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The isolation, characterization and sequence of two divergent β-tubulin genes from soybean (*Glycine max* L.)

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Abstract

Two divergent β-tubulin genes (designated Sβ-1 and Sβ-2) were isolated by screening a soybean genomic library with a *Chlamydomonas reinhardtii* β-tubulin cDNA probe. Restriction fragment analysis of the clones recovered, and of soybean genomic DNA, indicated that these represent two unique classes of structurally different β-tubulin genes in the soybean genome. However, it is possible that unidentified members of these classes or additional highly divergent classes of β-tubulin genes (thus far undetected) exist in the soybean genome. The Sβ-1 and Sβ-2 genomic clones were sequenced, revealing that both are potentially functional genes which would encode β-tubulins of 445 and 449 amino acids, respectively. A comparison of their derived amino acid sequences with β-tubulins from several organisms showed that they are most homologous to *Chlamydomonas* β-tubulin (85–87%), with lesser degrees of homology to β-tubulins of vertebrate species (79–83%), *Trypanosoma brucei* (80–81%) and *Saccharomyces cerevisiae* (66–68%). The amino acid sequences of Sβ-1 and Sβ-2 are as divergent from each other as they are from the *Chlamydomonas* β-tubulin. The amino acids at the diverged positions in Sβ-2 are nearly all conservative substitutions while in Sβ-1, 18 of the 69 substitutions were non-conservative. Both soybean β-tubulin genes contain two introns in exactly the same positions. The first soybean intron is located in the same position as the third intron of the *Chlamydomonas* β-tubulin genes. Codon usage in the two soybean β-tubulins is remarkably similar ($D^2 = 0.87$), but differs from codon usage in other soybean genes.

Introduction

Microtubules are filamentous polymers of the dimeric protein tubulin, whose two subunit polypeptides, designated α- and β-tubulin, have molecular weights near 50000 [20, 33]. A comparison of the amino acid sequences of the β-tubulins from numerous species, obtained by direct protein sequencing or derived from nucleotide sequences of their encoding genes or cDNAs, suggests that the tubulins are extensively conserved in evolution, and that the divergence between the β-tubulins is clustered primarily in one highly variable domain at the carboxyl terminus [9]. A constant region also has been identified

which shows nearly 100% conservation between different species (amino acid positions 401–425). The yeast *Saccharomyces cerevisiae* β-tubulin exhibits greater divergence than that of other species (approximately 70% conserved as compared to mammalian β-tubulins), but it has a similar pattern of highly conserved and diverged regions [31].

Plant cells have several microtubule arrays, with specialized functions, which are not found in animal cells. These include: 1) the cortical array, which may be involved in the orientation of cellulose microfibril deposition, 2) the preprophase band, which may orient the plane of cell division, and 3) the phragmoplast, which forms the cell plate during cytokinesis [15]. Recent studies have shown that higher plant tubulins differ significantly from vertebrate tubulins in their drug and herbicide-binding characteristics, immunological properties, and peptide maps [26–30]. For these reasons it is important to determine the sequence of higher plant tubulins and to compare these sequences with those of vertebrates and other organisms. Ultimately we hope to explain the unique functional properties of plant tubulin and the interactions of microtubules with other cellular structures in terms of the molecular structure of the tubulin monomers.

The tubulins are encoded by multigene families in nearly all eukaryotes examined thus far [9]. The exceptions are *Saccharomyces cerevisiae* [31], *Schizosaccharomyces pombe* [18] and *Neurospora* [32], each of which have a single β-tubulin gene, and *Tetrahymena,* with one α-tubulin gene [5]. Other eukaryotes exhibit from 2 to 20 different α- and β-tubulin genes or pseudogenes, although in humans many of these are pseudogenes [9]. While the number of functional tubulin genes usually varies from two to four in most lower eukaryotes, mammals have six functional α- and six functional β-tubulin genes [41, 42]. This paper describes the isolation and sequencing of two members of the β-tubulin gene family from a higher plant (soybean). Sequence analysis revealed that both genes contain regulatory signals found in most eukaryotic genes, indicating that they are potentially expressed. Analysis of the deduced amino acid sequences of these soybean β-tubulins demonstrates that they encode comparatively divergent proteins (for tubulins), both from each other and from the β-tubulins of other species.

Materials and methods

Soybean genomic library screening

A Charon 4a Lambda library was constructed in the laboratory of Dr R. Goldberg (Biology Dept., UCLA), from sized, randomly sheared genomic DNA of *Glycine max* cv. Forest, to which *Eco*RI linkers had been attached. The library was screened by plaque hybridization [1] to a 1.2-kb *Pst*I fragment from the *Chlamydomonas reinhardtii* β-tubulin cDNA (which was obtained from Dr C. Silflow, Dept. of Cell Biol. and Genetics, Univ. of Minnesota) [37]. The hybridizations were carried out at 42 °C for 16 h in a mixture of 3× SSC, 1× Denhardt's solution [10], 40% formamide, and 100 μg/ml salmon sperm DNA. Filters were washed in a series of 3×, 1× and 0.3× SSC (each with 0.1% SDS) at 55 °C for a total of 60 min.

Bacterial strains and plasmids

The *E. coli* strain KH802 was used as a Lambda host, while strains JM83 [25] and TB1 [T. O. Baldwin, Texas A&M Univ., personal communication] were used for cloning in pUC13 [40] and in SP6 plasmids [24]. Strain JM103 [44] was used for M13 phage vectors. The cells were made competent for DNA transformation by standard methods [17].

Nucleic acid extractions and analysis

DNA was extracted from *Glycine max* cv. Forest seedlings using the CTAB method [38]. Bacteriophage DNA was phenol-extracted from CsCl gradient-purified liquid lysate preparations [22]. Plasmid DNA was purified by alkali extraction [2], and large-scale plasmid preparations were further purified by CsCl density gradient ultracentrifugation [22]. Southern analysis was performed by separating DNA restriction fragments by agarose gel electrophoresis followed by transfer to nitrocellulose filters using standard techniques [22]. The blots were hybridized to labeled probes in 50% formamide, 3× SSC, and 10× Denhardt's solution at 42 °C, then washed at 50 °C in 0.1× SSC and 0.1% SDS for 2 h before autoradiography.

SP6 RNA probes

Fragments of Sβ-1 and Sβ-2 containing coding regions (a *Xho*I-*Bam*HI fragment from Sβ-1 and a *Bgl*II-*Sal*I fragment from Sβ-2) were subcloned into the vectors SP64 and SP65. Single-stranded, labeled RNA hybridization probes were obtained by transcribing the cloned sequence from the SP6 promoters of these vectors [24].

DNA sequencing

Fragments spanning the coding sequences and flanking regions of both Sβ-1 and Sβ-2 were isolated from the pUC13 subclones and either sequenced directly by the Maxam and Gilbert [23] method, or subcloned into M13mp18 or mp19, and sequenced by the Sanger dideoxy method [34, 36] (see Fig. 1). The reactions were electrophoresed on 0.4-mm-thick urea/polyacrylamide gels [35], then autoradiographed. Sequence data was analyzed with the software developed by the University of Wisconsin Genetics Computer Group [11], using a Digital Equipment Corporation VAX II computer. Sequences of other tubulin genes were retrieved from the Genbank and EMBL data bases.

Results

Isolation and characterization of soybean genomic fragments containing β-tubulin homologous sequences

Approximately 1.9 genome equivalents of soybean DNA in a Lambda Charon 4a library were screened with a 1.2-kb *Pst*I fragment from a *Chlamydomonas* β-tubulin cDNA designated β-37 [37]. Thirty-five Lambda clones were isolated which hybridized strongly to the heterologous probe. After three rounds of plaque purification, DNA was isolated

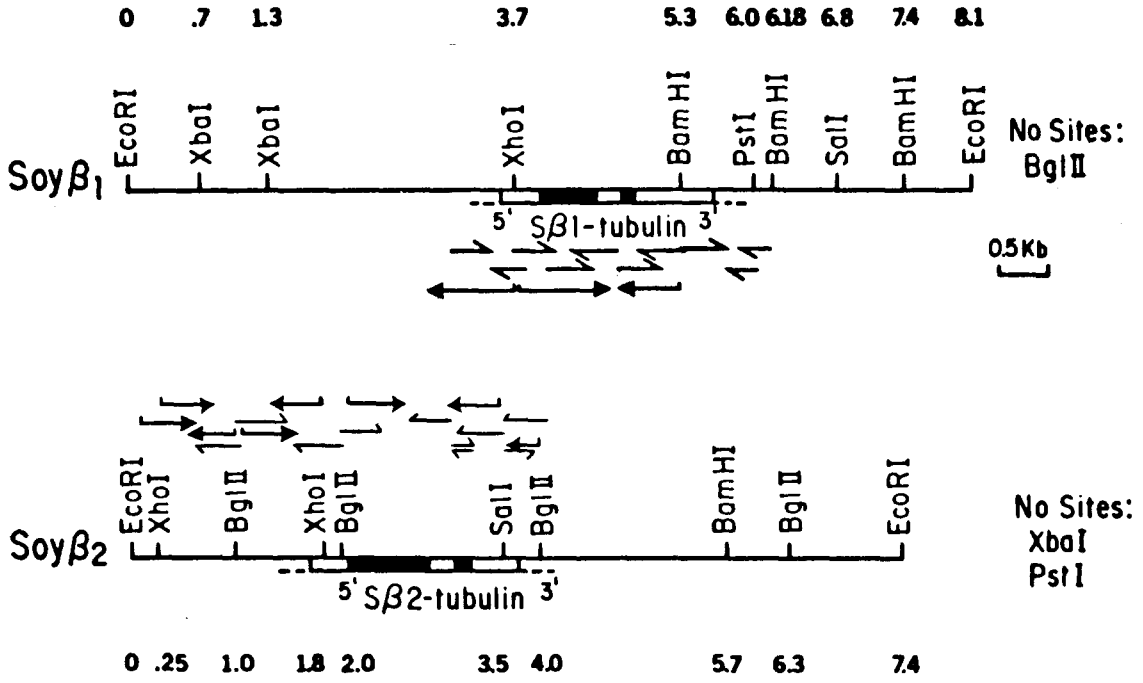

Fig. 1. Restriction maps of cloned soybean DNA fragments containing unique β-tubulin genes Sβ-1 and Sβ-2. The location of the coding sequences (open boxes), introns (solid boxes) and the direction of transcription (5′ to 3′) are indicated as deduced from hybridization to various *Chlamydomonas* β-tubulin cDNA fragments. Restriction fragments that were sequenced by the dideoxy method [36] are indicated with half-headed arrows, while those sequenced by the method of Maxam and Gilbert [23] are indicated with complete arrows.

from these clones and restriction mapped. The restriction fragments containing putative β-tubulin sequences were identified by hybridization to the labeled *Chlamydomonas* probe described above. Two Lambda clones (designated MG-2 and MG-5) were identified which contained sufficient homologous sequence to represent full-length β-tubulin genes. The selected clones also were representative of the two most common restriction patterns observed. All of the remaining Lambda clones which had been selected and mapped exhibited a pattern of β-tubulin positive restriction fragments which were identical to either all or a subset of the fragments observed in either MG-2 or MG-5 (data not shown).

The 8.1-kb and 7.4-kb *Eco*RI fragments from the Lambda clones MG-2 and MG-5, respectively, which contained the sequences hybridizing to the *Chlamydomonas* β-tubulin cDNA probe, were subcloned into the *Eco*RI site of pUC13 (plasmids designated pMG2Sβ1-8.1 and pMG5Sβ2-7.4). Restriction maps of these inserts show that the two sequences differ in both internal and flanking restriction sites (Fig. 1). These two putative soybean β-tubulin genes were designated Sβ-1 (from MG-2) and Sβ-2 (from MG-5). The 3′ ends, and thus the orientation of the genes, were determined by hybridization of restriction fragments from Sβ-1 and Sβ-2 with a cloned 0.46-kb *Sal*I-*Bam*I fragment from the 3′ terminus of the *Chlamydomonas* β-tubulin cDNA β-(8-31) [37].

To assess the number of β-tubulin genes, soybean genomic DNA and the plasmids containing the cloned SB-1 and SB-2 sequences were digested with *Hinf*I and analyzed by Southern hybridization (Fig. 2). Both the genomic blots and the blots of the cloned soybean sequences were probed with a nick-translated 2.3-kb *Xho*I-*Pst*I fragment of Sβ-1, while the blots of the cloned sequences were probed with the nick-translated 0.46-bp *Sal*I-*Bam*HI fragment from the β-(8-31) cDNA of *Chlamydomonas* as well. The probe from SB-1 includes nearly the entire β-tubulin homologous region, while the second probe contains the 3′ end of the *Chlamydomonas* β-tubulin coding sequence. The soybean genomic DNA exhibited four *Hinf*I fragments which hybridized to Sβ-1 probe, with sizes of 1.4, 1.0, 0.8 and 0.5 kb. Two *Hinf*I restriction fragments of the Sβ-1 DNA, a 1.4-kb fragment and a 0.8-kb fragment,

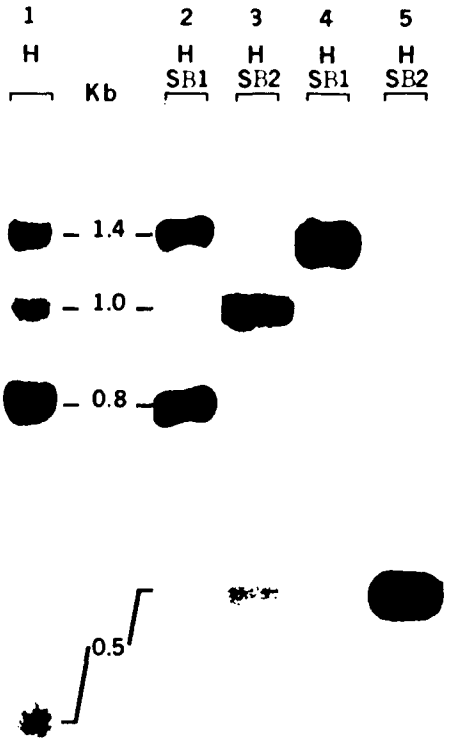

Fig. 2. Southern analysis of soybean genomic DNA and the cloned soybean β-tubulin genes. *Glycine max* cv. Forest genomic DNA (lane 1) and cloned plasmids containing the soybean β-tubulin genes Sβ-1 (pMG2Sβ1-8.1; lanes 2 and 4) and Sβ-2 (pMG5Sβ2-7.4; lanes 3 and 5) were restricted with *Hinf*I (=H). The restriction fragments were separated by agarose gel electrophoresis, and the gels were blotted to nitrocellulose and probed. Lanes 1–3 were probed with a nick-translated 2.3-kb *Xho*I-*Pst*I fragment from Sβ-1, while lanes 4 and 5 were probed with a nick-translated 460-bp *Sal*I-*Bam*HI fragment from the *Chlamydomonas* β-tubulin cDNA β-(8-31), which spans the 3′ end of the coding sequence. The blots were hybridized under the conditions described in Materials and methods. The autoradiogram of lane 1 was exposed for 28 days, while those of lanes 2–5 were exposed for 16 h. The molecular weights of the hybridizing fragments are indicated to the left of lane 2. The 0.5-kb markers are not aligned because lanes 2–5 were electrophoresed for less time than was lane 1.

hybridized to the Sβ-1 probe. The 1.4-kb fragment also hybridized to the *Chlamydomonas* probe. The *Hinf*I digest of Sβ-2 also exhibited two fragments which hybridized to the Sβ-1 probe, with sizes of 1.0 kb and 0.5 kb. One of these (the 0.5-kb fragment) also hybridized to the *Chlamydomonas* probe. Over-exposure of the filter revealed no additional bands. Thus, all four of the genomic bands are accounted for in the two subclones.

Sequence analysis

The nucleotide sequences of the two putative β-tubulin genes and flanking DNA were determined. The positions and orientations of the fragments sequenced are shown in Fig. 1. The nucleotide sequence of these two genomic fragments (Figs. 3 and 4) indicates that both contain potentially functional genes. Both sequences contain consensus TATA and CAAT boxes in the 5′ flanking DNA, and two introns. The proposed locations of the introns were determined by comparison of the possible open reading frames with known β-tubulin sequences and by the identification of consensus intron splice site dinucleotides at the appropriate positions [3]. The putative 3′ and 5′ splice junction and branch point sequences are shown in Table 1. The first intron in Sβ-1 is 508 bp and it interrupts codon 132, while the first intron in Sβ-2, also interrupting codon 132, is 723 bp. A second intron interrupts codons 222 in both genes and is 72 bp in Sβ-1 and 113 bp in Sβ-2. A comparison of the 5′ and 3′ flanking and intron sequences revealed no other significant homologies between them, with other tubulin genes, or with analogous sequences from various other plants genes.

Sβ-1 would encode a β-tubulin of 445 amino acids and a net negative charge of −22, while Sβ-2 would encode a protein 4 amino acids longer with a net negative charge of −26. The deduced amino acid sequences of the two soybean genes were compared with those of β-tubulins from five diverse organisms

Table 1. Conserved 5′ and 3′ splice junctions and putative branch points from the four introns in the two soybean β-tubulin genes, Sβ-1 and Sβ-2. The consensus splice junction and branch point sequences were derived by Brown [3] through a comparison of 177 plant introns. The splice site is indicated with a colon. For the branch point, numbers to the right of the sequences indicate the distance from the putative adenosine branch point to the 3′ splice junction. Positions which do not fit the consensus are underlined.

5′ Splice junction

Position	−3	−2	−1	:	1	2	3	4	5	6
Consensus	C/A	A	G		G	T	A	A	G	T
Sβ1IVS1	A	G	G		G	T	A	C	T	A
Sβ1IVS2	G	T	T		G	T	A	A	G	T
Sβ2IVS1	A	A	G		G	T	A	T	G	G
Sβ2IVS2	G	C	T		G	T	A	A	G	C

3′ Splice junction

Position	−15	−14	−13	−12	−11	−10	−9	−8	−7	−6	−5	−4	−3	−2	−1	:	+1
Consensus	T	T	T/Pu	T	T	T/Pu	T/Pu	T/Pu	T/Pu	T	T	G	C	A	G		G
Sβ1IVS1	T	T	T	G	A	T	G	A	T	C	A	A	C	A	G		G
Sβ1IVS2	C	T	T	T	A	A	C	A	A	T	T	G	C	A	G		T
Sβ2IVS1	T	T	C	T	T	C	T	T	G	T	T	G	C	A	G		G
Sβ2IVS2	C	A	T	T	T	T	T	G	T	G	T	G	C	A	G		T

Putative branch points

Position	−5	−4	−3	−2	−1	0	+1	
Consensus	T/Pu	T/Pu	C/T	T	Pu	A	Py	−16 TO −49
Sβ1IVS1	A	T	A	T	G	A	G	−25
Sβ1IVS2	G	A	G	T	A	A	T	−25
Sβ2IVS1	G	G	T	T	G	A	T	−26
Sβ2IVS2	T	A	T	T	G	A	T	−23

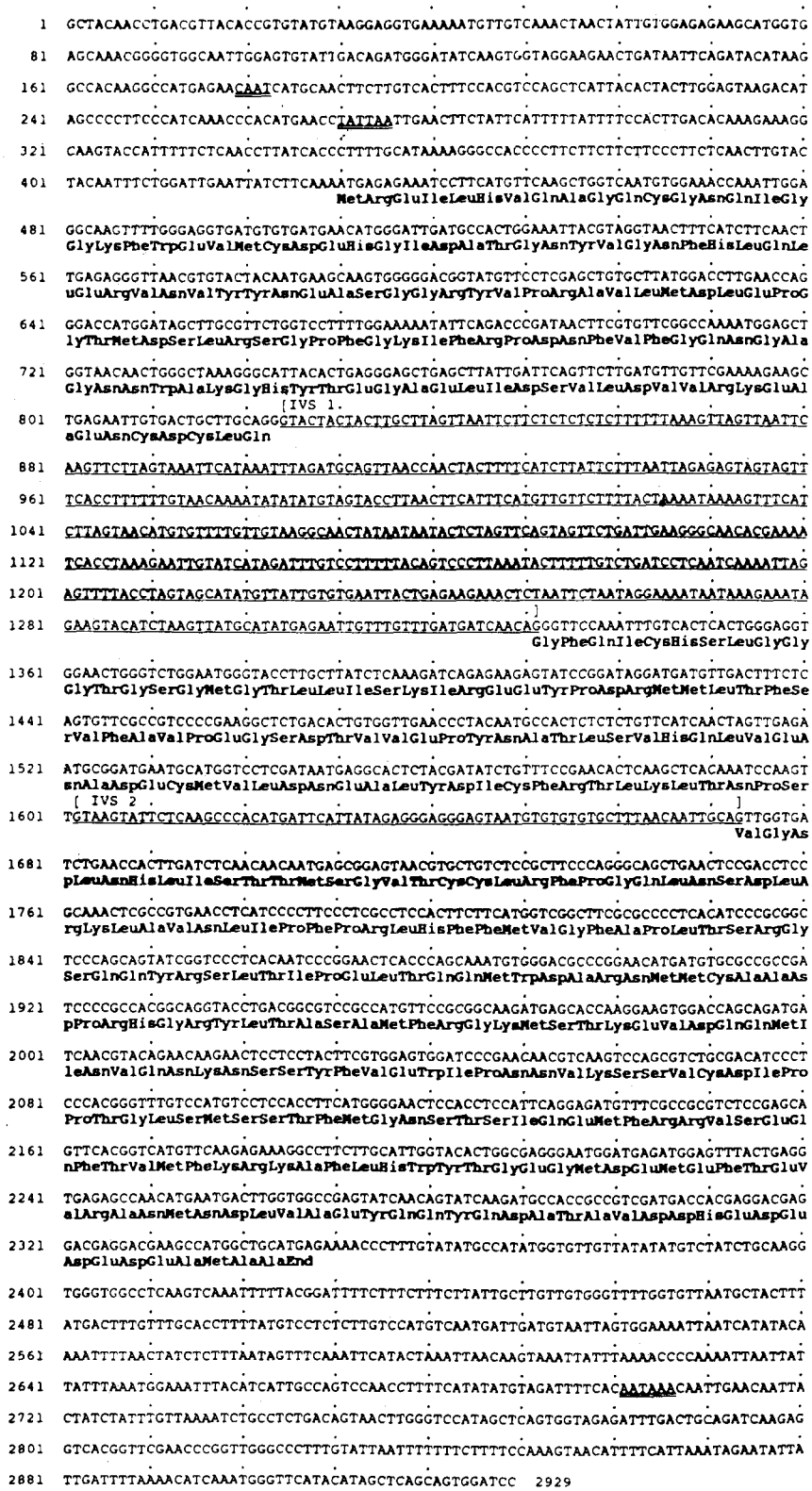

Fig. 3. The complete nucleotide sequence and deduced amino acid sequence of Sβ-1. The location and orientation of the overlapping restriction fragments that were sequenced are shown in Fig. 1. The intron sequences (IVS1 and IVS2) are marked with brackets and underlined, while putative regulatory signals (TATA and CAAT boxes as well as the possible polyadenylation signals) are indicated with a double underline. The coding regions were translated and the amino acids are shown on the line below the nucleotide sequence with standard three-letter abbreviations.

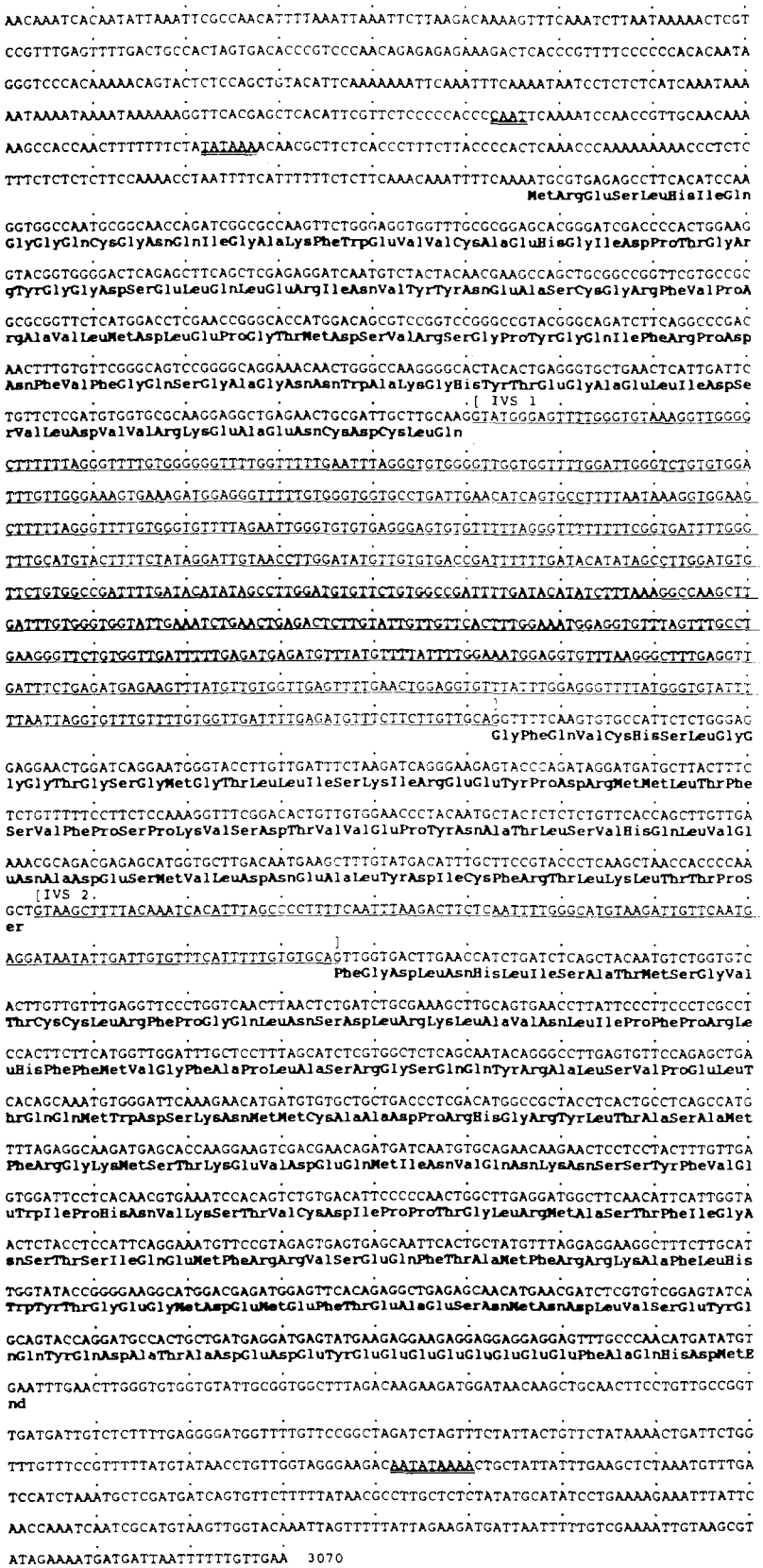

Fig. 4. The complete nucleotide sequence and deduced amino acid sequence of Sβ-2. The position and orientation of the overlapping restriction fragments that were sequenced are shown in Fig. 1. Other details are as shown in the legend for Fig. 3.

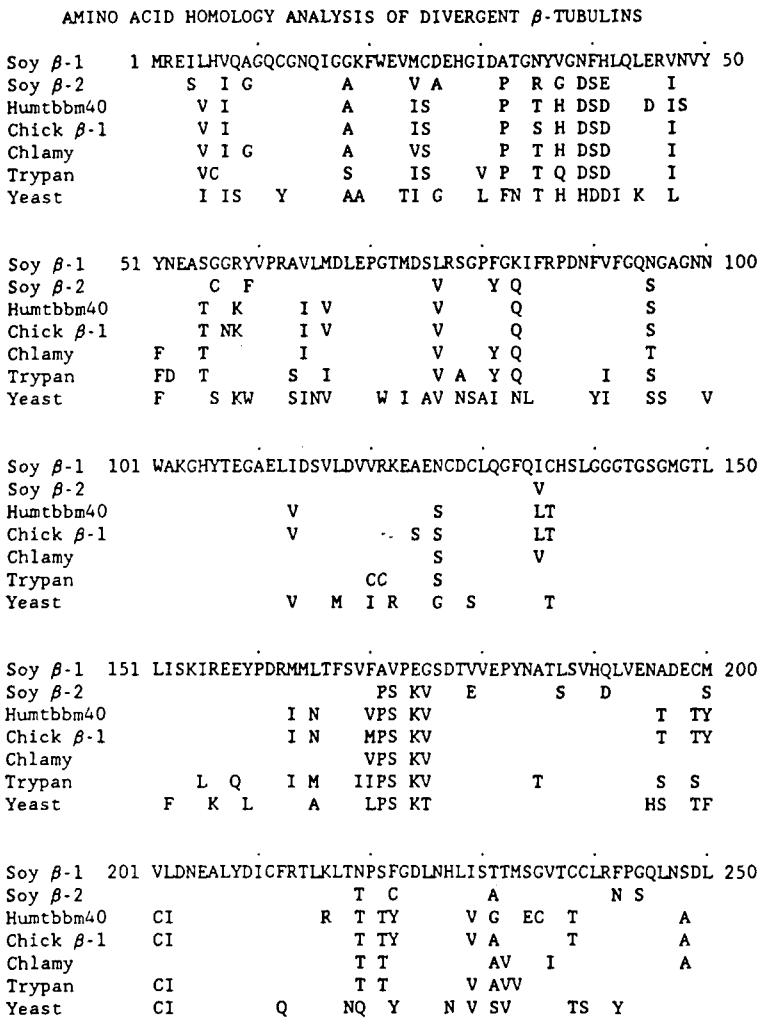

Fig. 5. See page 179 for legend.

(Fig. 5). Both soybean genes were from 66% to 87% homologous with these other β-tubulins. They are somewhat more homologous to the β-tubulins of *Chlamydomonas* (85% – 87%) than they are to vertebrate tubulins (79% – 83%). However, the soybean β-tubulins are as diverged from each other (87%) as they are from the *Chlamydomonas* tubulin. Although the Sβ-1 tubulin is only slightly more divergent overall than Sβ-2, most of the amino acid substitutions in Sβ-2 are conservative, whereas 18 out of 69 (26%) of the Sβ-1 residues that differed from the consensus are non-conservative changes that could be expected to affect the secondary of tertiary structure of the protein, particularly the clustered non-conservative changes at positons 39 – 41 and 171 – 175 (Table 2). In addition, Sβ-2 exhibits a cluster of 3 substitutions, one of which is non-conservative, within the region from position 401 to 425 which is very highly conserved in all other known β-tubulins [9].

A comparison of the hydropathy plots of the 7 β-tubulin proteins revealed a similar pattern of hydrophilic and hydrophobic regions in all except the yeast protein (data not shown). However, there were some notable differences. First, Sβ-1 exhibits a strongly hydrophobic region near residue 165. This region is less hydrophobic and shifted slightly toward the N-terminus in other tubulins. Secondly, the region near residue 315 is less hydrophobic in both soybean tubulins than it is in the other tubulins. Finally,

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Soy β-1  251 RKLAVNLIPFPRLHFFMVGFAPLTSRGSQQYRSLTIPELTQQMWDARNMM 300
Soy β-2                              A        A SV         SK
Humtbbm40          MV         P              A  V D    VF  K
Chick β-1          MV         P              A  V       F S
Chlamy                          T            A  V          K
Trypan              V         M              G SV        F  K
Yeast               V           Y    AI   SF    V        F  K

Soy β-1  301 CAADPRHGRYLTASAMFRGKMSTKEVDQQMINVQNKNSSYFVEWIPNNVK 350
Soy β-2                                    E                 H
Humtbbm40    A C         VA V   R  M     E  L
Chick β-1    A C         VA I   R  M     E  L
Chlamy                      L   R        E  L
Trypan       Q              L   R        E  L          I       I
Yeast        A     N      VA F    V V    EDE HK  S   D           Q

Soy β-1  351 SSVCDIPPTGLSMSSTFMGNSTSIQEMFRRVSEQFTVMFKRKAFLHWYTG 400
Soy β-2       T          R A   I                      A  R
Humtbbm40    TA       R  K AV  I     A    L K I      A  R
Chick β-1    TA       R  K  A  I     A    L  RI      A  R
Chlamy                K  K  A  I     A      K        A  R
Trypan                K  K AV  I   N C          G     L  R
Yeast        TA  SVA Q  D AA  IA         L K  GD   SA             S

Soy β-1  401 EGMDEMEFTEVRANMNDLVAEYQQYQDATAVDDHEDEDEDEAMAA*              445
Soy β-2                AES       S           DE EYE E E E F QHDM*        449
Humtbbm40              AES       S           EEEEDFGE A EE *             444
Chick β-1              AES       S           DEQG F E G EDE *            445
Chlamy                 AES       S         S EEEG F G E EA*              443
Trypan                 AES       S          IEEEG FDE EY*                441
Yeast             L  S AES       S       E  VE  E VDENGDFG PQNQDEPITENFEB* 458
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		Percent homology relative to;	
		Sβ-1	Sβ-2
Soy β-1	- Glycine max β-1	100%	87%
Soy β-2	- Glycine max β-2	87%	100%
Humtbbm40	- Homo sapiens β-M40	79%	82%
Chick β-1	- Gallus gallus β-1	81%	83%
Chlamy	- Chlamydomonas reinhardtii	85%	87%
Trypan	- Trypanosoma brucei	80%	81%
Yeast	- Saccharomyces cerevisiae	66%	68%

Fig. 5. A comparison of the deduced amino acid sequences of the soybean β-tubulins with those of five diverse organisms. The amino acid sequence encoded by Sβ-1 is given using the single-letter amino acid code. The sequences of the other β-tubulins compared are placed below Sβ-1, with an amino acid shown only if it differs from that of Sβ-1 in the same position. In addition to the two soybean genes, β-tubulins from chicken [39], human [16], yeast [31], *Trypanosoma brucei* [19] and *Chlamydomonas* [45] were compared.

the C-terminal 20 amino acids are more hydrophilic in the two soybean β-tubulins than in the other β-tubulins examined.

Codon usage in Sβ-1 and Sβ-2 is shown in Table 3. Codon usage in the β-tubulin genes of soybean and several other organisms were compared by the method of Grantham [13]. This method provides a statistical analysis of the similarity of codon usage between two genes by calculating the sum of the squares of the differences (D^2) in codon usage frequency. The smaller the value of D^2 the more similar the codon usage in the two genes compared. Although the two soybean β-tubulin genes exhibit very similar codon usage ($D^2 = 0.87$) this bias is not similar to the codon usage of tubulin genes in other organisms. Furthermore, although the codon usage of the soybean β-tubulin and certain other soybean genes is somewhat similar ($D^2 = 1.6$ for the small subunit of ribulose-1,5-bisphosphate decarboxylase; $D^2 = 3.8$ for soybean actin) codon usage in the soybean β-tubulins and soybean leghemoglobin is very different ($D^2 = 6.4$).

Table 2. Non-conserved substitutions in the amino acid sequences of the two soybean β-tubulin genes. The deduced amino acid sequences of the soybean Sβ-1 and Sβ-2 were compared with those of five other organisms, as shown in Fig. 5. The following is a listing of those residues in which the substitution observed in the sequence of Sβ-1 was not conservative.

Position	Amino acid in		Consensus amino acid	Number with consensus/ 7 β-tubulins
	Sβ-1	Sβ-2		
32	Ala	Pro	Pro	(5)
39	Asn	Asp	Asp	(5)
40	Phe	Ser	Ser	(5)
41	His	Glu	Glu	(5)
83	Lys	Gln	Gln	(5)
171	Ala	Pro	Pro	(6)
172	Val	Ser	Ser	(6)
174	Glu	Lys	Lys	(6)
175	Gly	Val	Val	(5)
231	Thr	Ala	Ala	(4)
283	Ser	Ala	Ala	(4)
328	Gln	Glu	Glu	(5)
359	Thr	Thr	Arg or Lys	(4)
362	Ser	Arg	Lys	(4)
412	Arg	Glu	Glu	(6)
420	Ala	Ser	Ser	(6)
431	Val	Asp	Glu	(4)
435	His	Glu	Glu	(3)

Discussion

The cloned soybean genomic fragments designated Sβ-1 and Sβ-2 each contain potentially functional β-tubulin genes. Homology analysis of the derived amino acid sequences demonstrated that the protein encoded by each of these genes would bear a high degree of homology to the β-tubulins of other organisms (see Fig. 5). Hydropathy profiles of these soybean β-tubulins also illustrated the remarkable degree to which they are similar structurally to β-tubulins from evolutionarily divergent organisms. At the same time, tubulins are considered to be one of the most highly conserved groups of proteins and the degree of amino acid sequence conservation of these higher plant β-tubulins does not appear to be as great as that of most other tubulins. For example, even *Chlamydomonas* β-tubulins are nearly 90% homologous with the most prevalent vertebrate β-tubulin isotype [9]. Also, there are six different mammalian β-tubulin isotypes and five of these six are 95% – 97% homologous to each other. On the other hand, the sixth mammalian β-tubulin is only 78% homologous with the other isotype [41, 42]. These considerations indicate that the soybean β-tubulins are among the more divergent β-tubulins, although the degree of their divergence is not unique.

Codon usage was remarkably similar in the 2 soybean β-tubulin genes (Table 3). This would indicate that the 2 genes had recently diverged from a common ancestral gene, particularly since there was little similarity in codon usage between the β-tubulin genes and other soybean genes. The fact that the intron positions were the same in the soybean β-tubulin genes tends to support this view, although there was no significant sequence homology in the intron sequences, beyond short regions of homology that most likely represent splice junction and branch point sequences [3]. However, the comparatively high degree of coding sequence divergence makes this explanation less than convincing. It is possible that there has been some selection pressure to maintain the codon bias exhibited by these genes.

The results presented here demonstrate that soybean β-tubulins are encoded by a small multi-gene family. Genomic Southern analysis of soybean DNA using high stringency conditions showed that when cut with the four-base recognition enzyme *Hinf*I, and hybridized to a β-tubulin probe, four bands of hybridization are observed. The cloned soybean β-tubulin genes, Sβ-1 and Sβ-2, give rise to *Hinf*I restriction fragments which can account for all hybridizing bands seen in the genomic DNA. Therefore, the two cloned genes (Sβ-1 and Sβ-2) represent two unique β-tubulin gene classes in soybean. The possibility that there may exist in the soybean genome multiple loci or alleles of these genes cannot be ruled out, nor can the possible existence of other highly divergent β-tubulin gene classes. Although the origin of the cultivated soybean is not known, because of the large number of chromosomes (40) it is unlikely to be true diploid species. If it is a tetraploid species, the genome could be expected to contain at least four β-tubulin genes, although their degree of divergence could not be predicted. As a result, our estimation that the soybean haploid genome contains two β-tubulin genes must be considered a minimum esti-

Table 3. Codon usage in the soybean β-1 and β-2-tubulin genes.

Amino acid	Codon	Number		Fraction		Amino acid	Codon	Number		Fraction	
		SB1	SB2	SB1	SB2			SB1	SB2	SB1	SB2
Gly	GGG	8	8	0.22	0.24	Trp	TGG	5	5	1.00	1.00
Gly	GGA	12	8	0.33	0.24	End	TGA	1	1	1.00	1.00
Gly	GGT	8	8	0.22	0.24	Cys	TGT	6	5	0.55	0.42
Gly	GGC	8	10	0.22	0.29	Cys	TGC	5	7	0.45	0.58
Glu	GAG	19	26	0.61	0.67	End	TAG	0	0	0.00	0.00
Glu	GAA	12	13	0.39	0.33	End	TAA	0	0	0.00	0.00
Asp	GAT	15	11	0.56	0.44	Tyr	TAT	5	4	0.36	0.27
Asp	GAC	12	14	0.44	0.56	Tyr	TAC	9	11	0.64	0.73
Val	GTG	11	13	0.35	0.45	Leu	TTG	8	9	0.24	0.27
Val	GTA	3	0	0.10	0.00	Leu	TTA	0	1	0.00	0.03
Val	GTT	9	11	0.29	0.38	Phe	TTT	6	8	0.25	0.35
Val	GTC	8	5	0.26	0.17	Phe	TTC	18	15	0.75	0.65
Ala	GCG	3	2	0.11	0.07	Ser	TCG	0	2	0.00	0.06
Ala	GCA	3	4	0.11	0.15	Ser	TCA	5	6	0.17	0.17
Ala	GCT	7	13	0.26	0.48	Ser	TCT	4	11	0.14	0.31
Ala	GCC	14	8	0.52	0.30	Ser	TCC	14	6	0.48	0.17
Arg	AGG	3	10	0.13	0.40	Arg	CGC	3	2	0.13	0.08
Arg	AGA	5	2	0.22	0.08	Arg	CGA	3	2	0.13	0.08
Ser	AGT	2	3	0.07	0.09	Arg	CGT	1	4	0.04	0.16
Ser	AGC	4	7	0.14	0.20	Arg	CGC	8	4	0.35	0.17
Lys	AAG	9	12	0.69	0.92	Gln	CAG	11	13	0.52	0.59
Lys	AAA	4	1	0.31	0.08	Gln	CAA	10	9	0.48	0.41
Asn	AAT	9	4	0.35	0.19	His	CAT	6	5	0.55	0.45
Asn	AAC	17	17	0.65	0.81	His	CAC	5	6	0.45	0.55
Met	ATG	24	22	1.00	1.00	Leu	CTG	4	4	0.12	0.12
Ile	ATA	1	0	0.06	0.00	Leu	CTA	1	1	0.03	0.03
Ile	ATT	5	9	0.31	0.53	Leu	CTT	8	8	0.24	0.24
Ile	ATC	10	8	0.63	0.47	Leu	CTC	13	10	0.38	0.30
Thr	ACG	4	0	0.16	0.00	Pro	CCG	3	3	0.18	0.16
Thr	ACA	6	5	0.24	0.21	Pro	CCA	3	5	0.18	0.28
Thr	ACT	8	11	0.32	0.46	Pro	CCT	4	6	0.24	0.33
Thr	ACC	7	8	0.28	0.33	Pro	CCC	7	5	0.41	0.28

mate. We propose that the two sequences described in this report are representatives of two different classes of β-tubulin genes. Each of these classes contains at least one, but possible more, members.

There is a report that the sequence of a cDNA for the B subunit of glyceraldehyde-3-phosphate dehydrogenase of mustard chloroplasts exhibits a high degree of homology to β-tubulin [7]. This raises the possibility that one of the two soybean β-tubulin genes we have characterized could in reality encode the B subunit of this chloroplast enzyme. However, since both Sβ-1 and Sβ-2 encode hydrophilic amino terminal regions which would be unlikely to function as transit peptides, this possibility seems remote.

Both α- and β-tubulins are encoded by multi-gene families in most species examined at this point [9]. The significance of this observation is obscure at the present time. Fulton and his co-workers proposed that the different microtubular arrays in eukaryotes were constructed from different tubulin isotypes [12]. This "multi-tubulin" hypothesis does not appear to be correct for lower eukaryotes, at least in the sense that the product of a unique tubulin gene must perform a function for which the product of another member of the same tubulin multi-gene family cannot substitute. While in some lower eukaryotes a specific tubulin gene may be expressed preferentially at a particular developmental stage, there is no evidence that the developmentally regulated tubulin gene encodes a tubulin subunit that is essential for the completion of the events of that developmental stage [4, 43].

The multi-tubulin hypothesis has not been adequately tested in higher eukaryotes. It is possible that function-specific tubulins have evolved in vertebrates and angiosperms. This is suggested in vertebrates by the observation that there appears to be strong selection pressure to conserve several β-tubulin sequences, both within a given species and among the vertebrates as a whole. Cleveland [8] noted that several β-tubulin isotype classes could be identified. A given isotype class, which may be expressed primarily in a specific tissue, shows a very high degree of sequence conservation, approaching 100%, in different mammalian species. Although far from a demonstration that a given isotype class is function-specific, it is difficult to account for the strong conservation of a specific isotype sequence in the absence of a specific function. On the other hand, Lewis *et al.* [21] used highly specific antibodies to demonstrate by means of immunofluorescence that three of the six β-tubulin isotypes were present in both mitotic spindle and cytoplasmic cytoskeletal arrays in mammalian cells. Furthermore, they demonstrated that, when HeLa cells were transfected with a cDNA for the highly divergent mammalian β-tubulin, M1, under the control of the SV40 T-antigen promoter, this hematopoietic-specific β-tubulin was synthesized and incorporated into all microtubule arrays, which continued to function normally. These observations argue against a specific function for the different tubulin isotypes, at least in cultured mammalian cells. However, even here it is possible that these individual tubulin isotypes may play some essential and unique role at a specific point in the life of the organism. This possibility was not addressed by these experiments. At the present time very little tubulin sequence data is available for higher plants. As a result, it is impossible to know if higher plants exhibit a similar pattern of intraspecies divergence and interspecies conservation of specific tubulin isotypes.

The pattern of expression of Sβ-1 and Sβ-2 during soybean development currently is under investigation in this laboratory. In addition, we have demonstrated that SB-1 is expressed when it is introduced into tobacco plants by means of Ti-mediated transformation [14].

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