CELL STRUCTURE AND FUNCTION 22: 291–298 (1997)

Characterization of The Carrot β-Tubulin Gene Coding a Divergent Isotype, β-2

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Key words: β-tubulin isotypes/carrot/colchicine/hydrophobicity profile/gene expression

ABSTRACT. **Four different β-tubulin clones were isolated from carrot genomic and cDNA libraries. Their nucleotide sequences were determined[1] and their predicted amino acids were compared with each other. The predicted amino acid composition of the C-terminal region of three of them (β-1, 3, 4) resembled one another, but that of one isotype (β-2) was divergent. The β-2 tubulin included two hydroxyl amino acids, serine and threonine, and consisted of a lower number of negatively charged amino acids than the others in the C-terminal region.**

The predicted hydrophobicity profile of the β-2 tubulin around the residue 200 is less hydrophobic than β-1, but it is still more hydrophobic than those of animal and fungal β-tubulins. The β-2 gene was transcribed in cultured cells and flowers, while the β-1 gene was ubiquitously transcribed in cultured cells, roots, shoots and flowers.

When the predicted amino acids of plant tubulin were compared with those of other organisms, substitutions from non-polar amino acids to those with hydroxyl group were conspicuous in the region corresponding to the third exon in the plant genes.

Unique distributions and functions of microtubules are known to exist in the plant cell during the process of cell division cycle and morphological differentiation (16). In plant cells, microtubules are formed as a preprophase band at G2, phragmoplast at G1 and cortical microtubules at interphase, in addition to universally observed microtubules as mitotic spindle and interphase cytoplasmic microtubules extending from near the surface of the nucleus to the cell periphery (11). On the other hand, aster, midbody and centriole microtubules, observed in animal, are generally not seen in plant cells. Some pharmacological properties are different between plant and animal cells. The sensitivity of plant tubulin to colchicine is low compared with animal tubulin, and podophyllotoxin does not inhibit the colchicine binding to carrot tubulin (23). These different functional and pharmacological properties would seem to be the result, at least in part, of the structural differences between tubulin molecules in plant and animal.

Some observations suggest specific expression or modification of certain isotype(s) at the specific phase in a cell cycle. α-Tubulin transcript in *Physarum* has been shown to increase its transcript before mitosis and decrease sharply after anaphase (25). In synchronous tobacco culture, acidic isoform increased before mitosis after which the main component shifted to a more basic one (20). Phosphorylation of β-tubulin during carrot cell growth was reported by Koontz and Choi (14). The class III β-tubulin in animal brain has serine at the C-terminal region which can be phosphorylated *in vivo* and *in vitro* (1, 7). However, the relationship of the isotypes and the different microtubule arrays in a cell cycling has not been established yet. Dawson and Lloyd (6) have reported that no obvious changes were observed in the isoform composition between growing and differentiating cells. Microinjected animal tubulin could be incorporated into all the microtubule arrays in living plant cell and the incorporated cells underwent normal mitosis (32).

The DNA sequences of β-tubulin gene determined to date were in 4 species of dicotyledonous and 3 species of monocotyledonous plant. Tubulins are encoded in a multigene family in plant cells as well as in animal cells. Snustad and his colleagues have investigated tubulin isotypes on *Arabidopsis* and *Zea mays* extensively and have suggested that certain isotypes are expressed organ-specifically and in a developmentally controlled manner (26, 30). Fosket and his colleagues reported the tissue-specific expression of one of the β-tubulin isotypes in soybean and its down regulation by light (4).

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[1] The nucleotide sequences reported in this paper has been submitted to GenBank/EMBL/DDBJ with accession numbers, U64029 (β-1), U63927 (β-2), U64430 (β-3), U64431 (β-4).

Microtubules in cultured carrot cells disassemble under cold within 1 hour. The free tubulin degrades gradually in four days in the cold and is restored to the pretreatment level within a day after treatment. The decay and recovery rates of the two β-tubulin isoforms were different (22). Chu *et al.* (5) reported the difference in expression of *Arabidopsis* β-tubulin genes and the stability of their transcripts at low temperature exposure.

We have previously cloned a cDNA fragment of carrot β-tubulin which encoded consensus and variable C-terminal regions and had 3′-flanking sequence (21). Using this cDNA as a probe, we obtained four different clones by screening carrot genomic and cDNA libraries. In this paper, we describe nucleotide and predicted amino acid sequences and the levels of transcript of the isotypes, mainly the divergent β-2, and discussed the isotype composition in carrot and differences in local hydrophobicity between animal and plant β-tubulins.

MATERIALS AND METHODS

Materials. The cell line, GD2, was isolated from a red carrot, *Daucus carota* cv. Kintoki and established as a suspension culture (19). It is maintained by transferring aliquots every week to a fresh Murashige and Skoog's medium (18). Plant materials for RNA extractions were grown from the seeds of carrot cultivar Kintoki (Takii Seed Corp., Kyoto). Seedlings were harvested at 14 days after the imbibition and divided into root and shoot segments. Whole flower tissues were harvested at the early stage of flowering. All materials were frozen in liquid N_2 immediately after harvest and stored at −80°C until use.

Construction of genomic and complementary DNA libraries. Genomic DNA was isolated by phenol chloroform method. Cells at early stationary phase (0.1 g) were collected by suction, rinsed briefly with saline and quickly frozen in liquid N_2. The frozen cells were ground with mortar and pestle in liquid nitrogen with the extraction buffer (100 mM TrisHCl (pH 8.0), 50 mM EDTA, 500 mM NaCl, 1 mM β-mercaptoethanol) which is previously frozen dropwise in the mortar. Powdered cells were put into 9.9 ml of extraction buffer kept at 75°C and dispersed quickly with a siliconated glass stick. After all the disrupted cells were transferred, the mixture was brought to a 65°C bath, and 667 μl of 20% SDS was added. The suspension was mixed gently and incubated at 65°C for 10 minutes. Then, 1.44 ml of 8 M $NaClO_4$ (pH 8) was added to the mixture and spum at 1,000 rpm for 5 minutes at room temperature. An equal volume of isopropanol was added to the supernatant and mixed gently with a glass stick. The fibrous precipitate was taken with the stick and rinsed in 50% isopropanol and then in 70% ethanol. The precipitate was dried in a desiccator and suspended in 1 ml of TE at 55°C. After that, 10 μl of 20 mg/ml RNase A was added and the suspension was incubated at 37°C for 1 hour. Then, 25 μl of 400 mM EDTA, 25 μl of 20% SDS and 10 μl of 10 mg/ml proteinase K were added and incubated at 55°C overnight. After digestion, 30 μl of 5 M NaCl, 15 μl of 1 M TrisHCl (pH 9.0) were added and extracted with phenol, phenol: chloroform and chloroform. The purified DNA was precipitated with ethanol.

An aliquot (30 μg) of genomic DNA was partially digested with *Sau*3A. Fragments of about 9–23 kb were collected by gel purification using low-melt agarose, ligated to lambda DASH II arms and packaged *in vitro* with GigaPack II (Stratagene, LaJolla, Ca. USA). The complexity was determined by measuring the number of plaque forming units on an aliquot of the library, and the library was used for screening without amplification.

Complementary DNA library was constructed as described before (21) from the polyA$^+$ RNA isolated from the cultured carrot cells.

Screening of the library. About 50,000 plaques were formed on a 135 mm petri dish with *E. coli* LE392 or SRB (Stratagene) as host bacteria. The plaques were lifted with nitrocellulose paper, denatured, neutralized and fixed at 80°C for 2 hours. In some experiments, charged nylon membrane was used with alkali-fixation instead of baking. The filter was hybridized with CB1 (a 180 bp 3′-fragment of carrot β-1 tubulin cDNA (21)) or C-β 1.8 (a 1.2 kbp fragment of genomic DNA corresponding to the *Bam*HI site in the first intron to the *Eco*RI site in the third exon of carrot β-1 tubulin gene) probe.

Southern hybridization. The carrot genomic DNA was digested with an appropriate enzyme and electrophoresed on 0.8% agarose. DNA in the gel was denatured and transferred to a nitrocellulose membrane with 20 × SSC. The membrane was washed once with 2 × SSC, baked at 80°C for 2 horus and used for the hybridization.

Hybridization was performed using C-β 1.8 as a probe with non-radioactive ECL direct nucleic acid labelling and detection systems (Amersham). The filter was incubated with 10 ng probe DNA/ml prehybridization mix at 42°C overnight. NaCl concentration in the prehybridization mix was adjusted to 0.5 M. The filter was washed twice in the primary wash buffer (6 M urea, 0.5 × SSC) at 42°C and once with the secondary wash buffer (2 × SSC) at room temperature.

Dot blot hybridization. Total RNA was prepared by phenol extraction and LiCl precipitation as described previously (20). FITC-labelled probes specific for each isotypes were prepared by amplifying 3′-untranslated regions by PCR. Hybridization and detection were performed with Gene Images labelling and detection systems (Amersham).

RESULTS

Isolation and characterization of λ clones carrying carrot β-tubulin DNA. In a previous paper (21), we have reported a cDNA fragment, CB1 encoding C-terminal region of a β-tubulin which was cloned by screening a carrot cDNA library with a monoclonal antibody

against chicken brain β-tubulin. Using CB1 as a probe, we obtained its genomic clone, λ-P1. However, this was truncated in the putative first intron. By southern blotting of carrot genomic DNA probed with a fragment of λ-P1 insert, C-β 1.8 that included about 2/3 of the coding region, we detected multiple positive bands, suggesting multiple isotype genes in carrot genom (Fig. 1). Another genomic library was therefore constructed using *E. coli* strain, SRB as a host and we could obtain 25 positive clones by its screening. These clones were classified into four groups by comparing the restriction maps and Southern blotting profiles.

We had other clones that contained fragments of β-tubulin genes by screening carrot cDNA library. From the predicted amino acid sequence corresponding to the C-terminal variable region, they were classified into three groups, λ-c1, -c11 and -c12. We assigned the genes in λ-c1 and λ-c12 to two of the genomic clones by Southern hybridization. The insert of one genomic clone, λ-8, did not have any corresponding sequence in the cDNA clones. On the other hand, the genomic DNA corresponding to λ-c11 was not found. One genomic clone, λ-6, was likely to have two different β-tubulin sequences, probably identical to the insert of λ-c1 and λ-c12. We nominated the isotype corresponding to CB1 as β-1, the insert in λ-8 as β-2, and the other two isotypes inserted in λ-c12 and λ-c11 as β-3 and β-4, respectively. They encoded unique C-terminal amino acid sequences as shown in Fig. 2. The predicted C-terminal amino acid was methionine in three clones (β-1, 3, 4) and had a consensus XQX′M sequence.

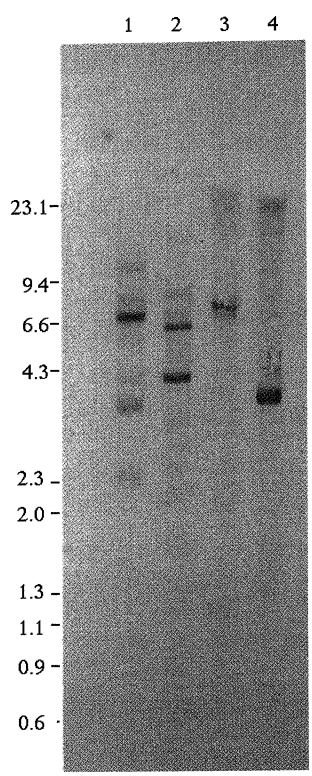

Fig. 1. Southern analysis of carrot genomic DNA. DNA (10 μg) from carrot cells was digested with *Eco*RI (lane 1), *Hin*dIII (lane 2), *Pst*I (lane 3) and *Bam*HI (lane 4), subjected to electrophoresis in a 0.8% agarose gel, transferred to nitrocellulose membrane and allowed to hybridize with the probe Cβ-1.8.

Analysis of the nucleotide sequence of the β-2 gene. The β-tubulin-homologous sequence was included in the center of 4 kb *Bam*HI fragment of λ-8 phage DNA. The fragment was subcloned into pUC 118 plasmid in both directions and the nucleotide sequence was determined. The nucleotide and deduced amino acid sequences are shown in Fig. 3. The sequence contained two putative introns at the same position as most of the other reported β-tubulin genes from higher plant. Both introns began by GT at the 5′ and ended by AG at the 3′.

The predicted amino acid sequence was similar to the β-tubulins of other higher plants except for the extreme C-terminal regions. The C-terminal acidic amino acid cluster was rich in aspartic acid rather than glutamic acid, and had two hydroxyl amino acids, threonine and serine. The sequence was shorter by three or four amino acids, and the extent of the negative charge in the cluster was less than the other three carrot β-tubulin isotypes (Fig. 2).

Expression of β-2 gene in cultured cells and differentiated organs. Specific probes for each isotypes were prepared using the 3′-untranslated regions and hybridized with total RNA from cultured cells, roots, shoots and flowers. Fig. 4 shows the result of dot blot hybridization. The β-2 transcript level was highest in flower and cultured cells, and very low in roots and shoots, while β-1 transcript level was highest in cultured cell and ubiquitously present in differentiated organs at the lower levels. The β-3 transcript level was highest in flowers and low in other cells and organs observed.

Plant-specific amino acid substitutions. A moderate number of amino acid substitution was found when the predicted amino acid sequences were compared with other organisms. Figure 5 shows the summary of the substitutions with reference to chick β-2 tubulin (29). Most of the substitutions occurred commonly in plant

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β1   421 EYQQYQDATADEEEYYEDEEEEEA-QGM 447
β2   421 EYQQYQDATAEEDDYDDGEGSTGD---- 444
β3   421 EYQQYQDATADEEGDYFEDEEEE-RQEM 447
β4   421 EYQQYQDATAEEEDYYEDEQEEEAHQDM 448
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Fig. 2. C-terminal sequences of carrot β-tubulin isotypes. Gray letters are the same amino acids in β-2,3 and 4 as those at the corresponding position in β-1.

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   1 aagctttcaatataaaatgtatagttatgtttccatagaggctcatgtttaatctgcttggcaacttttaattacctatgtaatccttgcatcggaggtttgcaagaaagtgttgaatcg  120
 121 ccctgttggctttttgagttcaattttgtaaatttgattatacaagttttgagggataaaattataataatcgagtcatttcatgtcaaatttgggttaaaaagtcatgttcgtgatgttt  240
 241 caagatatttatgtatgaatcgagtgaagtttcggttgtgttgatgtttgacatactaacgagcataactctgtaatagttcgtttagacctagttcgcgttcctcacactgatatgcga  360
 361 caactctgaggaaaagatcattaaccgcacagcaaactatacgactttccgaggcatatcagtatgctgatactactgaaataccatgtaaattttaacattttcatgattaagaacgca  480
 481 gcatgtgaaacacagagtagagataagtagtgatggggattggccggcgatcacaagatcaatgcccagtggcatgtcatatgattctcatttctcataaccaaatggtcaactaaatga  600
 601 cacagctaccatctctcagtaaacggttgcttctccgccgtccgatcaaattctactcctccaatccttaccttccaatccaactgcaggccagttattcaactgttatataccactta  720
 721 aaattctgtatgtccaacagtctcaactgcaagtacttaacatatttcttcaccatctggtccgtgatcacttcctcttacgagccaaaaATGCGCGAGATTCTTCACATCCAAGGAGGGC  840
                                                                                                  M  R  E  I  L  H  I  Q  G  G      10
 841 AATGCGGGAACCAAATTGGATCAAAGTTCTGGGAAGTTGTTTGTGATGAGCATGGCATTGACCCTACTGGTCAAGTACTGTCGGAATCTGACCTGCAGCTTGATAGAATCAATGTGTACT  960
       Q  C  G  N  Q  I  G  S  K  F  W  E  V  V  C  D  E  H  G  I  D  P  T  G  Q  V  L  S  E  S  D  L  Q  L  D  R  I  N  V  Y      50
 961 ACAATGAAGCTAGCGGCGGGAGGTACGTTCCACGGGCAGTGCTCATGGACCTCGAGCCAGGCACCATGGACAGCGTCAAGACAGGCCCGCATGGACAGATTTTCAGGCCTGATAACTTTA 1080
       Y  N  E  A  S  G  G  R  Y  V  P  R  A  V  L  M  D  L  E  P  G  T  M  D  S  V  K  T  G  P  H  G  Q  I  F  R  P  D  N  F      90
1081 TCTTCGGACAGTCAGGAGCTGGCAACAATTGGGCTAAGGGGCATTATACTGAGGGTGCTGAGCTTATTGACTCTGTTCTTGATGTTGTTAGAAAGGAAGCTGAGAATTGTGAATGTTTAC 1200
       I  F  G  Q  S  G  A  G  N  N  W  A  K  G  H  Y  T  E  G  A  E  L  I  D  S  V  L  D  V  V  R  K  E  A  E  N  C  E  C  L     130
1201 AAGgttagtgataactgatcagtgttacaagcttcagttcctgaatttacctactaattagcatatgtaatcagaagtcaatgaaaggttggaaagaccattattctgagtgctgataa t 1320
       Q
1321 tatttcagGTTTTCAAGTATGTCATTCTCTTGGAGGTGGCACAGGATCTGGAATGGGAACTTTGCTTATTTCAAAGATAAGGGAAGAATACCCTGACAGAATGATGCTTACTTTCTCTGT 1440
                G  F  Q  V  C  H  S  L  G  G  G  T  G  S  G  M  G  T  L  L  I  S  K  I  R  E  E  Y  P  D  R  M  M  L  T  F  S  V     169
1441 GTTCCCGTCTCCTAAGGTCTCCGACACGGTTGTAGAGCCCTACAATGCCACCCTCTCTGGTCATCAGCTGGTAGAGAATGCTGATGAGTGCATGGTGCTTGATAATGAAGCTCTTTACGA 1560
       F  P  S  P  K  V  S  D  T  V  V  E  P  Y  N  A  T  L  S  G  H  Q  L  V  E  N  A  D  E  C  M  V  L  D  N  E  A  L  Y  D     209
1561 TATCTGCTTCAGGACACTCAAACTATCCACTCCCAGCTgtaagtgttgctataatgtttgatcggccacataccattcagcttaagcttttacattatttgacaacattctgatctgatt 1680
       I  C  F  R  T  L  K  L  S  T  P  S
1681 tgtgtgtactaacagTTGGAGACTTGAAT CATCTGATTTCGGGTACAATGAGTGGAGTGACTTGCTGCTTGCGTTTTCCTGGTCAGCTGAATTCAGACCTCAGGAAGCTAGCAGTGAACT 1800
                      F  G  D  L  N  H  L  I  S  G  T  M  S  G  V  T  C  C  L  R  F  P  G  Q  L  N  S  D  L  R  K  L  A  V  N     257
1801 TGATTCCATTCCCAAGACTCCACTTTTTCATGGTGGGGTTTGCTCCCCTGACATCGAGAGGATCACAGCAGTACAGAACTTTGACTGTCCCGGAACTGACACAACAAATGTGGGATTCCA 1920
       L  I  P  F  P  R  L  H  F  F  M  V  G  F  A  P  L  T  S  R  G  S  Q  Q  Y  R  T  L  T  V  P  E  L  T  Q  Q  M  W  D  S     297
1921 AGAACATGATGTGTGCAGCTGACCCGCGACATGGTCGCTATCTGACAGCCTCAGCAATGTTTAGAGGCAAAATGAGCACCAAAGAGGTCGATGAACAGATCCTGAATGTGCAGAACAAAA 2040
       K  N  M  M  C  A  A  D  P  R  H  G  R  Y  L  T  A  S  A  M  F  R  G  K  M  S  T  K  E  V  D  E  Q  I  L  N  V  Q  N  K     337
2041 ACTCATCTTATTTTGTCGAGTGGATTCCCAACAATGTAAAGTCTAGTGTCTGTGACATTCCTCCAAGAGGGCTCTCTATGTCTTCCACATTCATTGGCAATTCAACCTCCATCCAGGAGA 2160
       N  S  S  Y  F  V  E  W  I  P  N  N  V  K  S  S  V  C  D  I  P  P  R  G  L  S  M  S  S  T  F  I  G  N  S  T  S  I  Q  E     377
2161 TGTTTCGACGAGTTAGTGAACAGTTTACAGCTATGTTCAGGCCAAAGGCTTTCTTGCATTGGTATACCGGAGAGGGAATGGATGAAATGGAATTCACTGAAGCCGAAAGCAATATGAATG 2280
       M  F  R  R  V  S  E  Q  F  T  A  M  F  R  P  K  A  F  L  H  W  Y  T  G  E  G  M  D  E  M  E  F  T  E  A  E  S  N  M  N     417
2281 ATCTTGTTTCGGAGTATCAGCAGTACCAAGATGCTACAGCTGAAGAGGATGACTATGATGATGGTGAAGGCAGTACTGGAGATTGAAgatgttccccaacattgttgatgcgtattttac 2400
       D  L  V  S  E  Y  Q  Q  Y  Q  D  A  T  A  E  E  D  D  Y  D  D  G  E  G  S  T  G  D  *                                  444
2401 gtgcagcaatgtatcataaactttaatttctcagaagttttattcttcctttatttcacttcaagtgcttcaatgagtccagtgatcaatgtgactctgtgatacacatatgtatggcct 2520
2521 gtcttcctatctatt 2535
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Fig. 3. The nucleotide and the deduced amino acid sequences of λ-8 insert (β-2). Two CAATs and TATA sequences at the 5′-untranslated region and CATAAA at the 3′-untranslated region are underlined.

and green algae, and it was noticed that substitutions of non-polar amino acids with hydroxyl or sulfhydryl amino acids frequently contributed to it. The cystein residues in animal tubulin at the positions 201 and 303 shifted two amino acids, and those in carrot and other plant tubulins at the position 239, one amino acid ahead.

The non-polar amino acids, alanine, methionine or phenylalanine at eight sites in the regions corresponding to the third exon, were substituted for hydroxyl-amino acids such as serine, threonine or tyrosine. The characteristics of the substitution were inverse in the region corresponding to the second exon.

Hydrophobicity profiles of carrot β-1 and β-2 tubulin. The hydrophobicity map of carrot β-tubulins was compared with that of chicken β-2 using the parameters of Kyte and Doolittle with eight windows (15). The hydrophobicity profile of carrot β-tubulins changed appreciably at the two regions (bars in Fig. 6). At the region near the ^{199}Cys which located two amino acids former than in animal β-tubulins, the hydrophobicity was in-

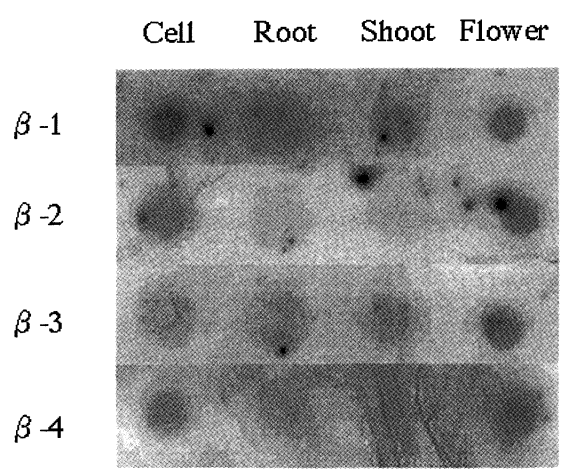

Fig. 4. Expression of β-tubulin isotypes in carrot cells and organs. Equal amounts of total RNA from cultured cells and various organs were dot blotted on a charged nylon membrane and hybridized with isotype-specific probes as described in Materials and methods. Hybridization was conducted at 65°C in 5×SSC, 0.1% (w/v) SDS, 0.5% dextrane sulfate and blocking reagent. Washings were done in 1×SSC, 0.1% SDS at 65°C and then, 0.1×SSC, 0.1% SDS at 65°C.

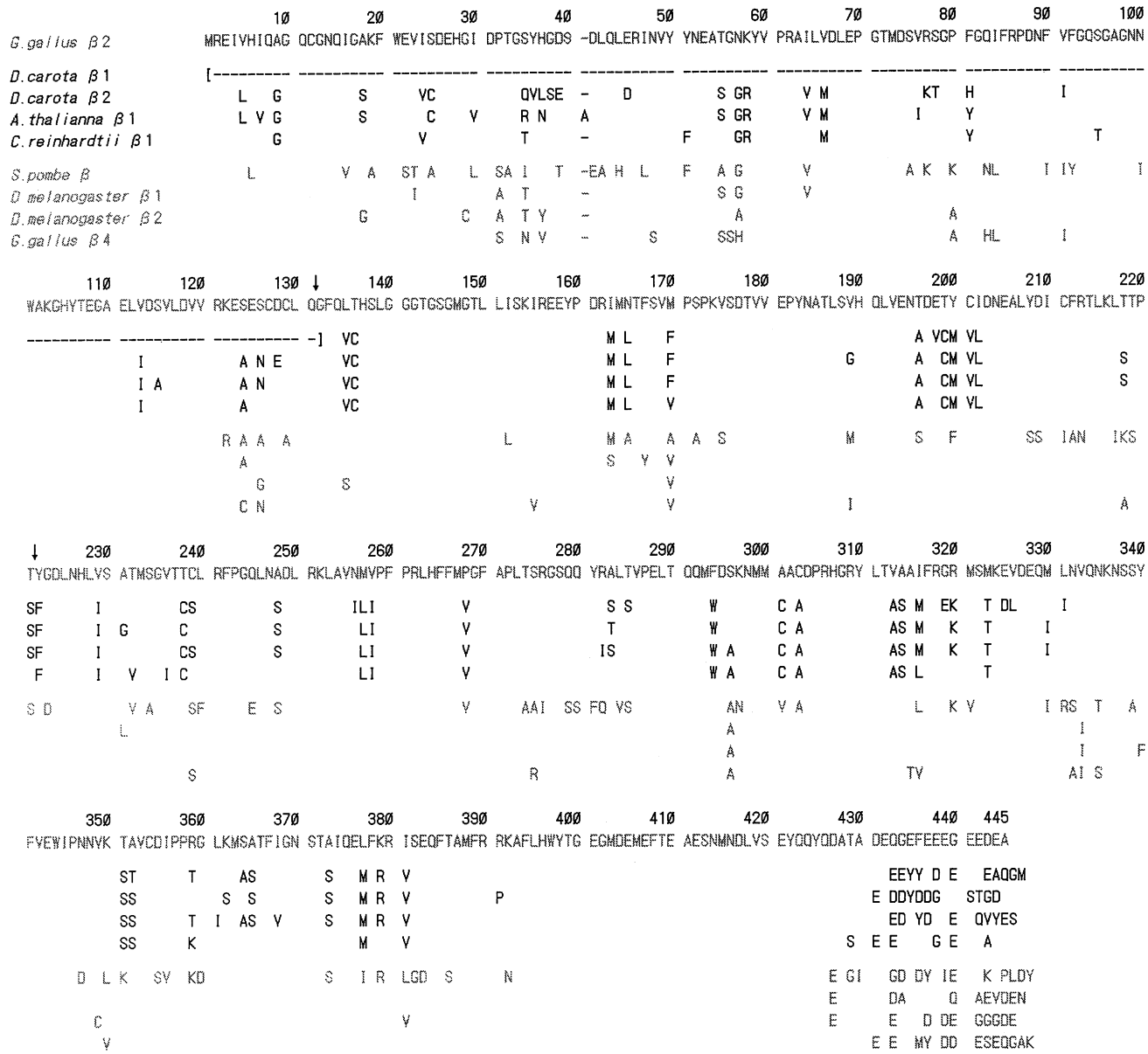

Fig. 5. Comparison of predicted β-tubulin amino acid sequences. The chicken β-2 tubulin sequence (29) is shown on the top line and the carrot β-1, β-2, *Arabidopsis* β-1 (24), *Chlamydomonus* (31) yeast (12), *Drosophila* β-1, β-2 (17), and chicken β-4 (28) are shown below in optimal alignment. Only amino acids differing from the corresponding amino acid sequence of chicken β-2 tubulin are shown. A gap is inserted between 40 and 41, because additional amino acid exists at this position in the *Arabidopsis* β-1 sequence. Black letters are sequences of higher plant and green algae, and gray letters are those of the other organisms. *Drosophila* β-2 and chicken β-4 sequences are shown as examples of divergent isotypes in each organisms. Arrows indicate the positions where intron is inserted in higher plant β-tubulins.

creased considerably. The degree of increase in hydrophobicity was significantly different between the two carrot isotypes. As is shown in Fig. 6, residue 195–205 was a fairly hydrophilic region in animal tubulin (Fig. 6A–D; broken line, Fig. 6C), but it turned to be hydrophobic in carrot β-1 (Fig. 6A; solid line). Carrot β-2 showed

Fig. 6. Hydrophobicity plot of carrot β-1, β-2, chicken β-4 and yeast β-tubulin. Each profile is compared with that of chicken β-2 (broken line; 29). The light-gray area represents the region where it is more hydrophobic than chicken β-2. The dark-gray area represents the region where it is more hydrophilic than chicken β-2. A: carrot β-1, B: carrot β-2, C: chicken β-4 (28) and D: yeast β (12). Bars are the region where the hydrophobicity profile is considerably different between plant and animal β-tubulins.

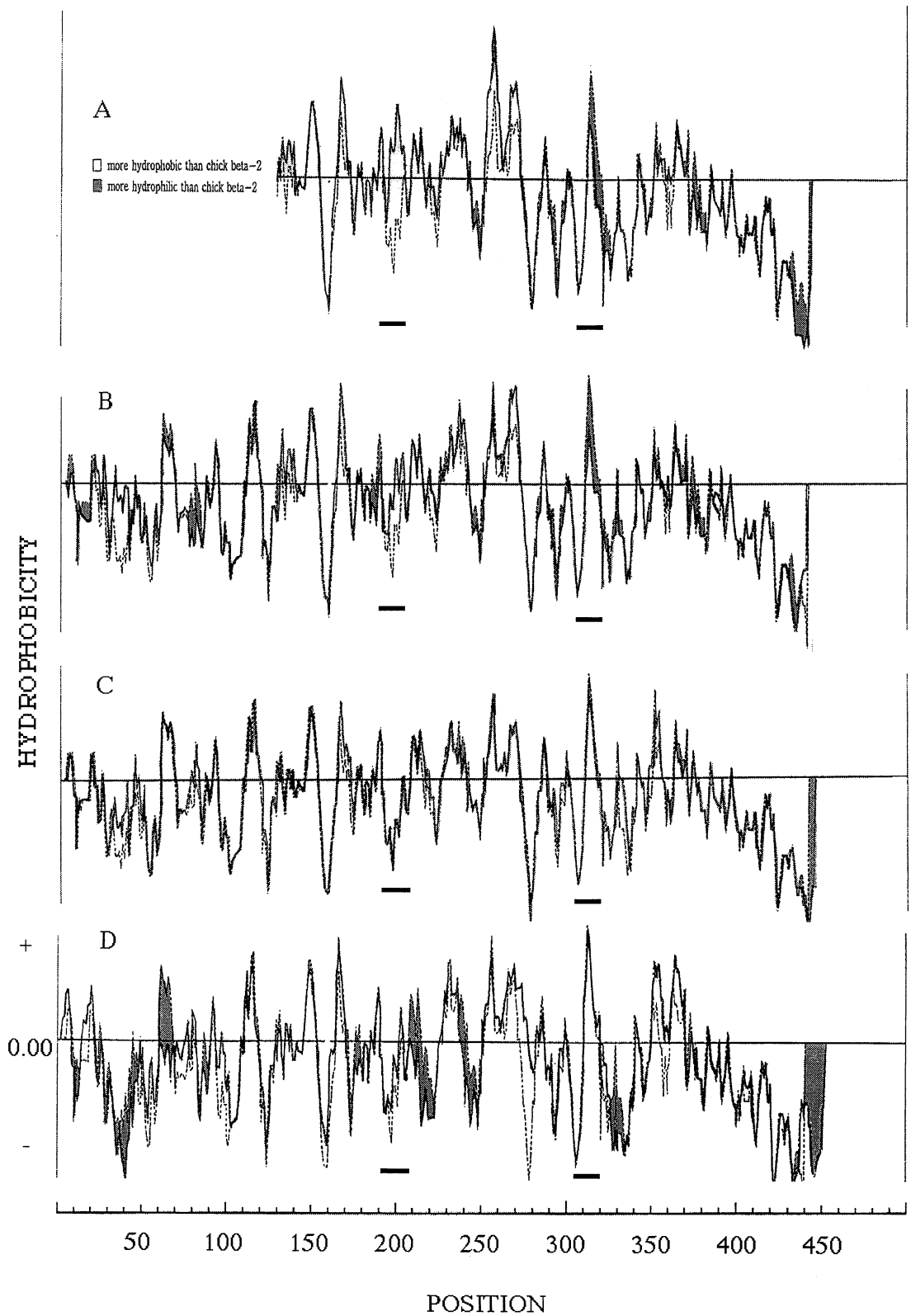
A
more hydrophobic than chick beta-2
more hydrophilic than chick beta-2
B
C
D
HYDROPHOBICITY
+
0.00
-
50
100
150
200
250
300
350
400
450
POSITION

Fig. 6.

an intermediate profile between animal and carrot β-1. The tendency of increase in hydrophobicity at this region was common to all the β-tubulin isotypes in plant (26), and also in fungal tubulin (12; Fig. 6D). In the class III β-tubulin, which is the vertebrate isotype with lower affinity to colchicine (β-4 (28) for chicken; Fig. 6C), the hydrophobicity around this region did not deviate from that of chicken β-2 (class II).

A relatively large hydrophobic area exists at 310–320 in animal tubulin. The hydrophobicity in that area decreased considerably in both carrot β-tubulins as compared with chick β-tubulins (Fig. 6A, B and C).

DISCUSSION

Two divergent classes of C-terminal structure in carrot β-tubulins. As is shown in Fig. 1, approximately nine β-tubulin copies are expected in a carrot genome. Four different β-tubulin genes were, however, cloned from carrot genomic and cDNA libraries. Three of the genomic clones contained the same sequence as found in the cDNA clones. The genomic clone corresponding to one of the cDNA clones, β-4, has not been found yet. Marked improvement in the cloning efficiency by using SRB as a host suggests that modifications, such as methylation of the genes or tight secondary structure, are present and would make the cloning of some genes still difficult.

Contrary to the case of the β-4 isotype, the β-2 sequence was not found in the cDNA library, though it is expressed in cultured cells (Fig. 4).

The C-terminal amino acid sequences were similar to each other among three isotypes (β-1, 3, 4), but it was considerably different in β-2. β-2 tubulin ended by aspartate, and had serine and threonine nearby, while others ended by methionine and have no hydroxyl amino acids in the corresponding region. β-2 tubulin is shorter and the number of the acidic amino acids it has in the region after 430 is eight, which is fewer than the others. As the extent of the negative charge at this region is suggested to be involved in the regulation of the interaction with MAPs and other ligands (2), the β-2 could provide a unique path of regulation. If the serine and/or threonine in the C-terminal region is phosphorylated, the minus charge at this region would increase and its electrostatic property would become similar to that of the other isotypes. It is therefore important to determine whether the serine/threonine becomes phosphorylated under certain physiological conditions. The Class III β-tubulin in vertebrate has serine at this region and has considerably different biochemical properties from other isotypes including the low affinity to colchicine. Serine at the C-terminal region of class III vertebrate tubulin is reported to be phosphorylated (2, 7). Class III β-tubulins have four non-acidic amino acid extensions at the extreme C-terminal region. It also contains tyrosine at 436 instead of phenylalanine in most of the vertebrate β-tubulin isotypes (28). These peculiar features in the C-terminal structure of the class III animal β-tubulins are apparently similar to carrot β-tubulins. In spite of these apparent similarities in the C-terminal region of carrot tubulins to chick β-4, the structure of other regions was quite different. The hydrophobicity profile of the class III β-tubulin did not show the characteristics of plant tubulins as discussed below and the shift of cystein was not observed.

Differences in hydrophobicity profile of plant β-tubulins. Many of the amino acid substitutions are commonly found at the same position in plants; dicotyledon, monocotyledon, fern and green algae. A conspicuous common feature is the shift of the position of cystein residue at 199, 238, and 301. Substitution of hydrophobic amino acids with the hydroxyl amino acids, also generally occurs in plant (9). The substitution is biased from hydroxyl to hydrophobic or vice versa depending on the regions. In the region corresponding to the second exon, T/Y, tended to be substituted by A/M/F, while in the region corresponding to the third exon, A/M/F, tended to be substituted by S/T/Y. As these substitutions are common in plants, it is conjectured that they originate before the branching of green algae and higher plants. The difference in local hydrophobicity may be evoked by the above mentioned type of substitutions and also by conservative substitutions of strongly hydrophobic isoleucine for less hydrophobic methionine. This type of substitution occurs relatively frequently between plants and animals. By the consequence of these substitutions, plant β-tubulins have considerably different local hydrophobicity. It is more hydrophobic than animal β-tubulins at the region between 190–270 and more hydrophilic at the region after 270. These differences in local hydrophobicity may affect the mode of folding leading to microenvironmental differences, which in turn result in differences in sensitivity of tubulin to antimitotics and other ligands.

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(*Received for publication, December 17, 1996*
and in revised form, February 28, 1997)