Identification and Partial Sequencing of Internal Control Gene of *Capsicum annuum*

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بسم الله الرحمن الرحيم

Dedicated

To

My Father and Mother

Specially to my Brother (Anas)

CERTIFICATE

This thesis, submitted by **Mr. Muhammad Awais** to the Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan, is accepted in its present form as satisfying the thesis requirement for the Degree of Master of Philosophy in Biochemistry/Molecular Biology.

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DECLARATION

I hereby declare that the work presented in the following thesis is my own effort and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree

Muhammad Awais

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LIST OF ABBREVIATIONS

β-Tub	Beta Tubulin
µl	Microliter
Adh	Alcohol Dehydrognase
BLAST	Basic Local Alignment Search Tool
Cat.	Catalog
cDNA	Complimentary DNA
CTAB	Cetyl tri-methyl ammonium bromide
Cyp	Cyclophilins
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide tri-phosphate
EDTA	Ethylene diamine tetra acetic acid
EF 1	Elongation factor-1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HCl	Hydrochloric acid
HDF	Hi Dye Formamide
Hell	Helicase
HPRT	Hypoxanthine phosphoribosyltransferase
hrs	Hours
Kda	Kilo dalton
mg	Miligram
$MgCl_2$	Magnesium chloride
Min	Minutes
ml	Millileter
mM	Milimolar
mRNA	Messenger Ribo nucleic acid
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information

ng	Nanogram
°C	Degree centigrade
PCR	Polymerase chain reaction
PVC	Poly Vinyl Chloride
RNA	Ribonucleic acid
RPM	Revolution per minute
rRNA	Ribosomal RNA
RT	Reverse transcription
Sec	Seconds
T.HCl	Tris hydrochloric acid
TE	Tris ethylene diamine tetra acetic acid

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Abstract

Proper functioning of an organism requires continuous expression of some genes called as housekeeping genes. The cell viability is thought to be governed by these genes. They are also used as reference genes for quantification of target gene transcript under particular experimental conditions. We have isolated and characterized a housekeeping gene in *Capsicum annuum*. The genomic DNA was extracted to amplify and sequence *β-tubulin* genes. Sequences of the said gene in different plants were downloaded from NCBI website and aligned. Primers were designed for the most conserved regions using primer3 software. *β-tubulin* genes was amplified using these primers designed for conserved regions. Approximately 230bp product was sequenced and analyzed using bioinformatics tools. *β-tubulin* gene sequence was used for BLASTn tool to find out other plant's genes based on similarity. We got *β-tubulin* gene sequences of monocot and dicot plants with 90% homology.

β-tubulin gene sequences of all plants were aligned using BioEdit software which showed high similarity. Phylogenetic tree was constructed using clustalW multiple alignment to check the evolutionary relationship among different plants based on *β-tubulin* genes. Plants from diverse groups were divided in two main groups of monocots and dicots. This newly isolated gene can be used as an internal control for gene expression studies in *Capsicum annuum*.

Introduction

Introduction

Reference gene should have constant expression under different experimental conditions (Wong and Medrano, 2005; Wan *et al.*, 2010). There is not any gene that can be expressed stably under different experimental conditions (Wong and Medrano, 2005; Gutierrez *et al.*, 2008).

Development is not determined by genome in most organisms. During body development, self-organization takes place due to cell to cell signaling and even within different tissues on greater scale. This affects the positioning and separation of the cells. There are certain problems which must be solved to arrange self-organizing processes in a single and consistent process to create an organism that functions like normal creature. Such as, how the selfish essentials can be excluded (Smith and Szathmáry, 1997)? How the developmental blast is decreased (Waddington, 1957; Novoplansky, 2002)?

Moreover, environment not only affects the growth but the development is also affected. Data collected from the environment can be used to empower the development (Novoplansky *et al.*, 1990; Oborny, 1994; DeWitt *et al.*, 1998; Alpert and Simms, 2002; Givnish, 2002)

1.1 Internal reference gene

Ideal reference gene which is also called as internal control should have constant expression under different experimental conditions (Thellin *et al.*, 1999; Schmittgen and Zakrajsek, 2000).. Normally identified internal control genes for example, *β-actin*, *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH)* and *18S rRNA* has enabled us to quantify transcript level of the gene. These genes may be used as internal controls with full care, as they are expressed inconsistently under different conditions (Dheda *et al.*, 2005; Nicot *et al.*, 2005). The reason for this variable expression is that these genes have role in metabolism control as well as in other cellular reactions (Singh and Green, 1993; Ishitani *et al.*, 1996). Reference genes for expression studies may be defined by different experiments on plants (Gutierrez *et al.*, 2008)

1.2 Some Important Housekeeping Genes

1.2.1 *Actin*

Actins are the most abundant proteins in eukaryotic cells. There are three main groups of isoforms i.e. α, β, γ. These proteins play key roles in cell motility and cytoskeleton maintenance (Hunter, 1977). *β-actin* and *γ-actin* are found in all cells, whereas *α-actin* is normally restricted to smooth muscle cells. *β-actin* is expressed constitutively and involved in basic housekeeping functions required for cell maintenance. Because of this, they are commonly used as endogenous controls to normalize gene expression. The use of these internal control genes in molecular biology is dependent on the assumption that their expression level remains constant within the cell and even under different treatment conditions (Biederman *et al.*, 2004).

1.2.2 *18S rRNA gene*

Ribosomes are very ancient structures that are involved in the synthesis of proteins and are commonly present in all types of cells. As far as the evolutionary distances regarding *18S rRNA* among eukaryotes are concerned, comparisons can be made on two levels based on the primary nucleotide sequences and the secondary structures of the rRNA (Torczynski *et al.*, 1983; Chan *et al.*, 1984; Connaughton *et al.*, 1984; Nelles *et al.*, 1984; Noller, 1984; Raynal, 1984).

Differences between the primary sequences can be seen among prokaryotic, eukaryotic, archaebacterial and organelle genes but the secondary structures have shown a marked preservation throughout evolution. The structurally conserved regions are separated by variable regions in which both sequence and structure have diverged among these major divisions. Several of these regions are restricted to only one group. Divergence rates between rRNA molecules are calculated separately for the "conserved" and "variable" regions (Raynal *et al.*, 1984).

1.2.3 Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a multifunctional enzyme It is a well studied protein for its role in cellular energy production as a glycolytic enzyme. However, current evidences reveal that this enzyme is actually a multifunctional protein displaying a number of diverse cellular functions which are unrelated to glycolysis (Cortez *et al.*, 2010).

Several reports have documented that the catalytic cysteine residue of *GAPDH* is susceptible to oxidative modifications by nitrating agents, and these modifications result in the loss of enzymatic activity. Molecular mass of *GAPDH* is 160 kDa due to this it is the subject of numerous investigations. Crystal structures of *GAPDHs* from *lobster, human, Bacillus stearothermophilus, Trypanosoma brucei, Thermatoga maritima, Thermus aquaticus and Escherichia coli* have been determined. Due to relatively high sequence identity among *GAPDHs*, the crystal structures of *GAPDHs* are also very similar. However, the orthorhombic structure of *L. mexicana GAPDH (LmGAPDH)* revealed certain unusual features (Kim *et al.*, 1993).

1.2.4 Ubiquitin

Ubiquitin (UBI), a small protein consisting of 76 amino acids, plays a key role in protein degradation processes, DNA repair, signal transduction, transcriptional regulation and receptor control by endocytosis (Christensen *et al.*, 1992). It is one of the most highly conserved proteins with 72 of the 76 amino acids invariant among fungi, plants, and animals (Callis and Vierstra, 1989). UBI-encoding genes are divided into two classes i.e. monomeric and polymeric (Binet *et al.*, 1991). Monomeric ubiquitin genes consist of 228 nucleotides (76 codons) with an additional C-terminal sequence that encodes ribosomal proteins either 52 or 76–81 amino acids in length (Garbarino and Belknap, 1994). Because of its constant expression this gene can be used as internal control.

1.2.5 Cyclophilins

Cyclophilins, or peptidyl-prolyl cis-trans isomerases, are enzymes belonging to the super family of immunophilins. Their biological significance is manifested by the catalysis of protein folding via peptide bond rotation on the amino side of proline residues (Fischer *et*

al., 1989). They play role as a chaperone for protein trafficking and nucleolytic degradation of the genome (Montague *et al.*, 1997). Most of the studies treat *CYP* isoforms as a single functional unit, however there is evidence that the expression of *CYP* isoforms is independent from each other (Billich *et al.*, 1997). Cyclophilin is commonly used as an internal control gene in plants expression experiments e.g. soya bean (Bo Jian1 and Han*2, 2008). These observations have profound implications regarding *cyclophilin's* use as an invariant control and highlight the fact that it is essential to treat *cyclophilin* isoforms as separate entities, rather than one functional unit. To minimize the risk of misleading data acquisition, it is suggested that *CYP* probes are designed not to cross-react with different isoforms.

1.2.6 Elongation Factor-1 Alpha

The *elongation factor-1 alpha* or *EF-1 alpha* is a ubiquitous protein that binds aminoacyl-transfer RNA to ribosomes during protein synthesis. It has been predetermined to be a good internal control (Dostal *et al.*, 1994). However, being an integral part of the translation apparatus, its expression can be modulated in areas of high protein turnover such as rapidly growing tissue, plant meristems and gametophytes.

1.2.7 Hypoxanthine phosphoribosyltransferase (HPRT)

Hypoxanthine phosphoribosyltransferase (HPRT) is involved in nucleotide metabolism and expressed at relatively low levels. Therefore, this gene is not suitable when using methodologies with low sensitivity. However, it has been shown to be invariably expressed when using both northern protocols and PCR based approaches as well as in studies of alveolar macrophages and spleen cells (Foss *et al.*, 1998).

1.2.8 *β -Tubulin*

Tubulin is one of the member globular proteins families. The *α-tubulin* and *β-tubulin* are the most abundant member of this family. Each of these has a molecular weight of about 55 kilo Dalton. α- and *β-tubulin* are arranged in dimeric form to construct a microtubule. They are somewhat acidic having 5.2 to 5.8 isoelectric point (Williams *et al.*, 1999). Microtubules are formed when the dimers of α- and *β-tubulin* come in association with GTP and aggregate on the +ve ends of microtubules (Howard and Hyman, 2003).

1.2.8.1 Tubulin Isotypes and Stochiometry

It is considered that plants regulate their dynamicity of microtubules by the various tubulin isoforms (Gull *et al.*, 1986). The left handed helical symmetry and the disturbed bilateral symmetry helps to distinguish the mutant forms. This changed symmetry is attained due to the negative mutation in the TUA6 or TUA4. As a result serine replaced with phenylalanine at position 183. More importantly a single replacement of amino acid in tubulin sequence can lead to a considerable structural damage (Thitamadee *et al.*, 2002).

The cell wall deposition is reliant upon the microtubule which in turn influences the sequence of amino acids in tubulin. Lefty mutants of microtubules show greater sensitivity to the anti-microtubular drugs like pronamide, dinitroaniline and taxol. Thus, the changes in universal morphology of a plant, cell-wall deposition, drug sensitivity and cortical microtubule arrangement can be caused by a single modification in tubulin amino acid sequence (Thitamadee *et al.*, 2002).

1.2.8.2 Multiple Tubulin isoforms in higher plants

In accordance with the occurrence of manifold tubulin genes in higher plants, many tubulin isoforms have been observed in numerous plants, including *Phaseolus vulgaris* root tips (Hussey and Gull, 1985), carrot cell suspensions (Dawson and Lloyd, 1985; Cyr *et al.*, 1987), carrot plant tissues (Hussey *et al.*, 1988), different organs and tissues of maize (Joyce *et al.*, 1992), *Arabidopsis* (Kopczak *et al.*, 1992; Snustad *et al.*, 1992), rye root tips (Kerr and Carter, 1990b) and spinach leaf cells (Bartolo, 1990). Certain tubulin isoforms are differentially present through various stages of development (Hussey *et al.*, 1988; Joyce *et al.*, 1992). The both a- and P-tubulin isoforms are changed in root tips of rye when seedlings are grown at low temperature for cold acclimation (Kerr and Carter, 1990). It was also established that P-tubulin isoforms are changed in spinach leaf cells in reply to low temperature (Bartolo, 1990).

Even if posttranslational modifications such as acetylation, tyrosination, glutamylation of a-tubulins while phosphorylation and glutamylation of P-tubulins cause tubulin diversity in animals (Joshi and Cleveland, 1990). None of these modifications has been confirmed in plant tubulins.

1.2.8.3 Structure of Tubulin

Collection of plant microtubules is different in fungi and animals because the formation of new microtubules does not take place from organizing centers like centrosomes (Shaw *et al.*, 2003). Microtubules might be centralized from isolated sites all along presented microtubules from complexes containing γ-tubulin (Wasteneys, 2002).

It is believed that these complexes are formed from minus ends of microtubules. Microtubule nucleation and cortical microtubule arrangements occur side by side and are dependent on inter-microtubule connections and few other protein activities, for example microtubule organization1 (Wasteneys, 2002).

The β and α-tubulin dimers are the primary structural components of microtubules. The α- and β-tubulin are arranged head to head in heterodimer form creating polarity. The orientation of heterodimers is that α-tubulin pointing towards negative end that polymerizes slowly and β-tubulin point towards the positive end. The animal cells have an organelle called centrosome that is present to assemble the microtubules. Centrosome is an organelle present in cell at pericentriolar matrix (PCM).

For the duration of interphase, the negative (slow-growing) ends of microtubules remain fixed in the PCM and positive (fast-growing) ends orient towards the cytoplasm. γ-tubulin forms a ring like arrangement in PCM. The diameter of γ-tubulin complex is nearly same to the microtubule diameter (Zheng *et al.*, 1995; Moritz and Agard, 2001). Additionally mitotic checkpoints are also controlled by the virtue of γ-tubulin (Prigozhina *et al.*, 2004).

Extreme C-termini of tubulin create the versatility in their structure. The C-terminus of beta-tubulin and alpha protrudes out from the surface of heterodimers. MT-associated proteins contact the tubulin by the c-terminus (Littauer *et al.*, 1986) and posttranslational modifications are also occurred here (MacRae, 1997).

1.2.8.4 Reasons to choose beta tubulin for gene expression

In order to determine the role of intron with respect to evolution and gene expression, there are significant reasons to choose plant tubulin genes:

1. Tubulin gene may be used as a marker for physlogenetic relationship among different plants. α- and β-tubulin gene has wide taxonomy and extensively identified in early deviating eukaryotic clad (Edgcomb *et al.*, 2001; Moriya *et al.*, 2001; Hampl *et al.*, 2005). Significant relations have been established on evolutionary level by tubulin introns productively in protozoa, fungi, flagellates, urochordates, endophytes and tuber genus (Hampl, *et al.*, 2005; Wong and Medrano, 2005). Production of various tubulin proteins is done by alternative splicing.
2. Introns of Tubulin did not spread randomly in eukaryotic genome but they are in the form of well defined up to 16 clusters. Eukaryotes have changed cluster combinations. But exceptions are there like Oikopleura and Caenorhabditis plants have short introns.
3. Beta-tubulin intron in higher plants is positioned at conserved sequence located within their corresponding genes.
4. In the tubulin gene, intron position is located near the 5-terminus, i.e., the gene expression is mainly controlled by this region.
5. Animal counterparts are less numerous than plant tubulin genes.
6. In some areas of plant biology, tubulin is considered responsible for gene expression.
7. Non-coding parts are related to major changes and coding regions are obviously stuck to severe functional requirements. That's why coding regions evolution is somewhat different than intron sequences.

1.3 Optimal choice of reference gene

Selection of the gene depends upon the range and type of the experiment (Thellin, *et al.*, 1999). Thus, the selection of most suitable reference gene is the key to overcome the inconsistency of samples for expression analysis like qRT-PCR (Dheda *et al.*, 2005)

A significant tip to identify appropriate reference genes is the selection of the first division of genes used in the validation method.

Recently, increasing amount of available articles reproduce the significance of internal reference genes and the requirement for authentication them for specific trial. Human and animal samples are dealt for these studies. Still, hardly few plants are concerned to this studies like tomato (Coker and Davies, 2003; Expósito-Rodríguez *et al.*, 2008), *Arabidopsis* (Czechowski *et al.*, 2005; Remans *et al.*, 2008), coffee (Barsalobres-Cavallari *et al.*, 2009), rice (Kim *et al.*, 2003; Ding *et al.*, 2004), potato (Nicot *et al.*, 2005), barley (Burton *et al.*, 2004), grape (Reid *et al.*, 2006), poplar (Brunner *et al.*, 2004), soybean (Jian *et al.*, 2008) and wheat (Paolacci *et al.*, 2009). Similarly, information are missing to choose the internal control genes for RT-qPCR analysis in peach plant.

1.4 Properties of reference genes

Housekeeping genes should have constant expression regardless of the cell in any organism. Such type of genes should have the following characters:

- It should be expressed in all the cells of an organism
- Such type of genes should have stable copy number in all cells of an organism
- Housekeeping genes should express with intermediate copy number for more accurateness (or alike copy number to gene of attention)

1.5 Reference genes as internal control

Constant expression of a reference gene is must for normalization of real-time PCR otherwise it leads to false results. Different genes such as (*GAPDH*), *β-TUBULIN* (*TUB*), *ACTIN* (*ACT*), *UBIQUITIN* (*UBQ*) and *18S rRNA* have been used as internal control genes due to their consistent expression. But some studies have confirmed that these genes show somewhat variable expression under the set of specific experimental conditions (Thellin *et al.*, 1999; Suzuki *et al.*, 2000; Lee *et al.*, 2002; Czechowski ,Stitt *et al.*, 2005).

1.6 Normalization of gene expression by internal control genes

Normalization of the genes with internal controls like *ACT* or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), is necessary for transcript quantification of the target gene (Bustin, 2002). Though as per rule the expression of housekeeping genes is kept stable. But sometimes it's expression can certainly differ with different developmental stages which leads to theatrical misapprehension of the expression outline of an intended gene (Lee *et al.*, 2002; Steele *et al.*, 2002). It is thus vital to recognize the housekeeping genes having expression regardless to the life stages which is necessary for data normalization in expression studies of transcript.

Statistical methods have been developed to study gene expression data (Vandesompele *et al.*, 2002; Vandesompele *et al.*, 2009). Usually gene expression profiles are normalized by single gene but different experimants may contain number of reference genes. Moreover, the accuracy of RT-qPCR normalization and the reference genes number are associated with one another.

1.7 Purpose of normalization

Proper normalization is necessary to obtain the accurate expression results which is independent from the technique selected. Specially when small differences in expression levels are to be calculated or when tissues of histological origin are under investigation (Huggett J *et al.*, 2006). Inconsistency among the samples may be developed by different steps of the experimental methods. e.g. Amount of the sample at start, recovery and integrity of RNA, cDNA synthesis efficacy and transcript level differences.

Among the normalization methods proposed (Thellin *et al.*, 1999; Suzuki ,Higgins *et al.*, 2000; Pfaffl, 2004; Argyropoulos *et al.*, 2006; Gilsbach *et al.*, 2006; Libus and Storchova, 2006) the most commonly applied technique is the selection of internal controls (Nolan *et al.*, 2006; VanGuilder *et al.*, 2008) as they are thought to participate in above mentioned variations. By the selection of suitable control, the highly relied expression profiles can be obtained (Huggett *et al.*, 2006). Otherwise the gene is considered impractical for experimental use (Dheda *et al.*, 2005)

1.8 Amplification by internal control genes

Transcript level of the gene is measured with the help of real time PCR. It also works where messenger RNA amounts are limited. But the choice of a standardized procedure is necessary to get accurate expression profiles (Freeman *et al.*, 1999; Bustin, 2002). The correction of non-specific results is key function of normalization.

Many complex cellular processes are approached by the study of gene expression that has increased our understanding about how life works in a cell. To date, the best technique to study the expression profiles at transcript level is the real-time RT-PCR. The key benefits of this method are greater sensitivity, specificity and wider range of quantification (Gachon *et al.*, 2004; Wong and Medrano, 2005; Nolan *et al.*, 2006; VanGuilder , *et al.*, 2008). Microarray results are also confirmed by Real-time RT-PCR (Chuaqui *et al.*, 2002; Canales *et al.*, 2006).

1.9 Work on *Capsicum annum* in past

In the past some years, many efforts have been done in order to sequence the genome of variety of organism, including *Arabidopsis* and rice (Goff *et al.*, 2002; Yu *et al.*, 2002). Although, entire genomic sequence of different plants existed, but "expressed sequence tags" (ESTs) and analysis of cDNA sequence remained invaluable in research of genomic, especially, for research with large genome size plant species. However, recent development in data analysis tool and DNA sequencing have made possible single pass cDNA sequencing to become considerable method which can be applied in genomics based research. In many plant species, ESTs has become method of choice for subsequent analysis of genes by temporal or special expression patterns. As an outcome, over a Million of ESTs have been reported from 30 plant varieties (http://www.ncbi.nlm.nih.gov/dbEST/).

Up till now hot pepper is under little consideration and NCBI contains the data base of less than 500 nucleotide sequence (http://www.ncbi.nlm.nih.gov/Taxonomy). A good source of vitamin is hot pepper and has medicinal significance for arthritis, reducer of pain and long term inflammation (Bosland and Votava, 2000).

1.10 Research Objectives

There are two main advantages to have internal control genes that they are;

1. helpful in targeting a specific gene
2. helpful in determining the expression of target gene.

For these reasons internal control genes are required to be identified and sequenced in different plant species under different sets of experimental conditions.

In this study we choose *C.annuum* to ,

- Identify *β-tubulin* gene in genome of *C.annuum*
- Sequencing of *β-tubulin* in genome of *C.annuum*
- Analysis of *β-tubulin* gene in genome of *C.annuum* by using bioinformatics tools.

Materials and Methods

MATERIALS AND METHODS

2.1 Plant material

Capsicum annuum, domestic specie, belongs to the Capsicum genus of plant. This genus is native to Northern South America and Southern North America. Evolution of its three species *C. annuum, C. frutescens and C. chinense* is considered from one common ancestor, located in the northwest Brazil - Colombia area. *C. annuum* is among the most common and widely cultivated of the five domestic capiscums.

The name of its species *annuum* means "annual" (from the Latin annus "year"), the plant is not considered as an annual plant but when there is no winter frosts it can stay alive up to a number of seasons and may grow as perennial shrub. Its flowers are of off-white (sometimes purplish) color but the stem is heavily branched and up to 60 centimeters (24 in) tall with green colored berry like fruit that becomes yellow or red upon ripening.

2.2 Classification

The classification of Capsicum is given below.

Kingdom	Plantae
(Unranked)	Eudicots
(Unranked)	Asterids
Order	Solanales
Family	Solanaceae
Genus	*Capsicum*
Species	*C. annuum*

2.3 Genomic DNA Extraction and Quantification

Genomic DNA extraction of *C. annuum* was done by method of CTAB (Zhang *et al.*, 2000). One gram of leaf tissue was taken and firstly washed with 70% ethanol and was homogenized in 3mls of extraction buffer (100mM T.HCl pH 8, 20mM EDTA, 1M NaCl,

2% PVP-40, 0.002% CTAB, 0.02% phenanthrolein, 0.2% β-mercaptoethanol). This mixture was then incubated for 1hr at 70°C. 3mls of chloroform and isoamylalcohol (1:24 ratio) was prepared and used with phenol in a ratio of 1:2 to purify the DNA. The samples were then centrifuged at 12,000 rpm for 15mins, the supernatant was taken after couple of washings in a separate falcon tube and an equal volume of ice cold isopropanol was added into the falcon tube. The mixture was kept at -20°C for overnight. Next day the frozen samples were thawed and solution was centrifuged at 6000 rpm for 12mins. The pellet was washed twice with 15mM ammonium acetate in 80% ethanol followed by 100% ethanol washings twice. The samples were then air dried and finally resuspended in 30µl 10mM Tris HCl. 5µl of the sample was loaded on 1% agarose gel and run at 80volts for 90mins.

After the extraction of genomic DNA and confirmation of its presence, its quantification was done and an instrument called as nanodrop was used for this purpose. The apparatus was calibrated to remove zero error with the help of T.E buffer in which DNA was dissolved and was taken as blank. 1µl from each aliquot was used in nanodrop for quantification and concentration was recorded in ng/µl units. Then different dilutions of stock genomic DNA was prepared in order to use required concentrations of DNA. 200ng/µl concentration of DNA was used for polymerase chain reaction (PCR).

2.4 Primer design

Some of the known sequences of *β-tubulin* from different selected plants were downloaded from NCBI database (http://www.ncbi.nlm.nih.gov/) and were aligned together by using Bioedit program.

As it is known that *β-tubulin gene* sequence is highly conserved in plants, the primers were designed from the highly conserved regions of the aligned sequences of *β-tubulin*. Following primers were used to amplify *β-tubulin gene* from genomic DNA of *C. annuum* (forward primer 5'-CGTAAGCTTGCTGTGAATCTCATC-3', reverse primer 5'CTGCTCGTCAACTTCCTTTGT-3') at annealing temperature 57°C.

2.5 Polymerase Chain Reaction

PCR amplification of *C. annuum* DNA was done by using forward and reverse primers given above. Genomic DNA was first denatured at 95°C, followed by 94°C for 45sec, 57°C for 45sec, 72°C for 1min, for a total of 35 cycles. Finally extension was done at 72°C for 5min followed by incubation at 15°C for 15min. 1.2% agarose gel was prepared with 2µl of ethidium bromide. 8µl PCR products were loaded on the gel and run at 80 volts for 90mins. UV transilluminator was used to take picture of gel.

2.6 Sequencing of *β-tubulin*

The PCR product was then purified and sequenced by the methods described as under:

2.6.1 PCR product purification

PCR products were purified using the rapid PCR Kit (Marligen, USA) as recommended by the manufacturer by adding 200µl of binding solution (HI) (Concentrated Guanidine HCl, EDTA, Tris HCl and Isopropanol) in to the 45µl of PCR product. The mixture obtained was transferred into the spin cartridge (column) containing silica based membrane where double stranded amplified DNA was selectively adsorbed with the column and was kept for 10min to allow solution to bind completely. Its adsorption and binding is dependent of buffer composition and temperature. Then 500µl of washing buffer (NaCl, EDTA, Tris HCl)) were used to remove Taq DNA polymerase, DNTPs and unused primers by centrifugation. The DNA kept bound with the silica column that was then eluted with the help of 15-20µl elution buffer (10mM Tris HCl (pH 8.0), 0.1mM EDTA) in which DNA bound to silica got dissolved that was free of many impurities at 65°C. This purified product was then checked at 2% horizontal gel electrophoresis for successful purification.

2.6.2 Sequencing PCR

Sequencing PCR reaction mixture was made by combining RRv3.1 (master mix of enzyme, dNTPs and MgCl2) (1µl), 5X sequencing buffer (1µl), *β-tubulin* F (forward primer) (1µl), template (1µl), water (6µl). Sequencing PCR was done at 95°C for 1min,

followed by 95°C for 30 seconds, 57°C for 30 seconds, 72°C for 4min, a total of 30 cycles were repeated followed by a final elongation at 72°C for 10min.

Finally sequencing PCR product was purified by transferring the reaction mixture in 1.5ml tube with 25µl of water + 65µl 100% ethanol (-20°C), vortexed and incubated at room temperature for 20min. The samples were centrifuged at 13000 rpm for 15min. Supernatant was immediately discarded and 150µl of 70% ice cold ethanol was added followed by centrifugation at 13000rpm for 15min. Supernatant was removed and sample was vacuum dried at 45°C. Pellet was resuspended in 20µl Hi Dye Formamide (HDF) for sequencing. Sequencing was done in Beckman CEQ 8800 sequencer by Sanger Dideoxy Chain termination method.

2.6.3 Trimming and Editing of sequences in Bioedit software

The sequences obtained of *C. annuum* after sequencing by Beckman CEQ-8800 sequencer were first analyzed in the Bioedit program and then both sequences were first trimmed to remove N's from the sequence for further analysis.

2.6.4 Data analysis

The newly isentified sequence of *β-tubulin* gene of *C. annuum* was then analyzed by the help of some bioinformatic tools. The details are as under;

2.6.4.1 BLAST

To find out the similarity of *β-tubulin* gene of *C. annuum* with other known plant *β-tubulin* sequences, we used bioinformatics tool, BLAST after adding the newly sequenced *β-tubulin* fragment of *C. annuum* in the FASTA format as an input, under "somewhat similar sequences (blastn) option". Number of plant *β-tubulin* sequences with their accession numbers and identity were downloaded from BLAST output and compared with the newly sequenced fragments.

2.6.4.2 Alignment of *β-tubulin* based on DNA sequences

Newly sequenced *β-tubulin* gene sequences and the other known downloaded sequences of different plants including *Medicago truncatula, Lotus japonicus, Glycine max,*

Results

Results

We selected *Capsicum annuum* for identification and sequencing of *β-tubulin gene* to use as internal controls for transcript analysis. A mature *C. annuum* plant is shown in Figure 3.1.

Fig 3.1: A mature *Capsicum annuum* plant

PCR and other PCR based technologies require the good quality genomic DNA. Genomic DNA of *C. annuum* was isolated by modified CTAB method (Zhang Z, 2000) as described in method section. Figure 3.2 shows the intact and good quality genomic DNA isolated from *C. annuum* in four replicates. There was no degradation in any of these replicates. This genomic DNA of *Capsicum annuum* was used as a template for Polymerase Chain Reaction (PCR) to amplify a conserved region of *β-tubulin* gene by using a set of primers as explained in material and methods.

The amplification of *β-tubulin* gene of *C. annuum* was done by using gene specific forward and reverse primers of *β-tubulin*. The PCR conditions were optimized to get the expected size band. A product of ~230bp was amplified at 57°C (annealing temperature), while no amplification was seen in negative control as shown in the Figure 3.3.

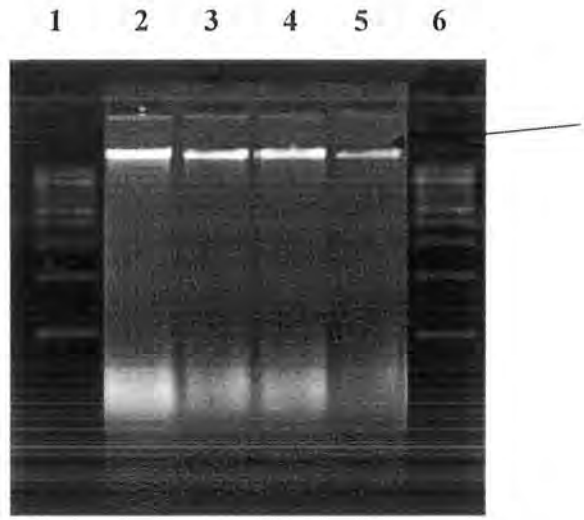


Figure 3.2: Agarose gel electrophoresis of isolated genomic DNA of *C. annuum*. Total genomic DNA was isolated from the leaf samples of *C. annuum* from four independent replicates. 8ul of DNA was loaded on ethidium bromide stained gel.

Lane 1 & 6 = 1Kb Ladder (Fermentas)

Lane 2, 3, 4 & 5 = Genomic DNA of *C. annuum*

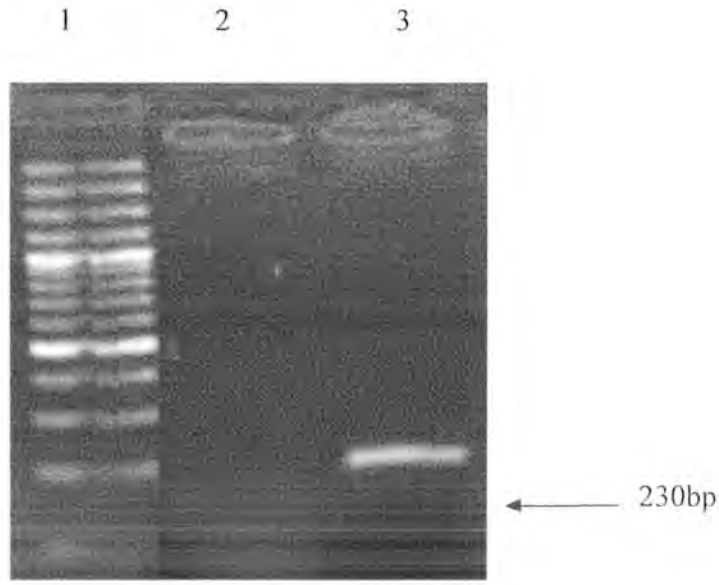


Figure 3.3: PCR Amplification of *β-tubulin* gene of *C.annuum*. 2% Agarose stained with ethidium bromide showing the PCR amplified product of *β-tubulin* gene of *C. annuum*

Lane 1 = 100bp ladder (Fermentas)

Lane2 = Non-template control amplification with *β-tubulin gene* primers.

Lane 3 = Capsicum DNA amplification with *β-tubulin gene* primers.

3.1 Sequencing of *β-tubulin gene*

To confirm the sequence of PCR product we sequenced the above product after product purification. Figure 3.4 shows the large scale amplification of about 200 to 250bp product which was then purified with rapid PCR Kit (Marligen, USA). Purified products were confirmed by running on 2% agarose gel at 80 volts for 1hr. Initially the purified product amplification showed non specific bands, when the sample was proceeded for sequencing in the Beckman CEQ-8800 sequencer, the results showed large number of N's indicating failure of sequencing. So we could not use these results to identify the PCR product.

The PCR was repeated by increasing the annealing temperature up to 57°C to reduce the non-specific amplification. Once the bands of exact size were observed the we again decided to to sequence this amplified product. For sequencing we need to amplify the same product on large scale, so the same PCR was repeated under same conditions with 50ul of volume as shown in Figure 3.4. This time we a single and specific band of approximate size of 200 to 250bp was observed as shown in Figure 3.5, indicating that the sample was good enough for sequencing.

Sequencing was done in Beckman CEQ-8800 sequencer by Sanger Dideoxy Chain termination method in our central facility. We were able to sequence only 185 bps with lots of N's. The resulting sequence was then trimmed to rempove N's by using Bioedit Software as shown in Figure 3.6 shows the sequence after editing.

The sequenced fragment was further analyzed by using different bioinformatics tools. BLAST is a bioinformatics tool, we used the sequence in the FASTA format as an input with "somewhat similar sequences (blastn)" option, Table 3.1 shows a list of plants showing the similarity with our newly sequenced *β-tubulin* fragment of *C. annuum* including *Medicago truncatula, Lotus japonicus, Glycine max, Gossypium hirsutum, Prunus salicina, Neosinocalamus affinis, Eleusine indica, Oryza sativa, Populus trichocarpa* and *Eucalyptus grandis* etc. with maximum homology of 90% according to Table 3.1.

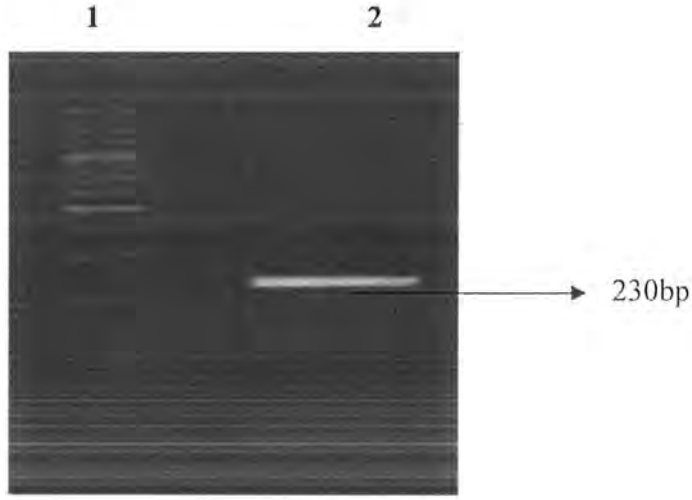


Figure 3.4: Large scale PCR amplification of genomic DNA of *C.annuum* for sequencing. PCR amplification of genomic DNA of *C. annuum* for sequencing, 50µl sample was run at 57°C annealing temperature with *β-tubulin gene* specific primers and was run on 1% agarose gel resulting in the PCR product of approximately 230bp.

Lane 1 = 100bp DNA ladder (Fermentas)

Lane 2 = PCR product of *C. annuum* (50µl).

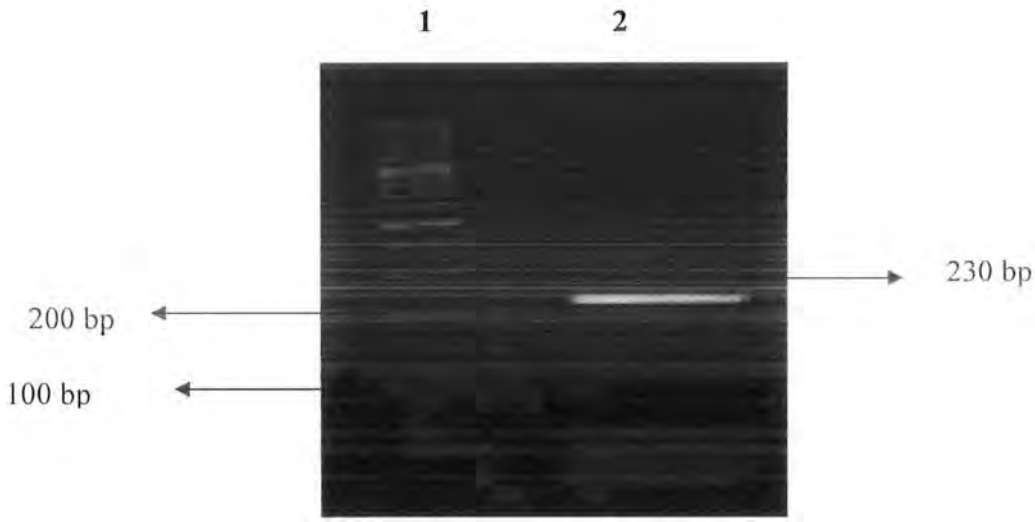


Figure 3.5: Purified PCR Product of *C. annuum* for sequencing. PCR products of *C. annuum* with *β-tubulin gene* specific primers were amplified and purified with rapid PCR Kit (Marligen, USA) followed by electrophoresis on 2% agarose gel

Lane 1 = 100bp ladder (Frementas)
Lane 2 = Purified PCR product of *C. annuum*

>***Capsicum annum***

GCATCCCAGCAGTATGGTGCTAATGACTGTACCTGAATTGACTCAGCAGAT
GTGGGATTCCAAGAACATGATGTCTGCTGCTGATCCACGCCCATGGCCGTT
ATGTGACTGCATCAGCAATGTTCCCGGTGGTAAGATGTAGCGACAAAGGAAG
TTGACGAGCAGAGTCACATAAGA

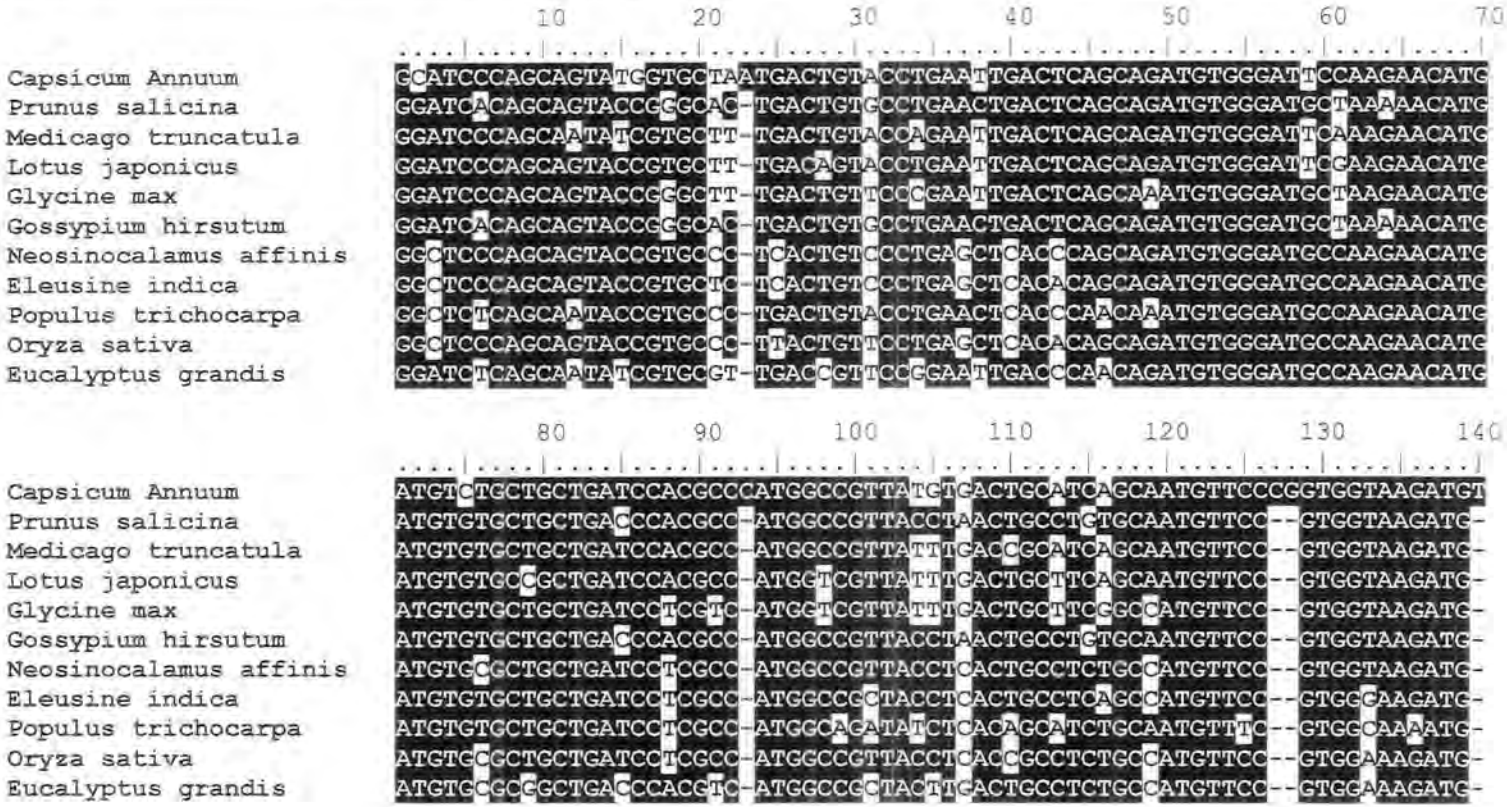
Figure 3.6: Newly identtfied sequence of *β-tubulin* of *Capsicum annuum.*

Table 3.1 BLAST results of newly sequenced *β-tubulin* gene of *C. annuum* showing similarity with other *β-tubulin* genes of different plants.

Accession	Description	Max score	Total score	Query coverage	e - value	Max ident
XM_003594548.1	*Medicago truncatula* Tubulin beta chain (MTR_2g031310) mRNA, complete sequence	206	206	92%	3e-50	90%
AB510590.1	*Lotus japonicus* LjTUB mRNA for tubulin beta chain complete sequence	194	194	92%	2e-46	90%
XM_003546228.1	PREDICTED: *Glycine max* beta-tubulin (LOC547917), mRNA	158	158	92%	1e-35	88%
AY345607.1	*Gossypium hirsutum* beta-tubulin 5 mRNA, complete cds	154	154	91%	2e-34	83%
FJ157349.1	*Prunus salicina* var. cordata beta-tubulin mRNA, complete cds	150	150	92%	2e-33	83%
EU246956.1	*Neosinocalamus affinis* clone 1 beta-tubulin mRNA, partial cds	150	150	92%	2e-33	83%
AF059288.1	*Eleusine indica* beta-tubulin 2 (TUB2) mRNA, complete cds	150	150	92%	2e-33	83%
XM_002299505.1	*Populus trichocarpa* tubulin, beta chain (TUB3), mRNA	147	147	92%	3e-32	82%
D30716.1	*Oryza sativa* Japonica Group mRNA for beta-tubulin, complete cds	139	139	92%	1e-29	81%
EF534222.1	*Eucalyptus grandis* beta-tubulin (TUB4) mRNA, complete cds	140	140	92%	4e-30	81%

Some of the above sequences were downloaded that showed homology up to 90% with our newly sequenced partial *β-tubulin gene* and were aligned with the newly isolated sequences of *C. annuum* by using Bio Edit software as shown in Figure 3.7.

Phylogenetic tree was constructed by using ClustalW multiple alignment that showed the evolutionary relationship of our newly sequenced *β-tubulin gene* with the *β-tubulin* sequences of other known plants. Figure 3.8 shows Phylogenetic tree that was constructed by using the sequences of *C. annuum*, *Medicago truncatula*, *Lotus japonicus*, *Glycine max*, *Gossypium hirsutum*, *Prunus salicina*, *Neosinocalamus affinis*, *Eleusine indica*, *Oryza sativa*, *Populus trichocarpa* and *Eucalyptus grandis* etc. The tree shows that *β-tubulin* of *Capsicum annuum* is higly conserved especially in this reigion among diverse group of plants.


10 20 30 40 50 60 70
Capsicum Annuum
Prunus salicina
Medicago truncatula
Lotus japonicus
Glycine max
Gossypium hirsutum
Neosinocalamus affinis
Eleusine indica
Populus trichocarpa
Oryza sativa
Eucalyptus grandis
80 90 100 110 120 130 140
Capsicum Annuum
Prunus salicina
Medicago truncatula
Lotus japonicus
Glycine max
Gossypium hirsutum
Neosinocalamus affinis
Eleusine indica
Populus trichocarpa
Oryza sativa
Eucalyptus grandis

Continued.....

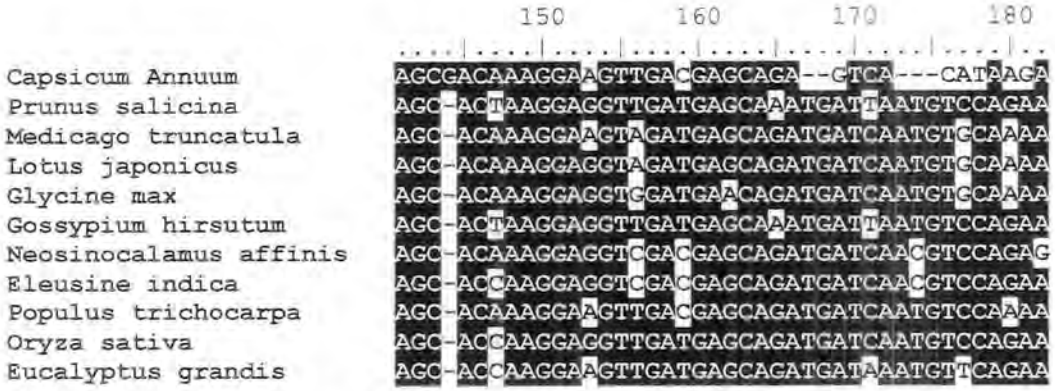


Figure 3.7: Alignment of newly identified partial sequence of *β-tubulin* gene from C. *annuum* with already known sequences of *β-tubulin*

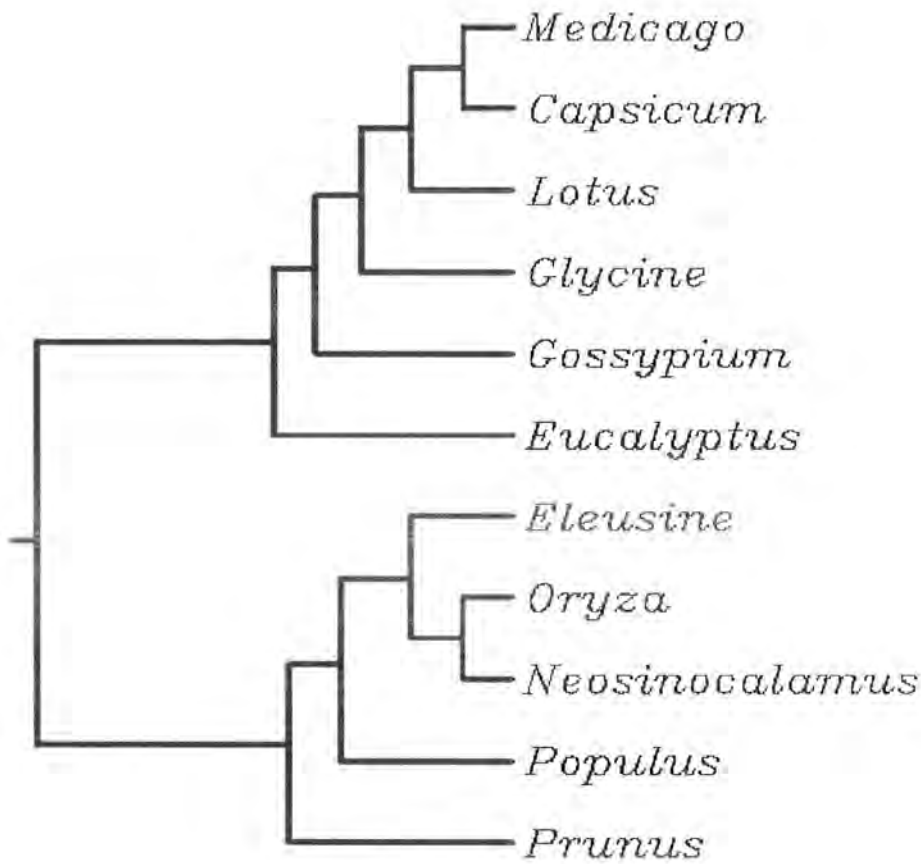


Figure 3.8: Phylogenetic tree based on *β-tubulin* sequence of plants including newly isolated *C. annum* with other *β-tubulin* genes from diverse group of plants downloaded from NCBI.

Discussion

Discussion

Those genes that have a constant expression profile regardless of tissue types and experimental conditions are called housekeeping genes. Due to their unique and constant expression among all the tissue samples they are widely used as internal controls for normalization of qPCR data. There are different types of housekeeping genes that can be used as internal control but here we only focused on *β-tubulin* gene of *C. annuum.*

Capsicum annuum of genus Capsicum was selected for identification and sequencing of *β-tubulin* gene. Genomic DNA from *C. annuum* was extracted by using CTAB method (Zhang Z, 2000). our data showed extraction of good quality genomic DNA of *C. annuum* as no DNA degradation was seen after optimization for this plant with high phenolic contents.

As *β-tubulin gene* sequence of *Capsicum annuum* was not known so *β-tubulin gene* sequences of some selected plants were down loaded from NCBI gene bank database and aligned together to see the similarities among the sequences of *β-tubulin gene*. Then we designed a set of (forward and reverse) gene specific primers for the *β-tubulin gene* of *Capsicum annuum* from the highly conserved region. Genomic DNA was used for PCR amplification with these primers to produce a product of approximately 230bp as no band was seen in the non-template control.

We sequenced the PCR product by using Beckman CEQ-8800 sequencer. Conformation of sequence was done using bio-informatics tools. Our newly identified sequences of *β-tubulin* gene were BLAST with the already known sequences of *β-tubulin* genes of various plants including monocots and dicots to identify the sequence homology and evolutionary relationship of our newly identified *β-tubulin* gene. The maximum homology was almost 90%.Some sequences were selected and downloaded to align with our newly sequenced *β-tubulin* gene by using Bio Edit software.

Dandogram was also constructed to check the evolutionary relationship among the *β-tubulin* of *C annuum* with other known *β-tubulin* sequences which showed the similarity among the sequences indicating the conserved nature of gene. In addition the newly

sequence of *β-tubulin* of *C. annuum* had similarity with other plants, showing that some changes have occurred during the evolution from a common ancestor. Usually housekeeping gene remains conserved during evolutionary history which allows stable expression under particular treatments. So we can use these housekeeping gene as internal control and a reference gene (Huggett, 2006) for transcript analysis.

References

References

Alpert P, Simms EL (2002). The relative advantages of plasticity and fixity in different environments: when is it good for a plant to adjust? Evolutionary Ecology 16 (3): 285-297.

Argyropoulos D, Psallida C, Spyropoulos CG (2006). Generic normalization method for real-time PCR. FEBS Journal 273 (4): 770-777.

Barsalobres-Cavallari CF, Severino FE, Maluf MP, Maia IG (2009). Identification of suitable internal control genes for expression studies in Coffea arabica under different experimental conditions. BMC molecular biology 10 (1): 1.

Bartolo ME (1990). Effects of freezing and cold acclimation on microtubules and tubulin in mesophyll cells of spinach. PhD thesis University of Minnesota, St. Paul.

Biederman J, Yee J, Cortes P (2004). Validation of internal control genes for gene expression analysis in diabetic glomerulosclerosis. Kidney international 66 (6): 2308-2314.

Billich A, Winkler G, Aschauer H, Rot A, Peichl P (1997). Presence of cyclophilin A in synovial fluids of patients with rheumatoid arthritis. The Journal of experimental medicine 185 (5): 975-980.

Binet MN, Weil JH, Tessier LH (1991). Structure and expression of sunflower ubiquitin genes. Plant molecular biology 17 (3): 395-407.

Bo Jian1, Bin Liu2, Yurong Bi1, Wensheng Hou2, Cunxiang Wu2 and, Han*2 T (2008). Validation of internal control for gene expression study in soybean by quantitative real-time PCR. BMC Molecular Biology 9 1-14.

Bosland PW, Votava E (2000). Peppers: vegetable and spice capsicums, Cabi.

Brunner AM, Yakovlev IA, Strauss SH (2004). Validating internal controls for quantitative plant gene expression studies. BMC plant biology 4 (1): 14.

Burton RA, Shirley NJ, King BJ, Harvey AJ, Fincher GB (2004). The CesA gene family of barley. Quantitative analysis of transcripts reveals two groups of co-expressed genes. Plant physiology 134 (1): 224-236.

Bustin S (2002). Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. Journal of molecular endocrinology 29 (1): 23-39.

Callis J, Vierstra R (1989). Ubiquitin and ubiquitin genes in higher plants. Oxford Surv Plant Mol Cell Biol 6 1-30.

Canales RD, Luo Y, Willey JC, Austermiller B, Barbacioru CC, Boysen C, Hunkapiller K, Jensen RV, Knight CR, Lee KY (2006). Evaluation of DNA microarray results with quantitative gene expression platforms. Nature biotechnology 24 (9): 1115-1122.

Chan YL, Gutell R, Noller H, Wool I (1984). The nucleotide sequence of a rat 18 S ribosomal ribonucleic acid gene and a proposal for the secondary structure of 18 S ribosomal ribonucleic acid. Journal of Biological Chemistry 259 (1): 224-230..

Christensen AH, Sharrock RA, Quail PH (1992). Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant molecular biology 18 (4): 675-689.

Chuaqui RF, Bonner RF, Best CJM, Gillespie JW, Flaig MJ, Hewitt SM, Phillips JL, Krizman DB, Tangrea MA, Ahram M (2002). Post-analysis follow-up and validation of microarray experiments. Nature genetics 32 (supp): 509-514.

Coker JS, Davies E (2003). Selection of candidate housekeeping controls in tomato plants using EST data. Biotechniques 35 (4): 740-749.

Connaughton JF, Rairkar A, Lockard RE, Kumar A (1984). Primary structure of rabbit 18S ribosomal RNA determined by direct RNA sequence analysis. Nucleic acids research 12 (11): 4731-4745.

Cortez LM AC, Bugeau CM, Farias RN, Morero RD, Chehin RN. (2009). Glyceraldehyde-3-phosphate dehydrogenase tetramer dissociation and amyloid

fibril formation induced by negatively charged membranes. FEBS 584(3) 625-630.

Cortez LM, Ávila CL, Torres Bugeau CM, Farías RN, Morero RD, Chehín RN (2010). Glyceraldehyde-3-phosphate dehydrogenase tetramer dissociation and amyloid fibril formation induced by negatively charged membranes. FEBS letters 584 (3): 625-630.

Cyr R, Bustos M, Guiltinan MJ, Fosket D (1987). Developmental modulation of tubulin protein and mRNA levels during somatic embryogenesis in cultured carrot cells. Planta 171 (3): 365-376.

Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR (2005). Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant physiology 139 (1): 5-17.

Dawson PJ, Lloyd CW (1985). Identification of multiple tubulin.s in

taxol microtubules purified from carrot suspension cells. EMEIO J 4 2451-2455.

DeWitt TJ, Sih A, Wilson DS (1998). Costs and limits of phenotypic plasticity. Trends in Ecology & Evolution 13 (2): 77-81.

Dheda K, Huggett J, Chang J, Kim L, Bustin S, Johnson M, Rook G, Zumla A (2005). The implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization. Analytical biochemistry 344 (1): 141-143.

Ding J, Jia J, Yang L, Wen H, Zhang C, Liu W, Zhang D (2004). Validation of a rice specific gene, sucrose phosphate synthase, used as the endogenous reference gene for qualitative and real-time quantitative PCR detection of transgenes. Journal of agricultural and food chemistry 52 (11): 3372-3377.

Dostal DE, Rothblum KN, Baker KM (1994). An improved method for absolute quantification of mRNA using multiplex polymerase chain reaction: determination of renin and angiotensinogen mRNA levels in various tissues. Analytical biochemistry 223 (2): 239-250.

Edgcomb VP, Roger AJ, Simpson AGB, Kysela DT, Sogin ML (2001). Evolutionary relationships among "jakobid" flagellates as indicated by alpha-and beta-tubulin phylogenies. Molecular Biology and Evolution 18 (4): 514-522.

Expósito-Rodríguez M, Borges A, Borges-Pérez A, Pérez J (2008). Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. BMC plant biology 8 (1): 131.

Fischer G, Wittmann-Liebold B, Lang K, Kiefhaber T, Schmid FX (1989). Cyclophilin and peptidyl-prolyl cis-trans isomerase are probably identical proteins.

Foss DL, Baarsch MJ, Murtaugh MP (1998). Regulation of hypoxanthine phosphoribosyltransferase, glyceraldehyde-3-phosphate dehydrogenase and β-actin mRNA expression in porcine immune cells and tissues. Animal biotechnology 9 (1): 67-78.

Freeman WM, Walker SJ, Vrana KE (1999). Quantitative RT-PCR: pitfalls and potential. Biotechniques 26 112-125.

Gachon C, Mingam A, Charrier B (2004). Real-time PCR: what relevance to plant studies? Journal of experimental botany 55 (402): 1445-1454.

Garbarino JE, Belknap WR (1994). Isolation of a ubiquitin-ribosomal protein gene (ubi3) from potato and expression of its promoter in transgenic plants. Plant molecular biology 24 (1): 119-127.

Gilsbach R, Kouta M, Bonisch H, Bruss M (2006). Comparison of in vitro and in vivo reference genes for internal standardization of real-time PCR data. Biotechniques 40 (2): 173.

Givnish TJ (2002). Ecological constraints on the evolution of plasticity in plants. Evolutionary Ecology 16 (3): 213-242.

Goff SA, Ricke D, Lan TH, Presting G, Wang R, Dunn M, Glazebrook J, Sessions A, Oeller P, Varma H (2002). A draft sequence of the rice genome (Oryza sativa L. ssp. japonica). Science 296 (5565): 92-100.

Gull K, Hussey P, Sasse R, Schneider A, Seebeck T, Sherwin T (1986). Tubulin isotypes: generation of diversity in cells and microtubular organelles. Journal of cell science. Supplement 5 243.

Gutierrez L, Mauriat M, Guénin S, Pelloux J, Lefebvre JF, Louvet R, Rusterucci C, Moritz T, Guerineau F, Bellini C (2008). The lack of a systematic validation of reference genes: a serious pitfall undervalued in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants. Plant biotechnology journal 6 (6): 609-618.

Hampl V, Horner DS, Dyal P, Kulda J, Flegr J, Foster PG, Embley TM (2005). Inference of the phylogenetic position of oxymonads based on nine genes: support for Metamonada and Excavata. Molecular Biology and Evolution 22 (12): 2508-2518.

Howard J, Hyman AA (2003). Dynamics and mechanics of the microtubule plus end. Nature 422 (6933): 753-758.

Huggett J, Dheda K, SA B (2006). Normalization. In Real-Time PCR Edited by: Dorak MT. New York BIOS Advanced Methods 83-91.

Hunter T GJ (1977). Characterization of the mRNAs for alpha-, beta- and gamma-actin. Cell 12 (767-781):

Hussey P, Lloyd C, Gull K (1988). Differential and developmental expression of beta-tubulins in a higher plant. Journal of Biological Chemistry 263 (11): 5474-5479.

Hussey PJ, Gull K (1985). Multiple isotypes of a- and P-tubulin in the plant Phaseolus vulgaris. FEBS Lett 181 113-118.

Ishitani R, Kimura M, Sunaga K, Katsube N, Tanaka M, Chuang DM (1996). An antisense oligodeoxynucleotide to glyceraldehyde-3-phosphate dehydrogenase blocks age-induced apoptosis of mature cerebrocortical neurons in culture. Journal of Pharmacology and Experimental Therapeutics 278 (1): 447-454.

Jian B, Liu B, Bi Y, Hou W, Wu C, Han T (2008). Validation of internal control for gene expression study in soybean by quantitative real-time PCR. BMC molecular biology 9 (1): 59.

Joshi HC, Cleveland DW (1990). Diversity among tubulin subunits: toward what functional end? Cell motility and the cytoskeleton 16 (3): 159-163.

Joyce CM, Villemur R, Snustad DP, Silflow CD (1992). Tubulin gene expression in maize (Zea mays L.)* 1:: Change in isotype expression along the developmental axis of seedling root. Journal of molecular biology 227 (1): 97-107.

Kerr GP, Carter JV (1990). Tubulin isotypes in rye roots are altered during cold acclimation. Plant physiology 93 (1): 83.

Kim BR, Nam HY, Kim SU, Kim SI, Chang YJ (2003). Normalization of reverse transcription quantitative-PCR with housekeeping genes in rice. Biotechnology letters 25 (21): 1869-1872.

Kim KS FJ, Woodward CK. (1993). Hydrogen exchange identifies native-state motional domains important in protein folding. Biochemistry. 32(37) 9600 9608.

Kopczak SD, Haas NA, Hussey PJ, Silflow CD, Snustad DP (1992). The small genome of Arabidopsis contains at least six expressed [alpha]-Tubulin genes. The Plant Cell Online 4 (5): 539-547.

Lee PD, Sladek R, Greenwood CMT, Hudson TJ (2002). Control genes and variability: absence of ubiquitous reference transcripts in diverse mammalian expression studies. Genome Research 12 (2): 292-297.

Libus J, Storchova H (2006). Quantification of cDNA generated by reverse transcription of total RNA provides a simple alternative tool for quantitative RT-PCR normalization. Biotechniques 41 (2): 156.

Littauer UZ, Giveon D, Thierauf M, Ginzburg I, Ponstingl H (1986). Common and distinct tubulin binding sites for microtubule-associated proteins. Proceedings of the National Academy of Sciences 83 (19): 7162.

MacRae TH (1997). Tubulin Post-Translational Modifications. European Journal of Biochemistry 244 (2): 265-278.

Montague JW, Hughes Jr FM, Cidlowski JA (1997). Native recombinant cyclophilins A, B, and C degrade DNA independently of peptidylprolyl cis-trans-isomerase activity. Journal of Biological Chemistry 272 (10): 6677-6684.

Moritz M, Agard DA (2001). γ-Tubulin complexes and microtubule nucleation. Current opinion in structural biology 11 (2): 174-181.

Moriya S, Tanaka K, Ohkuma M, Sugano S, Kudo T (2001). Diversification of the microtubule system in the early stage of eukaryote evolution: elongation factor 1a andα-tubulin protein phylogeny of termite symbiotic oxymonad and hypermastigote protists. J Mol Evol 52 6–16.

Nelles L, Fang BL, Volckaert G, Vandenberghe A, De Wachter R (1984). Nucleotide sequence of a crustacean 18S ribosomal RNA gene and secondary structure of eukaryotic small subunit ribosomal RNAs. Nucleic Acids Res 12 (23): 8749-8768.

Nicot N, Hausman JF, Hoffmann L, Evers D (2005). Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. Journal of experimental botany 56 (421): 2907-2914.

Nolan T, Hands RE, Bustin SA (2006). Quantification of mRNA using real-time RT-PCR. Nature protocols 1 (3): 1559-1582.

Noller HF (1984). Structure of ribosomal RNA. Annu Rev Biochem 53 119-162.

Novoplansky A (2002). Developmental plasticity in plants: implications of non-cognitive behavior. Evolutionary Ecology 16 (3): 177-188.

Novoplansky A, Cohen D, Sachs T (1990). How Portulaca seedlings avoid their neighbours. Oecologia 82 (4): 490-493.

Oborny B (1994). Growth Rules in Clonal Plants and Environmental Predictability--A Simulation Study. Journal of Ecology 341-351.

Paolacci A, Tanzarella O, Porceddu E, Ciaffi M (2009). Identification and validation of reference genes for quantitative RT-PCR normalization in wheat. BMC molecular biology 10 (1): 11.

Pfaffl MW (2004). Quantification strategies in real-time PCR. AZ of quantitative PCR 1 89-113.

Prigozhina NL, Oakley CE, Lewis AM, Nayak T, Osmani SA, Oakley BR (2004). γ-Tubulin plays an essential role in the coordination of mitotic events. Molecular biology of the cell 15 (3): 1374-1386.

Raynal FM, B. Bachellerie, J. P. (1984). Complete nucleotide sequence of mouse 18 S rRNA gene: comparison with other available homologs. FEBS Lett 167 (2): 263-268.

Reid K, Olsson N, Schlosser J, Peng F, Lund S (2006). An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. BMC plant biology 6 (1): 27.

Remans T, Smeets K, Opdenakker K, Mathijsen D, Vangronsveld J, Cuypers A (2008). Normalisation of real-time RT-PCR gene expression measurements in Arabidopsis thaliana exposed to increased metal concentrations. Planta 227 (6): 1343-1349.

Schmittgen TD, Zakrajsek BA (2000). Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. Journal of biochemical and biophysical methods 46 (1): 69-81.

Shaw SL, Kamyar R, Ehrhardt DW (2003). Sustained microtubule treadmilling in Arabidopsis cortical arrays. Science 300 (5626): 1715-1718.

Singh R, Green MR (1993). Sequence-specific binding of transfer RNA by glyceraldehyde-3-phosphate dehydrogenase. Science 259 (5093): 365-368.

Smith JM, Szathmáry E (1997). The major transitions in evolution, Oxford University Press, USA.

Snustad DP, Haas NA, Kopczak SD, Silflow CD (1992). The Small Genome of Arabidopsis Contains at Least Nine Expressed [beta]-Tubulin Genes. The Plant Cell Online 4 (5): 549-556.

Steele BK, Meyers C, Ozbun MA (2002). Variable expression of some. Analytical biochemistry 307 (2): 341-347.

Suzuki T, Higgins P, Crawford D (2000). Control selection for RNA quantitation. Biotechniques 29 (2): 332-337.

Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, Grisar T, Igout A, Heinen E (1999). Housekeeping genes as internal standards: use and limits. Journal of biotechnology 75 (2): 291-295.

Thitamadee S, Tuchihara K, Hashimoto T (2002). Microtubule basis for left-handed helical growth in Arabidopsis. Nature 417 (6885): 193-196.

Torczynski R, Bollon AP, Fuke M (1983). The complete nucleotide sequence of the rat 18S ribosomal RNA gene and comparison with the respective yeast and frog genes. Nucleic acids research 11 (14): 4879-4890.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome biology 3 (7): research0034.

Vandesompele J, Kubista M, Pfaffl MW (2009). Reference gene validation software for improved normalization. Real-time PCR: Current Technology and Applications 47-64.

VanGuilder HD, Vrana KE, Freeman WM (2008). Twenty-five years of quantitative PCR for gene expression analysis. Biotechniques 44 (5): 619.

Waddington CH (1957). The strategy of the genes. A discussion of some aspects of theoretical biology. With an appendix by H. Kacser. The strategy of the genes. A discussion of some aspects of theoretical biology. With an appendix by H. Kacser. ix+-262.

Wan H, Zhao Z, Qian C, Sui Y, Malik AA, Chen J (2010). Selection of appropriate reference genes for gene expression studies by quantitative real-time polymerase chain reaction in cucumber. Analytical biochemistry 399 (2): 257-261.

Wasteneys GO (2002). Microtubule organization in the green kingdom: chaos or self-order? Journal of cell science 115 (7): 1345-1354.

Williams Jr RC, Shah C, Sackett D (1999). Separation of tubulin isoforms by isoelectric focusing in immobilized pH gradient gels. Analytical biochemistry 275 (2): 265.

Wong ML, Medrano JF (2005). Real-time PCR for mRNA quantitation. Biotechniques 39 (1): 75.

Yu J, Hu S, Wang J, Wong GKS, Li S, Liu B, Deng Y, Dai L, Zhou Y, Zhang X (2002). A draft sequence of the rice genome (Oryza sativa L. ssp. indica). Science 296 (5565): 79-92.

Zhang Z, Wagner L, Miller W (2000). "A greedy algorithm for aligning DNA sequences.". J Comput Biol 7 203-214.

Zheng Y, Wong ML, Alberts B, Mitchison T (1995). Nucleation of microtubule assembly by a gamma-tubulin-containing ring complex. Nature 378 (6557): 578.

Electronic references

http://www.ncbi.nlm.nih.gov/dbEST/

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