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Characterization of β-tubulin cDNA(s) from *Catharanthus roseus*

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Abstract

Vinblastine and vincristine are anti-tumor drugs produced by different species of *Vinca*. Vinca alkaloids depolymerize microtubules and as a result interfere with the normal formation of microtubules during cell division. Extremely low amount of vinca alkaloids in Vinca species and increased demand for chemotherapy are motives to scale up its production by biotechnological approaches. Here, β-tubulin gene family in *Catharanthus roseus* (*vinca rosea*) was analyzed to provide enough information for further approaches. 24 β-tubulin coding sequences from plants, mainly *Arabidopsis thaliana*, were obtained from genebank and aligned to design gene specific primers. Total RNA from leaves of C. roseus were isolated, the cDNA(s) synthesized using specific reverse primers, the amplified fragments cloned in pJET cloning vector and sequenced. Eight clones containing partial β-tubulin cDNAs' of *C. roseus*; including an almost full-length coding sequence, were constructed. The comparison of sequencing results with β-tubulin mRNA from *Arabidopsis thaliana, Arabidopsis lyrata, Populus trichocarpa, Zea maize, Oryza sativa, Hordeum vulgare, Gossypium hirsutum, Nicotiana attenuate, Solanum tuberosum* and *lycopersicone sculentum* revealed about 85% similarity of nucleotide sequence and about 98% identities at amino acid level. The clustalW alignment analyses underlined a short peptide at position 359-367 with substitutions very specific to *C. roseus*. Based on our sequencing results, we predicted at least four members of β-tubulin gene family for C. roseus. TUB cDNAs' from *C. roseus* sequenced to reveal any differentiation in comparison with non-vinca alkaloid producer plants. It was proposed the sites would carry higher potential for any amino acid alteration providing self-protection in vinca alkaloids producer plants; e.g. *C. roseus*.

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Introduction

Catharanthus roseus; synonym *vinca rosea*, is a herbaceous perennial plant belongs to the family *Apocynaceae*. *C.roseus* is important as commercial medicinal plant because of its alkaloidal content. Whole plant contains about 130 alkaloids, those are classified as vinca alkaloids. Vincristine and vinblastine; two anti-microtubular agents of vinca alkaloids, are the first natural drugs utilized in cancer therapy (Aslam J *et al*., 2010). They depolymerize microtubules and as a result interfere with the normal formation of microtubules during cell division (McKean PG *et al*., 2001).

Microtubules (MTs) are involved in a broad range of cellular processes including maintenance of cell shape and formation of mitotic spindle which in turn discover the role of microtubules in cell cycle. Tubulins gene family encodes six distinct but highly conserved subfamilies; alpha-, beta-, gamma-, delta-, epsilon-, and zeta-tubulins in eukaryote cells, are subunits of microtubules (McKean PG *et al*., 2001). In eukaryotes, α-tubulins (*TUA*) and β-tubulins (*TUB*) are the most abundant proteins of MTs (Gupta S *et al*., 2003). Ends of a microtubule that terminates with β-tubulin is more dynamic than a free minus end, thus β-tubulins are targets for numerous anti-mitotic agents, including colchicine and anti-tumor drugs such as the taxanes, epothilones and vinca alkaloids; vinblastine and vincristine (Gupta S *et al*., 2003). Vinca alkaloids bind rapidly to one or two high affinity sites are dubbed as vinca binding domain (175-213aa β-tubulin) in addition to several low affinity-binding sites (Gupta S *et al*., 2003). *TUB* cDNA(s) of plants are 1335-1353 bp long, coding for 445-451 amino acids. The β-tubulin gene(s) is reasonably conserved; with at least 60% amino acid similarity between the most distantly related lineages (Jutti JT *et al*., 2005). Single *TUB* gene is always found in some fungi and many protists (Trivinos-Lagos L *et al*., 1993); however, β-tubulin (*TUB)* gene family exists in fungi, protists and nearly all plants and animals have been examined (MacKay RM *et al*., 2002). Evolutionary study of tubulin genes in plant discovered several duplications in α- and β-tubulin genes from one β-tubulin and two α-tubulin genes. Nine members for *TUB* gene family in *A. thaliana*, eight in barely, six in wheat and 20 in *P. trichocarpa* were found in NCBI Genebank (Jost W *et al*., 2004, Schroder R *et al*., 2000).

The expressions of some of β-tubulin isotype are tissue specific, developmental stage specific or correspond to internal and external stresses. Furthermore, variation in promoters and or modified tubulin coding sequence could possibly be addressed by some aspects of plant growth and resistance to herbicides, cold or the control of plant shape, differences in expression and post-translational modifications, drugs, resistance to anti-microtubular agents and or developmental signals (Breviario D *et al*., 2000, Huzil JT *et al*., 2007, Shalli K *et al*., 2005). Analyses of β-tubulin mutations in mammalian cell lines reveal at least 23 different alterations which confer resistance to anti-microtubular drugs (Huzil JT *et al*., 2007 and the references therein).

Vinblastine and vincristine are anti-tumor drugs produced by different species of *Vinca*. Vinca alkaloids depolymerize microtubules and as a result interfere with the normal formation of microtubules during cell division. Extremely low amount of vinca alkaloids in Vinca species and increased demand for chemotherapy are motives to scale up its production by biotechnological approaches. Microtubules are being studied in animals and *saccharomyces cerevisiae* for the effects of anti-microtubular agents including vinca alkaloids; however, no study has been reported for plants MTs' that produce anti-microtubular agents. To find any possible alteration as a species-specific β-tubulin (*TUB*) isotype, here the β-tubulin mRNAs from *C. roseus* (vinca) were sequenced and their predicted amino acid sequences were compared in plants. Analyses of β-tubulin gene family from *Catharanthus roseus* (*vinca rosea*) was aimed to provide enough information for engineering *Catharanthus roseus* cells for a higher amount of vinca alkaloids.

Material and methods

Plant : *C. roseus* was provided from faculty of Pharmacology, Isfahan Medicinal University and planted in our lab. The fresh leaves were utilized for RNA isolation.

Primers: 24*TUB* coding sequences data available in Genebank from *Arabidopsis thaliana*, *Zea mays* and *Oryza sativa*, were aligned by clustalW method provided in DNASTAR Lasergene software (EditSeq, Meg- Align, MapDraw; Version 5.00), GENEDOC (Multiple Sequence Alignment Editor & Shading Utility Version 2.5.000) (Nicholas KB *et al.*,1990) and tested for specificity with Genebank BLAST (Altschul SF *et al.*, 1990). The accession numbers are At1g75780 (*AtTUB1*), At5g62690(*AtTUB2*), At5g62700(*AtTUB3*), At5g44340(*AtTUB4*), At1g20010(*AtTUB5*), At5g12250(*AtTUB6*), At2g29550(*AtTUB7*), At5g23860(*AtTUB8*), At4g20890(*AtTUB9*); Zm.2878(*ZmTUB1*), Zm.79(*ZmTUB2*), Zm.7172(*ZmTUB3*), Zm.3257(*ZmTUB4*), Zm.4718(*ZmTUB5*), Zm.7750(*ZmTUB6*), Zm.16988(*ZmTUB7*), Zm.4718(*ZmTUB8*); Os.46904(*OsTUB1*), Os.5233(*OsTUB2*), Os.43313(*OsTUB3*), Os.11093(*OsTUB4*), Os.4326917(*OsTUB5*), Os.45929(*OsTUB6*) and Os.11155(*OsTUB8*).

Based on the most conservative parts of the alignments and the vinblastine binding, GTP hydrolysis and tubulin-tubulin interaction sites, 17 specific forward and reverse primers were designed (Table 1 and Fig. 1).

Table 1. Sequence and ID of the primers were designed in this study for isolation of *vinca TUB* coding sequence.

Location of primers on Fig 1	ID	Sequence
Box 1	P.BTU.I-FWD1	5'-ATG AGA GAA ATC CTT CA
Box 1	P.BTU.I-FWD2	5'-ATG CGT GAG ATT CTT CA
Box 1	P.BTU.I-FWD3	5'-ATG AGA GAG ATC CTT CA
Box 3	P.BTU.S-FWD1	5'-AAG AAC ATG ATG TGT G
Box 2	P.BTU.S-FWD2	5'-TGG GCC AAG GGA CAC TAC AC
Box 2	P.BTU.S-FWD3	5'-ATG ATG CTT ACC TTC TC
Box 3	P.BTU.S-FWD4	5'-AAG AAC ATG ATG TG
Box 2	P.BTU.S-FWD5	5'-ATT GAT TCT GTT CTT GA
Box 2	P.BTU.S-FWD6	5'-ATC GAT TCC GTT CTC GA
Box 3	P.BTU.S-REV1	5'-CAC ATC ATG TTC TT
Box 3	P.BTU.S-REV2	5'-CAC ATC ATG TTT TT
Box 2	P.BTU.S-REV4	5'-TCA AGA ACA GAA TCA AT
Box 2	P.BTU.S-REV5	5'-TCG AGA ACG GAA TCG AT
Box 4	P.BTU.S.REV15	5'-CCC TCA CCT GTG TAC CAA TG
Box 4	P.BTU.S.REV16	5'-CCT TCT CCT GTG TAC CAA TG
Box 4	P.BTU.S.REV17	5'-CCT TCA CCT GTG TAC CAA TG

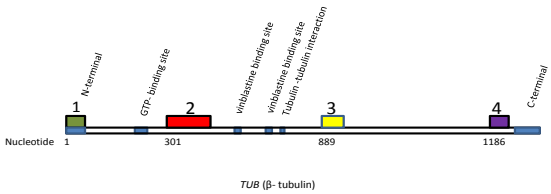

Fig. 1. Positions of the specific primers on plant β-tubulin coding sequence: Green box (1): P.BTU.I-FWD1, P.BTU.I-FWD2, P.BTU.I-FWD3 Red box (2): P.BTU.S-FWD2, P.BTU.S-FWD3, P.BTU.S-FWD5, P.BTU.S-FWD6, P.BTU.S.REV4, P.BTU.S.REV5 Yellow box (3): P.BTU.S-FWD1, P.BTU.S-FWD4,P.BTU.S.REV1, P.BTU.S.REV2, Purple box (4): P.BTU.S.REV15, P.BTU.S.REV16, P.BTU.S.REV17 and important regions as N-terminal, GTP-binding site, vinblastine binding site, Tubulin-tubulin interaction, C-terminal are demonstrated.

RNA isolation and cDNA amplification: Total RNA was isolated by RNeasy plant mini Kit (Qiagene). The cDNA(s) were synthesized using ″RevertAid™M-MuLV Reverse Transcriptase kit″ (#EP0441, Fermentas) and oligo d(T) 18mer, P.BTU.S-REV1, P.BTU.S-REV2,P.BTU.S-REV4, P.BTU.S-REV5, P.BTU.S.REV15, P.BTU.S.REV16 and P.BTU.S.REV17 primers (Table1) and amplified with a combination of primers listed in Table 2. The amplifications were driven 30 cycles at defined annealing temperature for every pairs of primers (T_m-5°C) and using *pfu* DNA polymerase (#EP0501, Fermentas). The program was ended by a final extension of 5-15 min at 72°Cupon the expected lengths (Fig.2 and Table 2).

Cloning and sequencing of Vinca *TUB*s: PCR products with the expected length were purified from 0.8% agarose gel using silica bead GEL extraction DNA kit (#K0513, Fermentas). The DNA fragments cloned in *pJET1.2*blunt end vector (#K1231, Fermentas) followed by introducing to *E. coli* (*DH5α*) via electroporation (Woodal CA , 2003). The plasmid DNAs, were isolated by alkaline lysis method (Ausubel F M *et al.*,1987, Sambrook J *et al.*,1989), and purified with 5MNH_4Ac. The clones from independent PCR reactions were sequenced utilizing Eurofins MWG Operon company service. Sequence analysis was done with DNASTAR Lasergene software (SeqManII, EditSeq, Meg- Align, MapDraw; Version 5.00), GENEDOC (Multiple Sequence Alignment Editor & Shading Utility Version 2.5.000)(Nicholas KB *et al.*,1990) and genebank BLAST (Altschul SF *et al.*, 1990).The sequencing data were submitted in NCBI genebank (http://www.ncbi.nlm.nih.gov/).The amino acid sequences were aligned with clustalW software (Chenna R *et al.*, 2003).*TUB* nucleotides and amino acid sequences aligned with *TUB* from other plants and analyzed (Fig.3). Positions of primers showed in Fig (1). PCR products with gel agarose1% exhibited in Fig (2).

Table 2. Forward and Reverse primers were used in combination to isolate *vinca TUB* partial cDNA and the expected lengths of PCR product.

Forward primer	Reverse primer	Expected length of PCR product
P.BTU.I.FWD3	P.BTU.S-REV2	355bp
	P.BTU.S.REV15	1200bp
P.BTU.S FWD1	P.BTU.S.REV15	300bp
	P.BTU.S.REV16	
	P.BTU.S.REV17	
P.BTU.S-FWD2	P.BTU.S.REV15	900bp
P.BTU.S-FWD6	P.BTU.S.REV15	850bp
	P.BTU.S.REV16	
	P.BTU.S.REV17	

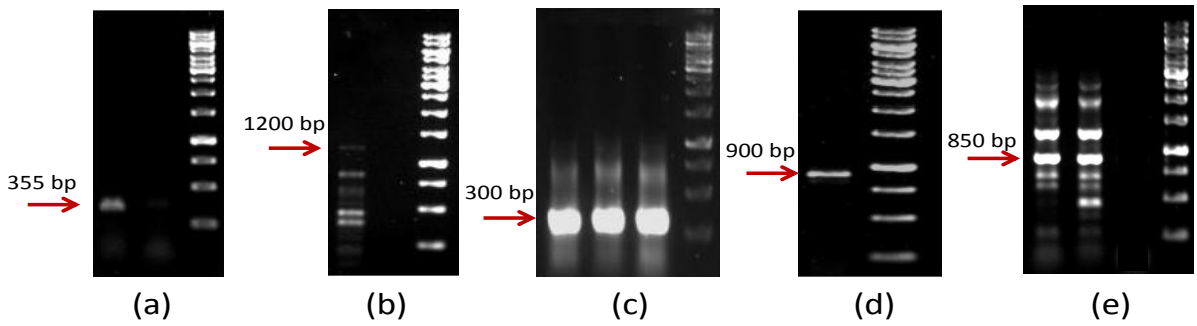

Fig. 2. β-tubulin coding sequences from *C. roseus* were amplified using primer pairs as: a)P.BTU.I-FWD3 and P.BTU.S-REV2, b)P.BTU.I-FWD3 and P.BTU.S.REV15, c)P.BTU.S-FWD1 and P.BTU.S.REV15, P.BTU.S-FWD1 and P.BTU.S.REV16, P.BTU.S-FWD1 and P.BTU.S.REV17, d)P.BTU.S-FWD2 and P.BTU.S.REV15, e)P.BTU.S-FWD6 and P.BTU.S.REV15, P.BTU.S-FWD6 and P.BTU.S.REV16 and P.BTU.S-FWD6. 1Kb ladder (Fermentas) was used as DNA size marker.

Results

To find out any probable changes in β- tubulin(s) (*TUB*s) from *C. roseus*, *TUB* partial cDNA's were sequenced and analyzed. Using the primers shown in Table 1 and 2 a gene-specific amplification was achieved for all cDNA fragments (Fig. 2). The PCR products were different in lengths respecting to the primer positions on *TUB* consensus (Fig.1,2 and Table 2). The 355, 1200, 300, 900 and 850 bp DNA fragments of vinca *TUB* were cloned and sequenced. The sequences of all fragments were submitted to the NCBI database (Genbank accession numbers: *TUB*cds1-353 (pZF11): JK265506, *TUB* partial cds337-1205 (pZF73): JK265507, *TUB* partial cds337-1205 (pZF85): JK265508, *TUB* partial cds1-1205 (pZF87): JK265509, *TUB* partialcds301-1205 (pZF89): JK265510, *TUB* partial cds889-1205 (pZF100): JK265511, *TUB* partialcds889-1205 (pZF103): JK265512, *TUB* partialcds889-1205 (pZF106): JK265513).

Comparison of the partial coding sequences with EST data confirmed that all fragments were a member of *TUB* gene family. Nucleotide BLAST search against the eight clones displayed that they were 82-89% similar to plants' *TUB*; while, the identity increased to 95-99% in protein BLAST alignments. Translated DNA from pZF11was 97% similar to β-tubulin of *Populus trichocarpa*, *Oryza sativa* and *Ricinus communis*. Pblast against pZF100 and pZF103 showed a 98% and 96% identity to an unknown protein in *Zea mays* respectively. The protein sequence from pZF106 was 99 and 97% identical to *Gossypium hirsutum* Tub4 and Tub2. Both clones pZF73 and pZF85, encoded proteins 99% similar to β-tubulin from *Nicotiana attenuate* and *Ricinus communis*. DNA product from pZF87 was 99% identical to Tub5 in *Zea mays*. Ninety nine percent identity was found for pZF89 protein to Tub6 in *Theobroma cacao* (data not shown). Alignment analyses were done using clustalW method among partial *TUB* translated mRNA's from *C. roseus* and corresponding sequences from *Arabidopsis thaliana*, *Arabidopsis lyrata*, *Oryza sativa*, *Triticum aestivum*, *Populus trichocarpa*, *Gossypium hirsutum*, *Salix arbutifolia*, *Solanum tuberosum*, *Solanum lycopersicom* and *Hordeum vulgare*. A part of the alignments is depicted in Fig. 3. Same as all β-tubulins have been known from all organisms, Tub from *C. roseus* started with the motif MERLI (data not shown). No amino acid substitutions were detected in vinca alkaloid binding domain located at positions180-218 and 244-266 (Fig. 3, black boxes) and GTP hydrolysis site at amino acids 146-153 (Fig. 3, green box). Amino acids 377-391 are conserved in plants (Fig. 3, blue box). According to alignment analyses were done in this study in companion with same previous studies, a species-specific short motif was detected at about C-terminal amino acids 359-367 (Fig. 3, the red box). More than 100 β-tubulins in

12 plants were compared for the nine amino acid sequences (Table 3).

Table 3. Classification of plants based on variation at amino acids 359-367 in β-tubulins.

Plant	Non-specific sequence	Semi-specific sequence	Species-specific sequence	β -tubulins in genebank
Arabidopsis thaliana	IPPKGLKMA IPPTGLKMA IAPRGLSMA		IAPTGLKMA IAPKGLKMA	9
Hordeum vulgare	IAPRGLSMA IPPRGLSMA	IPPTGLSMA	IPPTGLKMS MPPRGLKMA	5
Populus trichocarpa	IPPKGLKMA IPPTGLKMA IPPRGLKMA	IPPRGLAMA IPPKGLSMA IPPTGLQMA IPPTGLAMA IPPTGLAMS IPPTGLTMA SPPTGLKMA	IPPKGLKMS IAPKGLTMA IPPIGLAMA IPPNGLSMA	20
Salix arbutifolia	IPPTGLKMA	IPPTGLQMA IPPRGLAMA IPPTGLAMA IPPTGLAMS IPPTGLTMA SPPTGLKMA	IPPRGLKMS IPPIGLSMA VPPKGLTMS IPPTGLAIS IAPGGLTMS	20
Gossypium hirsutum	IPPKGLKMA IPPTGLKMA IPPRGLSMA IPPRGLKMA		IPPSGLKMA IPPKNLRMA	16
Oryza sativa	IPPIGLKMA IPPRGLSMA IPPRGLKMA IPPIGLKMA		IPPNGLKMA IPPRGLSMG	8
Triticum astivum	IPPTGLKMA IAPRGLSMA	IPPTGLSMA	IPPVGLAMA MPPRGLKMS IPPRGLSMS	6
Solanum tuberosum	IPPTGLKMA			1
Ricinus communis	IPPKGLKMA IPPTGLKMA IPPRGLSMA	IPPKGLSMA	IPPTGLSMS	11 (putative)
Nicotiana attenuata	IPPTGLKMA			1
Solanum lycopersicum	IPPTGLKMA IPPRGLSMA		IPPRGLSIS	4
Catharantus roseus	IPPIGLKMA		IAPKGLSMA IPPTGLRMA	8

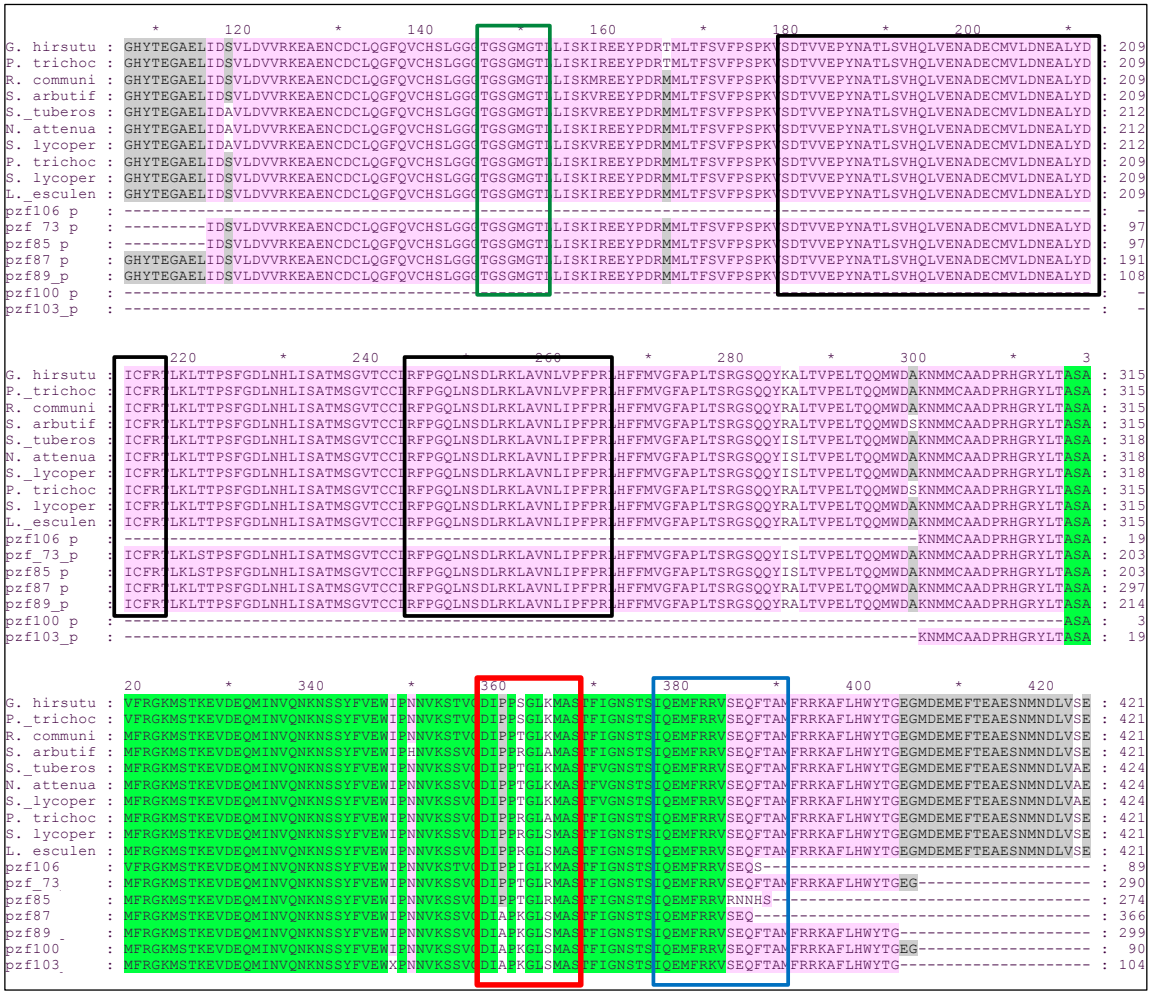

Fig. 3. Amino acid sequence alignment of *C. roseus* (vinca) β-tubulin clones created in this study (pZF 100, 103, 106, 73, 85, 87, 89) and corresponding sequences from *Gossypium hirsutum* (ABY866661.1), *Populus trichocarpa* (ABK94896.1), *Ricinus communis* (XM002509734.1), *Salix arbutifolia* (AGH08229.1), *Solanum tuberosum* (ACH68564.1), *Nicotiana attenuata* (AAR37366.1), *Solanum lycopersicum* (ABB13293.1), *Populus trichocarpa* (EF146853.1), *Solanum lycopersicum* (AK325964) as are appeared in this figure. The amino acids are presented in green box demonstrate GTP binding site, the black boxes show vinblastine binding domain and crosslink with vinblastine sites respectively, the amino acids are surrounded in blue box are conserved in plants. The short polypeptides are enclosed in red box show species-specific amino acid sequence. More explanation is provided in the text.

Different substitutions were classified as non-specific; same substitutions in three or more species, semi-specific; same substitutions in two species and species-specific; unique sequences for every species (Table 3). The sequences IPPTGLKMA, IPPRGLSMA and IPPKGLKMA were observed very often among β-tubulins of plants; although, the sequences IPPIGLKMA and IPPRGKLMA were seen infrequently in the analyzed β-tubulins. IPPTGLSMA was detected only in *H. vulgare* and *T. aestivum*. The sequences IPPRGLAMA, IPPTGLQMA, IPPTGLAMA, IPPTGLAMS, IPPTGLTMA and SPPTGLKMA were specific to *P. trichocarpa* and *S. arbutifolia*. Two amino acid sequences; IAPKGLSMA and IPPTGLRMA, marked to be specific for β-tubulins of *C. roseus*. Twenty four out of 109 β-tubulin sequences were quite specific to species. The variations in substitutions of C-terminus position 359-367 were directly related to number of β-tubulin gene members (known from genebank) in every plant (Table 3). The

amino acids, His6, Glu198 and Phe170 involved in benomyl sensitivity were also conserved in vinca Tubs. Amino acids 53-58 that interact with C-terminus were conserved (data not shown). The cysteine residues; C12, C25, C127, C201, C211 and C354, were without any changes in *C. roseus*. Among cds were characterized here, the clones pZF85 and pZF73 were fully identical. The clones; pZF103, pZF100, pZF89 and pZF87, showed same substitutions at position 359-367 and pZF106 was unique at this position. pZF11 contained a cds fragmentwith120 amino acids from start codon. pZF85 and pZF11 varied from each other at position 19-45. According to our results, β-tubulin gene family had at least four members in *C. roseus*.

Discussion

TUB cDNAs' from *C. roseus* sequenced to reveal any differentiation in comparison with non-vinca alkaloid producer plants.β-tubulins' partial cds from *C. roseus* (vinca) were isolated using specific primers designed for vinca alkaloid binding, GTP hydrolysis and tubulin- tubulin interaction sites. It was proposed the sites would carry higher potential for any amino acid alteration providing self-protection in vinca alkaloids producer plants; e.g. *C. roseus*.

Sequencing analyses leaded to eight partial cds of β-tubulin representing four isotypes from strat codon to amino acid about 400 in *C. roseus*. The fragments lack about 50 amino acids in C-termini.

A high degree of conservation was observed within the *TUB*-family of *C. roseus*. Absence of the last 50 amino acids raised up the identity to 99% in comparison to data from complete Tub protein alignments. (Oakley RV *et al*., 2007) showed twenty β-tubulins from *P. tremuloides* share 76% to 96% nucleotide identity with a variable 3' UTR (less than 68% sequence identity) and a highly conserved proteins; 89–98% sequence similarity, excluding the hyper-variable C-termini. This is compatible with our founding on vinca β-tubulins'.

In β-tubulins from *C. roseus*, vinca alkaloid binding, GTP hydrolysis and tubulin-tubulin interaction sites were same as other plants (Fig. 3). Evidences indicated vinca alkaloids bind sulfhydryl group (Gupta ML Jr *et al*., 2001). Based on mutations in the cysteine residues suggested that C12, C354 play roles in tubulin function *in vivo,* C354 mutants appeared to be completely stable to low temperature in *S. cervisiae* and the C239- C354 crosslinking is enhanced by vinblastine (Gupta ML Jr *et al*., 2001). The cysteine residues; C12, C25, C127, C201, C211 and C354, were highly conserved in *C. roseus* as well as other plants (Fig. 3). Furthermore, mutation of L240 to I240 in *Homo sapience TUB*s' is known to confer resistance against vinca alkaloids (Kavallaris M *et al*., 2001). Such an alteration was not detected in any of eight clones created in this study.

The tetra-peptide motif MREI at the N-terminus, which is a character for plant and animal β-tubulins' was occurred in vinca β-tubulin as well. The motif plays a regulatory role to control tubulin accumulation under anti-miotic drugs stress (reviewed in 4). Moreover, (Jost W *et al*., 2004) based on their investigation on *physcomitrella patens* declare that the extreme C-termini of plant α- and β-tubulin gene isotypes are responsible for differentiation in *TUB*s expression.

TUB families in populous (Oakley RV *et al*., 2007), Arabidopsis (Snustad DP *et al*.,1992, Cheng Z *et al*., 2001), rice (Yoshikawa M *et al*., 2003), cotton (Whittaker DJ *et al*., 1999 , Li L *et al*.,2007) and many other plants contain distinct isotypes with similarity about 85-98%. The isotypes show variable expression during development, in different tissues or under environmental stresses. Despite *TUB* families are highly conserved among species and organisms, the isotypes differ from each other in their very C-termini (Sackett DL , 1995, Breviario D *et al*., 2000, Jost W *et al*., 2004). Yet, no sequence (amino acid or nucleotide) comparisons of extreme C-termini of β-tubulins within plant species have been reported. All the eight partial coding sequences isolated from *C.*

roseus lack 50 amino acids at the C-termini; however, the alignments showed a variable short motif at position 359-367. Our study revealed that the sequence was variable among β-tubulin isoforms of species and plants (Table 3). Nearly in all 12 plants were compared for more than one β-tubulins isotypes, at least one specific sequence at amino acid 359-367 were found (Table 3). The short peptides at 359-367 in β-tubulins isotypes from *Populus trichocarpa* and *Salix arbutifolia*; two species belong to two genera in family salicaceae, shared six same substitutions as semi-specific sequences. A same phenomenon was observed in two species of family graminaceae; *Hordeum vulgare* and *Triticum aestivum,* where they had one identical β-tubulin isoform regarding position 359-367. Therefore it is reasonable to suppose a role for the short peptide (amino acids 359-367) either in gene and protein of β-tubulin or its function.

Since no differences were detected in the vinca alkaloid binding sites, the tolerant of vinca alkaloids in *C. roseus* must be based on other mechanisms than the presence of specific resistance conferring alleles of *TUB*. Mutations in α-tubulin proteins, allele specific regulation of gene expression or MT dynamics under vinca alkaloids stresses with focus on the species-specific motif at 359-366 are the most possible mechanisms to be addressed. The motif may play a regulatory role or provide a specific 3D structure which confers tolerant to its own alkaloids.

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