vinyl medical gloves,USA.
Glass wares	Pyrex brand	USA
Micropipettes	0.5-10 , 0.5-10µl,5-20µl, 10-100µl, 50-1000µl	Labsystems, Finland.
Gel kit and power	Model H5 Horizontal	BRL, Life technologies Inc USA.
Autoclave machine	Model HL-42A	E Hirayama Mfg Corp. Japan.
Micro-centrifuge	Eppendorf centrifuge5415C	Germany
DNA thermal cycler		GeneAmpPCR System 9700, Applied Bio-systems.
Vortex	Model 1190-1	Labline Instruments,USA.
Reciprocal shaking bath	Model 25	Precision Scientific.
Circulating water bath	Model260	Precision Scientific.
UV transilluminator	Model T2201	SIGMA Chemical CO., USA.

P^H meter		Orion Research, USA.
Tube rack		SIGMA, USA.
Balance machine		Mettler AE 100, Switzerland
Magnetic stirrer		Corning, Uk.
Incubator shaker	Innova 4300	New Brunswick Scientific Nalgene.
Centrifuge tube		USA.
Refrigerated superspeed centrifuge	Model RC 5B	Sorvall
Freeze drier	Model 12525	VirTis Comp.USA.
Refrigerator	-80°C	Barka Profiline
Microwave		Emerson, Korea.
Biodoc System	Kodak EDAS 290	Japan
Spectrophotometer	Analytikjena Specord 50	Germany
Nanodrop	NanoDrop 100	Fischer Scientific, USA