Pak. J. Bot., 45(2): 507-512, 2013.

MOLECULAR AND COMPARATIVE ANALYSIS OF NEWLY ISOLATED BETA-TUBULIN PARTIAL GENE SEQUENCES FROM SELECTED MEDICINAL PLANTS

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Abstract

Dynamic nature of beta tubulin (β-tubulin) gene is unleashed by recent studies reporting that apart from being a reliable reference gene serving for normalization purposes in gene expression analysis, β-tubulin encodes for structural proteins playing important role in cell cytoskeleton, microtubules and regulation of cell networking. This study is focused on the identification, isolation and characterization of 6 novel β-tubulin genes isolated from diverse range of 6 medicinal plants including *Ficus carica, Pisum sativum, Capsicum annum, Capparis decidua, Maytenus royleana* and *Eruca sativa*. The genomic sequences of newly isolated β-tubulin genes were analyzed and confirmed by using different bioinformatics tools followed by submission to genebank. We used ClustalW for pairwise alignment of these novel sequences with other known 18S rRNA sequences to find out their phylogenetic relationships. Our results have shown conserved nature of β-tubulin with some variable regions might be landmarks of some historical signals. Being housekeeping gene, β-tubulin can be used as good marker and internal control for several types of molecular analysis followed by validations of their consistent expression in the several plant species in future. This study will provide a platform for the molecular biologist interested in studying novel mechanisms of different medicinal plants.

Introduction

An essential pillar in maintaining dynamic microtubule network and wider aspects disclosed in recent researches are the factors making β-tubulin a gene of interest for future studies. Tight regulation of the dynamic behavior and function of the microtubule cytoskeleton is essential for axonal transport, organelle positioning, formation and assembly of cilia and flagella (Dutcher, 2001), cell motility, transport and maintenance of cell shape (Berrieman *et al.*, 2004) as well as for the development and survival of neurons (Tischfield & Engle, 2012) due to their involvement in various cellular processes (Jaglin & Chelly, 2009; Jaglin *et al.*, 2009). Alpha and β-tubulin heterodimers are the major components of the microtubules (Maccioni & Cambiazo, 1995; Dutcher, 2001).

β-tubulin is a GTP binding protein having 445 amino acids residues in sequence (Luduen, 1998; Wood *et al.*, 2001), encoded by four exons of β-tubulin gene (Diaz & Andreu, 1993). After their formation alpha and β-tubulin heterodimers polymerize in a head to tail array to form protofilaments, which assemble forming microtubules (Hesse *et al.*, 1987) containing different α- and β-tubulin isotypes each encoded by distinct genes (Lopata & Cleveland, 1987). Microtubule targeting agents bind to the β-tubulin subunit of the α/β-tubulin heterodimers forming microtubules (Stengel *et al.*, 2010). Microtubules needs to polymerize and depolymerize for performing its functions properly during cell division (Jordan & Wilson, 1998). Further researches on β-tubulin revealed new aspects of this gene. Recently many studies have shown differences in the β-tubulin gene expression is linked with drug resistance in cancer patients (Correnti *et al.*, 1995; Kavallaris *et al.*, 1997; Hasegawa *et al.*, 2003; Mozzetti *et al.*, 2005; Seve *et al.*, 2005; Urano *et al.*, 2006; Tommasi *et al.*, 2007). It has distinct multiple isotypes having dynamic properties (Banerjee *et al.*, 1992; Panda *et al.*, 1994) and all of these isotypes are conserved among different species (Berrieman *et al.*, 2004). However small differences in the properties of β-tubulin isotypes can influence the structure or assembly of microtubules, e.g., β_{III} -depleted tubulin polymerizes and assembles into microtubules at a faster rate than the unfractionated tubulins (Banerjee *et al.*, 1990; Ranganathan *et al.*, 1998; McKean *et al.*, 2001). Future studies that examine the role of each isotype in specific organism will greatly impact our overall understanding of microtubule function and behavior, and may provide avenues for future therapeutic intervention (Tischfield & Engle, 2012).

Moreover β-tubulin is also reported as a significant housekeeping gene to be used as internal control for gene expression analysis, because several studies account it as a very reliable reference gene for data normalization (Brunner *et al.*, 2004; Liu & Xu, 2006; deAlmeida *et al.*, 2010; Fernandez *et al.*, 2011). Those genes which encode the transcripts involved in basic cellular processes and cell survival, shows consistent expression in altering experimental conditions, so they are preferred for normalization of data (Czechowski *et al.*, 2005). β-tubulin is an example of such gene as it encodes for proteins playing role in structure of cytoskeleton, thus it is recommendable for normalization purposes. These wider angels of β-tubulin gene make us to carry out its identification and analysis in diverse group of medicinal plants.

This paper attempts to identify sequence and characterize novel isoforms of β-tubulin genes from six plants including *Ficus carica, Pisum sativum, Capsicum annum, Capparis decidua, Maytenus royleana* and *Eruca sativa*. These species are reported to be medicinally important (Duke, 1981; Konyalioglu *et al.*, 2005; Lynn *et al.*, 2006; Mahla *et al.*, 2010; Rauf *et al.*, 2012) this is a follow up of our earlier studies on *18s Ribosomal RNA* of these species (Banaras *et al.*, 2012).This wide range of β-tubulin gene sequences from non-model plants for their potential use as an internal control gene for future studies related to above mentioned plants.

Material and Methods

Plant material: Six diverse plants i.e., *F. carica, P. sativum, C. annuum, C. decidua, M. royleana* and *E. sativa* were collected from different parts of Pakistan for identification and sequencing of different homologues of β-tubulin gene. Their brief description is as follows;

F. carica commonly known as 'fig' belongs to family Moraceae and has many medicinal benefits. Its leaves and fruits are very famous as having laxative, stimulant, antitussive and emullient properties. They are also very effective against various throat diseases (Konyalıoglu *et al.*, 2005). Its latex also possesses potent pharmacological activities, the most important being the anticarcinogenic and antioxidant properties (Oliveira *et al.*, 2010). Moreover its methanolic extracts can lower the serum level of alanine aminotransferase, aspartate aminotransferase, bilirubin and malondialdehyde equivalents as an index of lipid peroxidation (Mohan *et al.*, 2007).

P. sativum belongs to family Fabaceae and is commonly called as 'pea'. It is cool season vegetable crop commonly used for culinary purposes. Its seeds contain trypsin and chymotrypsin that can be used as ecbolic, contraceptive, fungistatic and spermicide (Duke, 1981).

C. decidua belongs to family Capparaceae and is important drought resistant plant. It grows as dense, tufty and xerophytic shrub with significant medicinal value besides many socioeconomic and ecological benefits. It possesses many pharmacological properties like hypercholesterolemic, anti-inflammatory and analgesic, antidiabetic, antimicrobial, antiplaque, antihypertensive, antihelmintic & purgative activities (Mahla *et al.*, 2010; Singh *et al.*, 2011). Its intake also results in reduction in plasma triglycerides, total lipids and phospholipids concentration (Goyal & Grewal, 2003).

M. royleana belongs to family Celastraceae and grows in foot-hill zones. It is drought tolerant plant and can grow in arid or semi-arid areas. Its bark and leaves are involved in medicinal uses mainly for the treatment of bone fractures (Rauf *et al.*, 2012).

E. sativa belongs to Brassicaceae rocket species that are commonly used as salads vegetable and spice throughout the world (Lamy *et al.*, 2008). It possesses various therapeutic and medicinal properties like inhibition of tumorigenesis, anti-ulcer, hepatoprotective, stimulant, aphrodisiac, diuretic and in treatment of stomach diseases (Lynn *et al.*, 2006; Alqasoumi *et al.*, 2008; Rafatullah *et al.*, 2008) as well as owns dynamic anti bacterial activity (Gulfrazi *et al.*, 2011).

The fresh leaves of these plants were taken for the extraction of genomic DNA.

Genomic DNA extraction: Genomic DNA was extracted by CTAB (Cetyl Trimethyl Ammonium Bromide) method. Leaves tissues were washed with distilled water and 70% ethanol. One gram of washed leaves tissues from each plant was ground into fine powder in presence of liquid nitrogen in triplicates. The finely ground tissues were homogenized completely with 3ml of extraction buffer (E.B) in falcon tube and kept at room temperature. The composition of E.B was 100mM Tris HCl with pH 8, 20mM EDTA, 1M NaCl, 2% PVP-40, 0.002% CTAB, 0.02% phenanthrolein and 0.2% β-mercaptoethanol. The mixture was kept at 65°C in incubator for one hour with regular shaking and then was kept at the room temperature (25°C). Chloroform and isoamyl alcohol (1:24 ratio) was added in phenol (1:2 ratios) and 3 ml of this mixture was added in the falcon tube. All the samples were centrifuged at 12,000 rpm for 15mins to separate out the debris. The supernatant was taken after careful washing and poured into new falcon tubes. An equal volume of ice cold iso-propanol was added. The mixture was kept at -20°C overnight. Next day, the mixtures were centrifuged at 8000 rpm for 12mins and the pellets were washed twice with 15mM ammonium acetate in 80% ethanol first and then with 100% ethanol. The pellets were air dried and dissolved in 30µl 10mM Tris + EDTA (TE Buffer). Then at last, in order to check DNA quality, 5µl of each sample was loaded on 1% agarose gel and run at 90volts for 70mins. The gel was then checked on gel doc system at proper resolution and image was saved.

DNA quantification was done by NanoDrop-1000 spectrophotometer (ND/-1000 V3.7.1, Thermoscientific). The instrument 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).

Polymerase chain reaction (PCR): Genomic DNA of all the six plants was amplified by using gene specific primers under following conditions. First denaturation was done at 95°C for 5 min, followed by 35 cycles denaturation for 45 sec at 94°C, annealing at 57°C for 1 min followed by extension for 1 min at 72°C. Final extension was done for 10 min at 72°C. PCR products were checked on 1% agarose gel and UV trans-illuminator was used to scan the gel.

Sequencing of β-tubulin gene: Sequencing PCR products were purified by using Axygen prep kit (Tischfield *et al.*, 2010) according to the manufacturer's

instructions. Sequencing was performed by using Beckman CEQ 8800 sequencer. Sequencing PCR reaction mixture was made by adding RRv3.1 master mix as recommended by suppliers. Sequencing PCR was done by denaturation at 95°C for 1min followed by 30 cycles of denaturation at 95°C, annealing at 57°C (β-tubulin gene) for 30 seconds each and extension at 72°C for 4min followed by final extension at 72°C for 10min.

Analysis of sequence: The β-Tubulin gene sequence from genomic DNA amplification obtained after complete analysis by Beckman CEQ-8800 sequencer was then analyzed in Bioedit software to remove N's from the sequence and BLASTn software was used for alignment. This alignment information was then used for deducing phylogenetic information among these plants by constructing dandogram in clustalW multiple alignment application.

Results and Discussion

We selected 6 different plants (*Ficus carica, Pisum sativum, Capsicum annum, Capparis decidua, Maytenus royleana, Eruca sativa*) having significant medical importance in order to disclose β-tubulin gene identification, sequencing and evolutionary analysis. Good quality and quantity of DNA was extracted by CTAB method which was then quantified by Nano Drop. After DNA extraction, β-tubulin gene was amplified using forward and reverse primers which were designed from the conserved regions of known β-tubulin genes from NCBI database. The amplified product of ~250 bp of β-tubulin gene from respective plants is shown in the Fig. 1. We sequenced each product individually by Sanger dideoxy chain termination method in Beckman CEQ-8800 sequencer and β-tubulin partial gene sequences of *F. carica, P. sativum, C. annum, C. decidua, M. royleana, E. sativa* were submitted to genebank (Genebank group-grp 3974929). Initial analysis of these sequences by BLAST showed high degree of similarities with previously known homologues of β-tubulin genes of a number of other dicot plants, hence confirmed that the identified and sequenced gene is β-tubulin gene of selected medicinal plants. The dicot plants showing similarity in sequences, with isolated and sequenced β-tubulin gene were *B.napus, A.thaliana, G.hirsutum, C.maxima, S.tuberosum, P.trichocarpa, R.Communis, L.albus, P.pyrifolia, P.salicina, A.pyhllitidis, E.grandis, D.carota, T.tetragonioides, M.truncatula*. Sequences showing similarity were aligned with the new isolated sequences of respective medicinal plants, by using Bio Edit software as shown in Fig. 2. Alignment results indicate that β-tubulin gene sequence is highly conserved. These results are consistent with reports substantiating the conserved nature of β-tubulin gene (Burns & Surridge, 1990; Guenette *et al.*, 1991; Lai *et al.*, 1994).

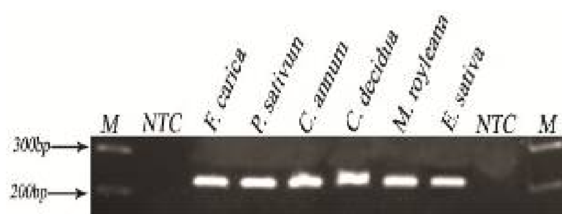

Fig. 1. Gel picture showing amplified products of β-tubulin gene in respective medicinal plants (*F. carica, P. sativum, C. annum, C. decidua, M. royleana* and *E. sativa*) obtained by polymerase chain reaction with 57°C annealing temperature and using gene specific primers. No bands were shown in negative control samples (NTC), 100 base pair marker (M) indicates amplified products of 250 base pairs approximately.

Our data suggests that this partial fragment of ~250bp of β-tubulin gene is highly conserved among the selected plant species in this study. Most of these sequences have less variability as compared to other sequences.

Phylogenetic tree was constructed based on DNA alignment generated by ClustalW. Result of phylogenetic tree showed evolutionary relationships of isolated β-tubulin gene with β-tubulin gene sequences of other known dicot plants as shown in Fig. 3. It clearly indicates that β-tubulin gene in these dicot plants remained conserved during evolution and is placed with the other dicots in the tree with slight changes in the sequence. Analysis of these newly isolated partial β-tubulin gene patterns and evolution in angiosperms fully or partially supports the clades of phylogenetic analysis of several previous studies (Banaras *et al.*, 2012).

This work can be further extended to study of expression profiles of β-tubulin gene as done previously with other genes (Aman *et al.*, 2012). β-tubulin being a reliable reference gene (Brunner *et al.*, 2004; Liu & Xu, 2006; deAlmeida *et al.*, 2010; Fernandez *et al.*, 2011) can serve for normalization purposes so this work can be proceeded to its validation in repective plants *Ficus carica, Pisum sativum, Capsicum annum, Capparis decidua, Maytenus royleana* and *Eruca sativa.*

To participate in exploring dynamic β-tubulin gene, we identified and characterized it in 6 different medicinal plants, we hope study of β-tubulin to flourish and further unleash its potentials in future. Newly revealed dynamic nature of β-tubulin proposes to examine the role of its each isotype, which will greatly impact our understanding of microtubule function and behavior, in relation to other structural proteins of the cell. For all of the future studies to explore different pathways and for gene analysis in the selected set of medicinal plants as internal control after appropriate validations for a given set of experimental conditions. This may provide avenues for future therapeutic intervention and growth of scientific knowledge of major/ minor mechanisms of plant life and physiology.

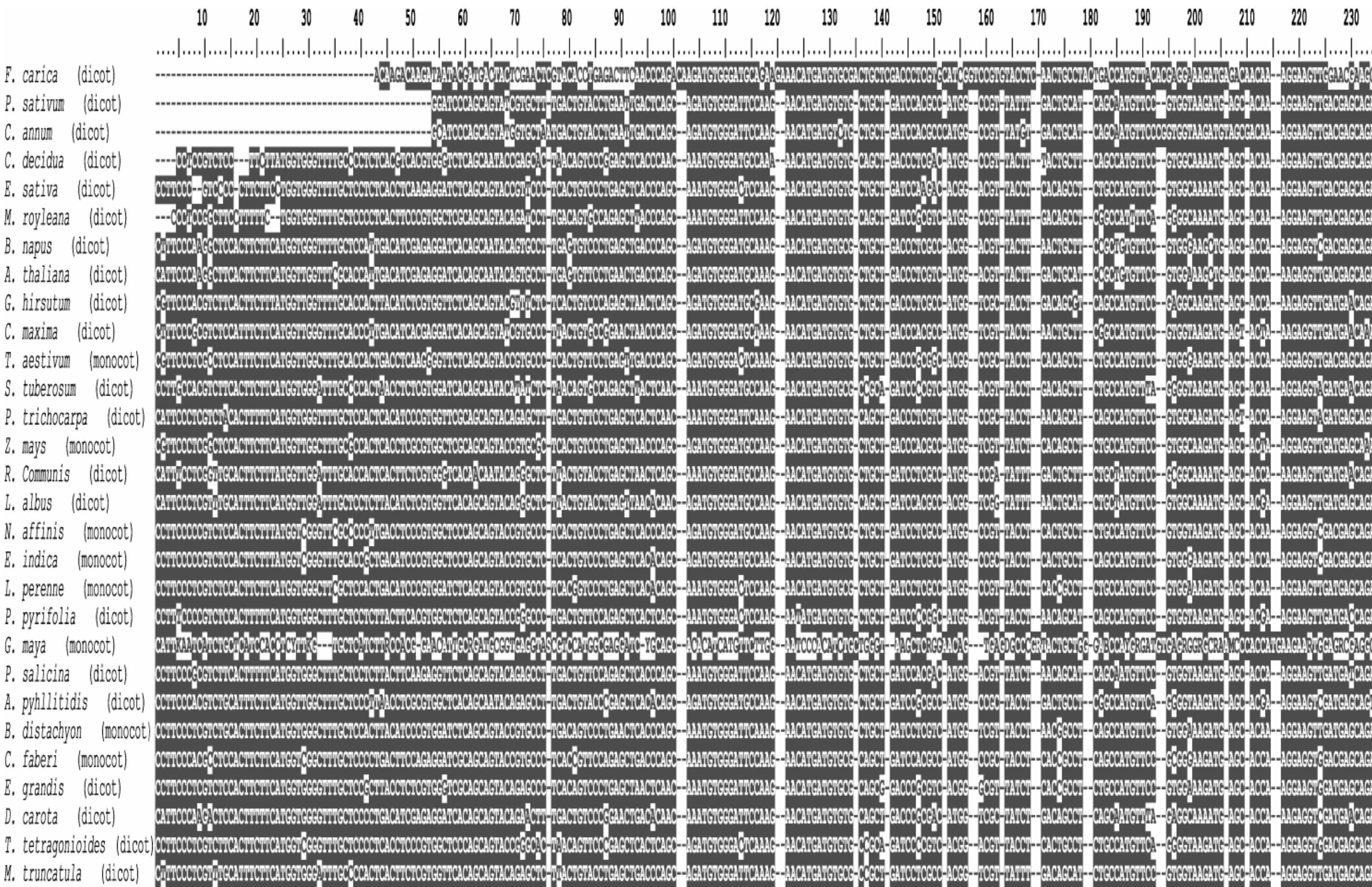

Fig. 2. Alignment of newly sequenced β-tubulin gene of selected plants including, *F. carica, P. sativum, C. annum, C. decidua, M. royleana* and *E. sativa* with related sequences downloaded from NCBI database. Amino acids conserved throughout all sequences are marked by a black background, which shows sequences of plants under study to be highly conserved.

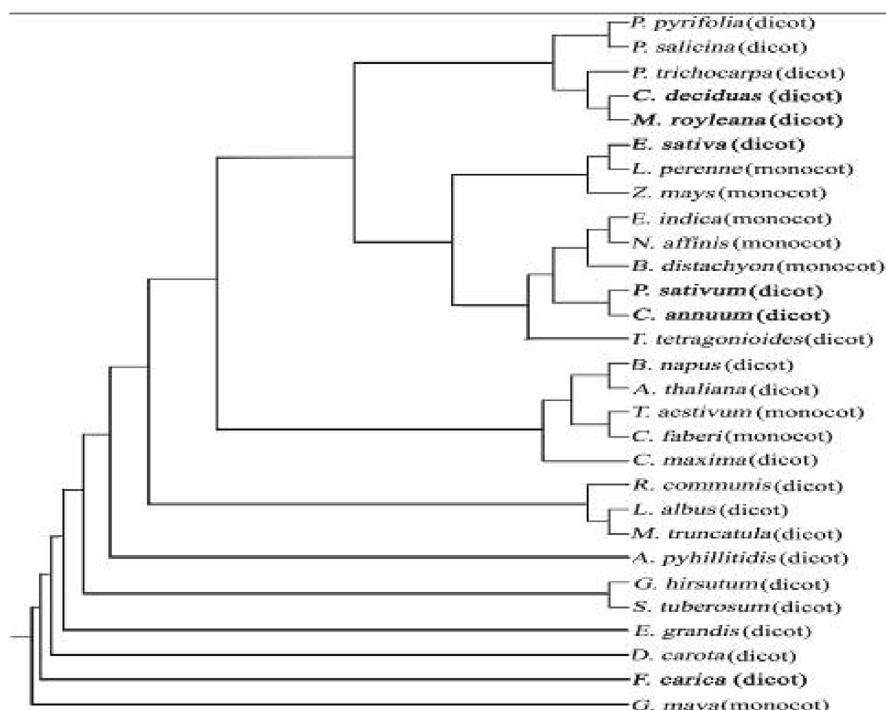

Fig. 3. Phylogenetic tree generated by using newly isolated sequences of β-tubulin gene of respective plants including, *F. carica*, *P. sativum*, *C.annum*, *C. decidua*, *M. royleana* and *E. sativa* with already known sequences of β-tubulin gene downloaded from NCBI.

Acknowledgments

This research was supported by a QAU Research Grant for 2010 to SNS at Quaid-e-azam University, Pakistan.

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(Received for publication 10 December 2011)