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The Small Genome of Arabidopsis Contains at Least Nine Expressed β-Tubulin Genes

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The small genome of Arabidopsis contains at least nine expressed β-tubulin (*TUB*) genes, in contrast to the large genomes of vertebrate animals, which contain a maximum of seven expressed β-tubulin genes. In this study, we report the structures of seven new *TUB* genes (*TUB2*, *TUB3*, *TUB5*, *TUB6*, *TUB7*, *TUB8*, and *TUB9*) of Arabidopsis. The sequences of *TUB1* and *TUB4* had been reported previously. Sequence similarities and unique structural features suggest that the nine *TUB* genes evolved by way of three branches in the plant β-tubulin gene evolutionary tree. Two genes (*TUB2* and *TUB3*) encode the same β-tubulin isoform; thus, the nine genes predict eight different β-tubulins. In contrast to the α-tubulin (*TUA*) genes with their divergent intron patterns, all nine *TUB* genes contain 2 introns at conserved positions. Noncoding 3′ gene-specific hybridization probes have been constructed for all nine *TUB* genes and used in RNA gel blot analyses to demonstrate that all nine genes are transcribed. Two-dimensional protein immunoblot analyses have resolved at least seven different β-tubulin isoforms in Arabidopsis, indicating that most, if not all, of the *TUB* transcripts are translated.

INTRODUCTION

The important roles of microtubules in a large number of basic cellular processes and in morphogenesis of higher plants have been discussed in Kopczak et al. (1992) and in several previous reviews (Hepler and Palevitz, 1974; Gunning and Hardham, 1982; Tiwari et al., 1984; Lloyd, 1987; Silflow et al., 1987; Fosket, 1989; Hussey et al., 1991). Yet, the genetic control of microtubule-based processes remains largely unstudied in higher plants. The basic structural unit of microtubules is the heterodimer containing one molecule of each of two proteins, α-tubulin and β-tubulin, both with a molecular weight of approximately 50,000. In multicellular eukaryotes, both proteins are usually encoded by small gene families (Cleveland and Sullivan, 1985; Silflow et al., 1987; Fosket, 1989; Hussey et al., 1991). The tubulin gene families of higher animals have been studied in considerable detail (reviewed by Cleveland and Sullivan, 1985). In contrast, little is known about the tubulin gene families of higher plants (Silflow et al., 1987; Fosket, 1989; Hussey et al., 1991). In Kopczak et al. (1992), we described the α-tubulin (*TUA*) gene family of Arabidopsis. In this study, we describe the β-tubulin (*TUB*) gene family of Arabidopsis. Together, these two gene families represent about 0.05% of the Arabidopsis genome.

The genomes of higher animals contain many sequences that hybridize to tubulin coding sequence probes; however, at least in mammals, many of these sequences are pseudogenes (Lee et al., 1983; Wang et al., 1986). The maximum number of expressed β-tubulin genes reported in any species is seven in chickens (Monteiro and Cleveland, 1988). Although multiple β-tubulin isoforms have been demonstrated in a few plant species (reviewed by Hussey et al., 1991), the sequences of only five plant β-tubulin genes have been published to date. These include two from soybean (Guiltinan et al., 1987), two from Arabidopsis (Marks et al., 1987; Oppenheimer et al., 1988), and one from maize (Hussey et al., 1990). In addition, Hussey et al. (1990) reported the sequence of a distinct β-tubulin cDNA from maize. Thus, six plant β-tubulin coding sequences are known, with no more than two sequences available in any one species. In this study, we report the cloning, sequencing, and initial analysis of expression patterns of seven additional *TUB* genes of Arabidopsis, bringing the total number of expressed *TUB* genes in Arabidopsis to nine, which is two more than in any other species studied to date. Our collection of clones contains the entire complement of coding sequences detected by hybridization of β-tubulin probes to genomic DNA on gel blots and permits us to examine the diversity of β-tubulin coding information in a single higher plant species.

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RESULTS

The *TUB* Gene Family in Arabidopsis

The sequences of two *TUB* genes, *TUB4* and *TUB1*, were reported by Marks et al. (1987) and Oppenheimer et al. (1988),

respectively. Four additional *TUB* genes, designated *TUB2*, *TUB3*, *TUB5*, and *TUB6*, were isolated from a λ *sep6 lac5* genomic library (Pruitt and Meyerowitz, 1986). Two more genes, designated *TUB7* and *TUB8*, were isolated from a cosmid vector pOCA18 genomic library (Olszewski et al., 1988). A DNA gel blot comparison of the available *TUB* clones and genomic fragments that hybridized to *TUB* coding sequence probes showed that two EcoRI fragments with homology to *TUB* sequences were not represented in our collection of clones. Clones of these two fragments were subsequently isolated from libraries prepared from size-fractionated genomic restriction fragments (see Methods) and shown to harbor *TUB9*.

Figure 1 shows a DNA gel blot cataloging the *TUB* genes present in the Arabidopsis genome and relating the respective genomic EcoRI fragments to the cloned *TUB* genes that they harbor. *TUB4* was not cloned in our laboratory; however, the location of *TUB4* on the DNA gel blot shown was determined by hybridization of the DNA on the gel blot to a 3′ noncoding gene-specific probe constructed from a *TUB4* clone (Marks et al., 1987). *TUB1*, *TUB4*, *TUB5*, *TUB7*, and *TUB8* are each located on single EcoRI fragments (approximately 11.7, 9.7, 9.4, 3.6, and 3.2 kb, respectively). Genes *TUB3*, *TUB6*, and *TUB9* are each located on two EcoRI fragments as follows: *TUB3*, 5′, 2.6 kb, 3′, 1.6 kb; *TUB6*, 5′, 1.6 kb, 3′, 3.4 kb; *TUB9*, 5′, 7.4 kb, 3′, 1.5 kb. *TUB2* is present on three EcoRI fragments of 4.5, 0.8, and 2.6 kb (5′ to 3′). *TUB2* and *TUB3* are linked as direct tandem repeats (Oppenheimer et al., 1988) such that the 3′ end of *TUB2* and the 5′ end of *TUB3* are present on the same 2.6-kb EcoRI fragment (shown only in the lane containing the *TUB3* clone in Figure 1). The DNA gel blot results are consistent with the possibility that all of the *TUB* genes of Arabidopsis have been cloned.

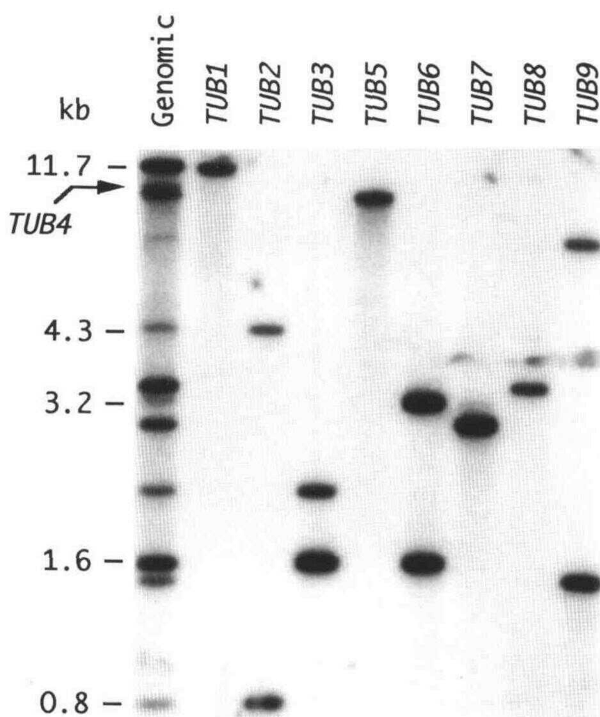

Figure 1. DNA Gel Blot Comparison of Cloned and Genomic *TUB* Genes of Arabidopsis.

All DNAs analyzed were digested with EcoRI, and the representative DNA samples on the gel blot are as follows: genomic DNA, 7.5 μg of EcoRI-digested total DNA; and *TUB1* through *TUB3* and *TUB5* through *TUB9*, agarose gel-purified EcoRI fragment inserts of the respective clones. The quantities of inserts loaded were adjusted based on fragment length to be equivalent to the number of single-copy sequences in 7.5 μg of genomic DNA. The DNAs on the gel blot were hybridized to a mixture of two restriction fragments containing most of the *TUB1* gene and the 3′ coding sequence of the *TUB5* gene (see Methods). The genomic fragment (arrow) containing *TUB4* (Marks et al., 1987) was identified by hybridization with a 3′ noncoding gene-specific probe (see Methods) constructed from a *TUB4* genomic clone.

The Nine *TUB* Genes Predict Eight β-Tubulin Isoforms

The EcoRI fragments containing genes *TUB2*, *TUB3*, *TUB5*, *TUB6*, *TUB7*, *TUB8*, and *TUB9* were subcloned in pUC119 (Vieira and Messing, 1987) in both orientations, and nested sets of deletion subclones were sequenced by the dideoxy chain termination method of Sanger et al. (1977). The complete nucleotide sequences of the eight *TUB* genes sequenced in our laboratory were submitted to EMBL, GenBank, and DDBJ; the accession numbers are given in Methods. The results of sequence comparisons of the *TUB* genes of Arabidopsis showed that the coding sequences of the nine *TUB* genes are highly conserved, with nucleotide sequence identity ranging from 76 to 98% for the various gene pairs. Figure 2 shows a comparison of the predicted amino acid sequences of the β-tubulins encoded by the nine *TUB* genes of Arabidopsis. The nine *TUB* genes predict eight different β-tubulin isoforms; two genes, *TUB2* and *TUB3*, encode the same isoform. The eight isoforms exhibit 89 to 96% amino acid sequence identity; they range in length from 444 to 450 amino acids and have predicted pIs of 4.41 to 4.55. A potentially important feature of the eight β-tubulin isoforms predicted by the sequences of the nine *TUB* genes is the striking diversity of the hydrophilic C termini. Given the involvement of the C-terminal regions of tubulins in interactions with microtubule-associated proteins (MAPs) (reviewed by Wiche et al., 1991), the divergent termini of the predicted β-tubulin isoforms could have functional significance.

Each of the Nine *TUB* Genes Contains Two Introns at Conserved Positions

Each of the nine *TUB* genes contains 2 introns at precisely conserved positions, suggesting that introns were present at these positions in a common ancestral gene. As is characteristic for Arabidopsis, most of the introns are quite small (14 range from 80 to 211 bp); however, a few are relatively large for Arabidopsis

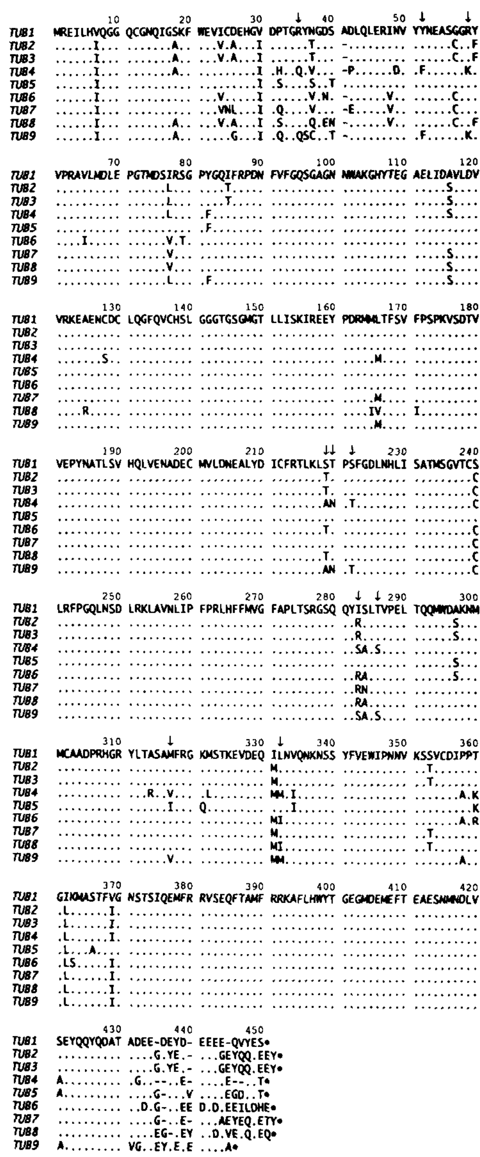

Figure 2. Comparison of the Predicted Amino Acid Sequences of the Nine β-Tubulins of Arabidopsis.

The complete amino acid sequence deduced for the *TUB1* gene product (β1-tubulin of Arabidopsis) is given on the top line. The predicted amino acid sequences of the other β-tubulins of Arabidopsis (*TUB2* through *TUB9*) are represented on the lines below the β1-tubulin sequence; only those amino acids that differ from those at the corresponding positions in the β1 sequence are shown. Amino acids having identity with those shown for the β1 sequence at top are indicated by dots, which are included as an aid in alignment of sequences. Dashes indicate where gaps were introduced to permit optimal sequence alignment. Translation termination codons are represented by bold periods. The arrows above the β1-tubulin sequence denote positions where the amino acid residues of the β4-tubulin and β9-tubulin are identical and differ from those present in the other seven *TUB* gene products. The sequences shown for *TUB1* and *TUB4* were obtained from Oppenheimer et al. (1988) and Marks et al. (1987), respectively.

(e.g., 1020 bp for intron 1 of *TUB5*). Although the codons interrupted by the 2 introns in *TUB1* and *TUB5* are at positions 133 and 223 rather than 132 and 222 as in the other seven *TUB* genes, these introns are at homologous sites within the genes. The positions are shifted downstream by one codon in *TUB1* and *TUB5* because of the unique insertion of three nucleotide pairs specifying alanine at position 41 in the β-tubulins encoded by these two genes.

Origin of the Nine *TUB* Genes: Three Branches in the Evolutionary Tree

Sequence comparisons and unique structural features in specific genes suggest that the nine *TUB* genes have evolved by way of three different branches in the plant β-tubulin gene evolutionary tree. Nucleotide sequence similarities and direct amino acid sequence comparisons (Figure 2) suggest that *TUB1* and *TUB5* evolved from one ancestral gene, *TUB4* and *TUB9* originated from a second ancestral gene, and the other five *TUB* genes evolved from a third ancestral gene. Of course, these three putative ancestral genes almost certainly evolved from an earlier common progenitor.

The strongest evidence that genes *TUB1* and *TUB5* have evolved from a common progenitor is that both genes contain a unique nucleotide pair triplet insertion specifying an alanine at position 41 in the predicted β-tubulins. This alanine codon insertion is not present in any other β-tubulin gene sequenced to date in any species. In addition, the coding sequences of *TUB1* and *TUB5* share 86% nucleotide sequence identity, and the predicted β1-tubulin and β5-tubulin isoforms exhibit 96% amino acid identity. Lastly, the amino acid sequences of the β1 and β5 isoforms are identical from amino acid 83 through amino acid 296; all of the differences between these two proteins occur in the N- and C-terminal regions.

The evidence for a second ancestral gene that gave rise to *TUB4* and *TUB9* also is compelling. Their coding sequences have 87% nucleotide sequence identity, and their predicted protein products have 96% amino acid sequence identity. At 11 positions (indicated by arrows in Figure 2), the β4-tubulin and β9-tubulin share a diagnostic amino acid that is not present at that position in any of the other β-tubulins of Arabidopsis. The coding sequences of *TUB4* and *TUB9* exhibit only 76 to 80% nucleotide identity with the coding sequences of the other seven *TUB* genes. Finally, the *TUB4* and *TUB9* coding sequences are both 444 codons long, 3 to 6 codons shorter than those of the other *TUB* genes.

The other five genes—*TUB2*, *TUB3*, *TUB6*, *TUB7*, and *TUB8*—may all have evolved from a third ancestral gene, but the evidence supporting this possibility is less convincing than that for the *TUB1*/*TUB5* and *TUB4*/*TUB9* ancestral genes. The *TUB2* and *TUB3* genes are almost certainly the products of a fairly recent (on the evolutionary scale) gene duplication. These two genes are located in tandem separated by 1 kb of noncoding sequence, and their coding sequences have 98% nucleotide sequence identity and encode the same β-tubulin

isoform. The predicted *TUB2/TUB3* gene product has 96 and 94% amino acid sequence identity with the predicted products of *TUB7* and *TUB8*, respectively, and the latter two proteins share 94% amino acid sequence identity. The *TUB6* gene is the most divergent member of this group; it encodes a protein sharing 92 to 94% amino acid sequence identity with the predicted products of the other genes in this group. However, the five genes in this group all encode proteins of 449 to 450 amino acids in length, 2 to 6 amino acids longer than the products of *TUB1*, *TUB4*, and *TUB9*. (The *TUB1* relative *TUB5* does not fit the pattern here in that it predicts a product 449 amino acids in length.) Lastly, there are three diagnostic amino acid positions (24, 57, and 283) where the products of *TUB2*, *TUB3*, *TUB6*, *TUB7*, and *TUB8* are all identical and differ from the products of the other four *TUB* genes. Thus, *TUB6* seems most likely to have evolved from the same progenitor as *TUB2*, *TUB3*, *TUB7*, and *TUB8* despite its somewhat more divergent sequence.

All Nine *TUB* Genes Are Expressed

Noncoding 3′ gene-specific hybridization probes were used to investigate the expression of all nine *TUB* genes. The gene-specific probes were hybridized to RNAs on gel blots of total RNA isolated from roots, leaves plus petioles, and whole flowers excised from 21-day-old plants. Figure 3 shows a summary of the RNA gel blot results obtained using a *TUB* coding sequence probe and each of the nine *TUB* gene-specific probes. The results demonstrate that all nine of the *TUB* genes are transcribed and suggest that the transcripts of certain genes preferentially accumulate in certain parts of the plant. The transcripts of several *TUB* genes seem to accumulate somewhat preferentially in flowers, which contain pollen, a tissue rich in microtubules. The transcripts of *TUB5* and *TUB6* were found to accumulate preferentially in leaves plus petioles. Despite the close evolutionary relationship between *TUB1* and *TUB5*, these genes have very different expression patterns; the *TUB1* transcript was shown to accumulate primarily in roots as previously reported (Oppenheimer et al., 1988).

To determine whether the β-tubulin heterogeneity predicted by the nine *TUB* coding sequences exists in vivo, two-dimensional protein immunoblots were performed with an anti-β-tubulin antibody on total proteins isolated from various plant organs and/or tissues. Figure 4 shows a two-dimensional protein immunoblot of the β-tubulin isoforms present in whole flowers. Seven major spots on the immunoblot suggest the presence of seven separable β-tubulin isoforms. The calculated pI for the β2/3 and β6 isoforms is 4.45, and their predicted molecular weights are 50,737 and 50,589, respectively, suggesting that these two isoforms might not be resolved on our immunoblots. Because the sequences of the nine *TUB* genes predict eight different isoforms, it seems likely that all of the *TUB* transcripts are translated. Two minor spots also are evident on the immunoblot shown in Figure 4. These could represent post-translationally modified proteins such as the phosphorylated β-tubulins characterized in animal systems

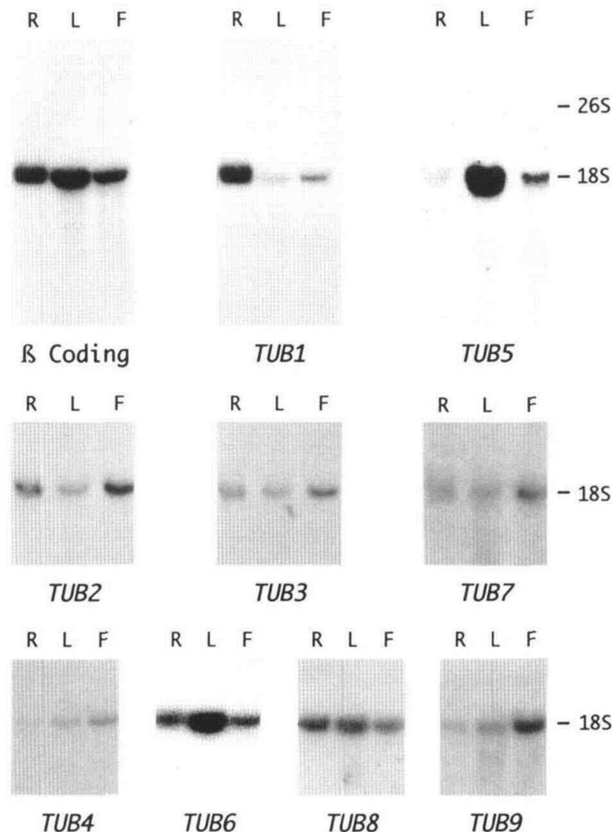

Figure 3. Accumulation of *TUB* Gene Transcripts in Roots, Leaves, and Flowers.

Total RNAs were isolated from whole roots (R), leaves plus petioles (L), and whole flowers (F) at all developmental stages of 21-day-old plants. Samples of these RNAs (10 μg per lane) were analyzed by RNA gel blot hybridizations using a *TUB1* coding sequence probe and noncoding sequence probes specific for each of the nine *TUB* genes (see Methods). Two RNA gel blots were used to obtain the data shown. Sequential hybridizations were done first with the coding sequence probe and then with the gene-specific probes; the RNA gel blots were stripped completely of probe between hybridizations. Approximate size markers are provided by the positions of the Arabidopsis 18S and 26S rRNAs indicated at the right.

(Gard and Kirschner, 1985); however, additional studies are needed to determine whether these spots identify distinct isoforms or are merely artifacts.

DISCUSSION

Evidence for Nine Expressed *TUB* Genes in Arabidopsis

In this study, we have demonstrated that the small genome of Arabidopsis contains nine expressed *TUB* genes, two more

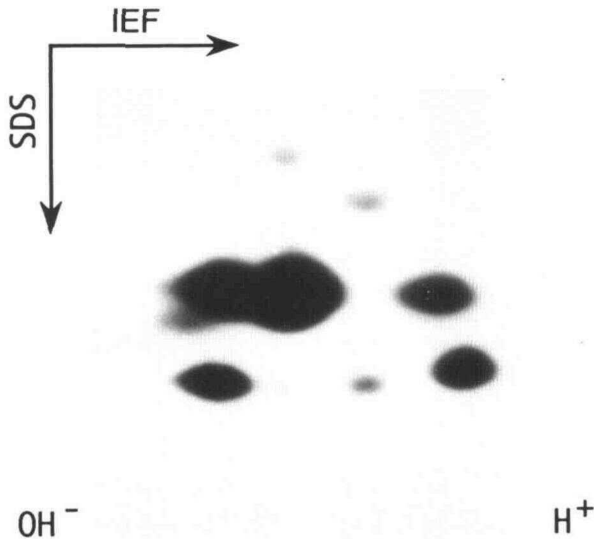

Figure 4. Two-Dimensional Protein Gel Blot Analysis of the β-Tubulins in Arabidopsis Flower Tissues.

The anti-β-tubulin monoclonal antibody 2-10-B6 was used upon a two-dimensional immunoblot containing proteins isolated from 21-day-old plants. An enlargement of the region of the immunoblot containing the β-tubulins is shown. The directions of SDS-PAGE (SDS) and isoelectric focusing (IEF) are indicated by the arrows.

than in any other species characterized to date. Although we cannot exclude the possibility of additional *TUB* genes on other co-migrating EcoRI fragments or of divergent *TUB* genes that cross-hybridize with the cloned *TUB* genes too weakly to be detected under our hybridization conditions, it seems likely that all of the Arabidopsis *TUB* genes may now have been cloned and sequenced.

Because vertebrates contain only six or seven expressed β-tubulin genes (Wang et al., 1986; Monteiro and Cleveland, 1988), the large number of *TUB* genes in the small Arabidopsis genome was unexpected. Perhaps large tubulin gene families will prove to be a characteristic of higher plants. In fact, Hussey et al. (1990) have estimated that the maize genome contains a minimum of nine β-tubulin genes, with perhaps as many as 16 genes present, and A. Colón and D. Fosket (personal communication) have evidence for 15 β-tubulin genes in soybean. In addition, Marks et al. (1987) concluded that of nine plant species examined, all but one, cucumber, had about as many β-tubulin genes as Arabidopsis, or more. If, indeed, plant genomes do contain more tubulin genes than the large genomes of higher animals, one must ask whether tubulin isoforms perform additional, possibly unique, functions in plants or whether tubulin genes of plants perhaps respond to specialized demands for tubulin synthesis that require additional regulatory circuits. In any case, the divergent hydrophilic C termini of the eight predicted β-tubulin isoforms could prove important since these regions of α- and β-tubulins are known to be involved in their interaction with various MAPs in animals and lower eukaryotes (reviewed by Wiche et al., 1991). Perhaps certain of the β-tubulin isoforms of Arabidopsis interact with specific MAPs based on their unique hydrophilic C-terminal regions.

Conserved Intron Positions in *TUB* Genes

In striking contrast to the variable number and positions of the introns in the *TUA* genes of Arabidopsis (Kopczak et al., 1992), each of the nine *TUB* genes was found to contain 2 introns at precisely conserved positions. Whether this difference between the two tubulin gene families is significant or just fortuitous remains unknown, but this should become clear as the tubulin gene families of other plants are characterized. The two sequenced β-tubulin genes of soybean also contain 2 introns at the same positions as those of the Arabidopsis *TUB* genes (Guiltinan et al., 1987). However, the one sequenced β-tubulin gene of maize contains only a single intron at the position of the first intron in the Arabidopsis and soybean β-tubulin genes (Hussey et al., 1990). As with the *TUA* genes (Kopczak et al., 1992), the structures of the Arabidopsis *TUB* genes provide little support for the proposal of Marchionni and Gilbert (1986) that introns were in place before the divergence of plants and animals. The third intron of the two *Chlamydomonas reinhardtii* β-tubulin genes (Youngblom et al., 1984) is located at the same position as the first intron in the plant β-tubulin genes. In addition, the single intron (separating codons 131 and 132) in the β2-tubulin gene of Drosophila (Rudolph et al., 1987) is located very close to the position of the first intron (interrupting codon 132; 133 in *TUB1* and *TUB5*) of the plant β-tubulin genes. However, none of the introns in the β-tubulin genes of vertebrate animals occurs at the same location as any of the introns in the plant β-tubulin genes.

Structural and Functional Divergence of β-Tubulin Genes

Plant tubulins are known to differ from animal and fungal tubulins in several respects (for discussions of these differences, see Morejohn and Fosket, 1986; Silflow et al., 1987; Fosket, 1989; Hussey et al., 1991). For example, plant microtubule-based processes are much less sensitive to colchicine than the analogous processes in animals (Morejohn and Fosket, 1986). On the other hand, microtubule-dependent phenomena in animals are not inhibited by low concentrations of dinitroaniline and phosphoric amide herbicides that completely arrest these processes in plants and algae (reviewed by Morejohn and Fosket, 1986; Silflow et al., 1987). Thus, structural comparisons of plant and animal tubulins are of interest because regions of divergence between the tubulins of these two groups must be responsible for their distinct functional properties. As would be expected for important functional domains, some sequences are conserved in essentially all β-tubulins. For example, amino acid sequences 148 to 163, 177 to 196, 204 to 218, 270 to 282, and 392 to 411 (Arabidopsis β1-tubulin

positions) are highly conserved in the β-tubulins of both plants and animals. Other sequences are conserved in all of the plant β-tubulins, but differ in animal β-tubulins. Perhaps the most obvious difference between the plant and animal β-tubulins occurs in amino acid sequence 197 to 203, where all the plant sequences currently available are identical and differ from the animal sequences at five of seven positions. Interestingly, the region from amino acid 192 to 203 is one of two segments where plant and animal β-tubulins exhibit major differences in their hydropathy profiles, with the animal sequences being considerably more hydrophilic than the plant sequences (Silflow et al., 1987). Other regions where the plant β-tubulins are completely conserved and differ from the animal β-tubulins are amino acid sequences 248 to 269 and 374 to 382. Whether or not these regions of the β-tubulins play any role in the observed differences between plant and animal microtubules will have to be determined by direct functional analyses. Nevertheless, sequence comparisons are useful in identifying possible structural bases for the known functional differences between plant and animal microtubules.

Developmentally Regulated Expression of *TUB* Genes

Another interesting question is whether structurally related tubulins exhibit similar patterns of expression in different plant species. The complete predicted amino acid sequences are now available for 13 plant β-tubulins: nine from Arabidopsis (Marks et al., 1987; Oppenheimer et al., 1988; this paper), two from maize (Hussey et al., 1990), and two from soybean (Guiltinan et al., 1987). When these sequences are compared, amino acid sequence identity is found to range from 81 to 100%. In vertebrates, all of the characterized β-tubulins fit into four conserved isotype classes that differ primarily in their C-terminal regions (Cleveland, 1987). Such isotype classes are not apparent in comparisons of the available plant β-tubulin sequences (Figure 2); however, isotype classes may be identified once more plant β-tubulin genes have been sequenced.

In addition to transcriptional regulation, the synthesis of β-tubulins in animal cells is autoregulated by modulation of mRNA stability by a translation-coupled recognition of the N-terminal tetrapeptide (Yen et al., 1988a). This translation-coupled modulation of mRNA stability was shown to be absolutely dependent on the Met-Arg-Glu-Ile N-terminal peptide of the nascent translation product (Yen et al., 1988b). Although there is no evidence for autoregulation of β-tubulin synthesis in Arabidopsis, the Met-Arg-Glu-Ile tetrapeptide is conserved in all nine of the predicted *TUB* gene products. Thus, the same mechanism of autoregulation of β-tubulin synthesis is possible for all the *TUB* genes of Arabidopsis.

Biological Significance of Multiple β-Tubulin Genes

Two major hypotheses have been proposed to explain the existence of multiple tubulin genes in eukaryotes. Fulton and Simpson (1976) proposed that the tubulin isoforms were functionally divergent, with different isoforms fulfilling distinct biological functions. Raff (1984) proposed that the different isoforms might be functionally equivalent, but also be the products of duplicated genes that have acquired distinct regulatory elements allowing the genes to respond to different demands for tubulins during the complex pathways of differentiation in higher eukaryotes. As it has turned out, at least in animals, both hypotheses are correct: some tubulin genes encode functionally divergent products (Asai and Remolona, 1989; Joshi and Cleveland, 1989; Kimble et al., 1989; Savage et al., 1989; Hoyle and Raff, 1990; Matthews et al., 1990), and other differentially regulated genes encode structurally and functionally equivalent, or at least very similar, products (see reviews by Cleveland, 1987; Raff et al., 1987; Hussey et al., 1991). Given the apparently even greater complexity of plant tubulin gene families, it seems likely that some tubulin genes will encode functionally divergent isoforms and others will encode equivalent isoforms with different temporal and spatial patterns of synthesis. Finally, the large sizes of the plant tubulin gene families might be related to the unique microtubule arrays—cortical microtubules, preprophase bands, and phragmoplasts—of higher plants. The utilization of isoform-specific antibodies should lead to definitive information about tubulin isoform usage in the various microtubule arrays of higher plant cells in the near future.

METHODS

Plant Material

All plant materials are as described in Kopczak et al. (1992).

Isolation of Genomic Clones

Clones of the *TUB2*, *TUB3*, *TUB5*, and *TUB6* genes were isolated from an *Arabidopsis thaliana* Columbia genomic DNA library prepared in λ *sep6 lac5*. The library was screened by in situ plaque hybridization (Benton and Davis, 1977) as previously described (Oppenheimer et al., 1988) using a *Chlamydomonas reinhardtii* β-tubulin cDNA probe (*C. reinhardtii* β1 clone 9-12; Youngblom et al., 1984). Clones of the *TUB7* and *TUB8* genes were isolated from an Arabidopsis (Columbia ecotype) genomic DNA library prepared in cosmid vector pOCA18 (Olszewski et al., 1988). This library was constructed using DNA from the chlorsulfuron-resistant mutant line GH50 (Haughn and Somerville, 1986). The cosmid library was screened by in situ colony hybridization as described in Kopczak et al. (1992), except that the hybridization probe was a 1.9-kb HindIII restriction fragment containing most of the coding sequence of the Arabidopsis *TUB1* gene (Oppenheimer et al., 1988) and the probe was labeled by the random-primer protocol of Feinberg and Vogelstein (1983). Clones of genomic EcoRI restriction fragments containing the 5′ and 3′ segments of the *TUB9* gene were isolated from libraries constructed in pUC119 (Vieira and Messing, 1987) and λ ZAPII (Stratagene), respectively, from agarose gel-fractionated restriction fragments prepared and screened as described by Kopczak et al. (1992) with the *TUB1* HindIII fragment as probe.

Subcloning and DNA Sequencing

The subcloning and sequencing methods used were described by Kopczak et al. (1992). The GenBank accession numbers for the eight *TUB* genes sequenced in our laboratory are M20405 (*TUB1*), M84700 (*TUB2*), M84701 (*TUB3*), M84702 (*TUB5*), M84703 (*TUB6*), M84704 (*TUB7*), M84705 (*TUB8*), and M84706 (*TUB9*).

DNA Gel Blot Analysis

DNA isolations, restriction endonuclease digestions, electrophoresis, transfers to membranes, and blot hybridizations were performed as described by Kopczak et al. (1992). The DNA gel blot shown in Figure 1 was hybridized to a mixture of two gel-purified restriction fragments: a 1.9-kb HindIII fragment of *TUB1* (Oppenheimer et al., 1988) and a 280-bp 3′ coding sequence fragment (EcoRV-Sau3A fragment; from 279 bp 5′ to the T of the TGA termination codon) of *TUB5*.

RNA Gel Blot Analysis

Isolation of total RNA from leaves plus petioles, roots, and flowers, formaldehyde–agarose gel electrophoresis, transfers of RNA to membranes, RNA gel blot hybridizations, and DNA gel blot tests of the specificity of 3′ noncoding sequence probes were carried out as described by Kopczak et al. (1992). All hybridization probes were single-stranded template DNAs prepared in pUC119 and labeled by the primer extension protocol of Hu and Messing (1982). The *TUB* coding sequence probe was the 1.9-kb HindIII restriction fragment of *TUB1* (Oppenheimer et al., 1988). All gene-specific probes were subclones containing primarily 3′ noncoding sequences as follows: *TUB1*, an approximately 430-bp fragment extending from the G of the TGA codon to about 430 bp 3′ of the termination codon; *TUB2*, a 305-bp fragment extending from 2 bp 5′ to 300 bp 3′ of the TGA codon; *TUB3*, a 298-bp fragment extending from 2 bp 5′ to 293 bp 3′ of the TGA codon; *TUB4*, a 435-bp fragment extending from 8 bp 5′ to 424 bp 3′ of the TAA codon; *TUB5*, a 180-bp fragment extending from the G of the TGA codon to 178 bp 3′ of the termination codon; *TUB6*, an approximately 490-bp fragment extending from 10 bp 5′ to about 480 bp 3′ of the TGA codon; *TUB7*, a 215-bp fragment extending from 26 bp 3′ to 240 bp 3′ of the TGA codon; *TUB8*, a 335-bp fragment extending from 5 bp 3′ to 339 bp 3′ of the TAA codon; and *TUB9*, a 107-bp fragment extending from 31 bp 5′ to 73 bp 3′ of the TAA codon.

Two-Dimensional Protein Gel Immunoblot Analysis

Proteins were extracted, separated by two-dimensional polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and reacted with antibodies as described by Hussey et al. (1988). The monoclonal antibody 2-10-B6 was raised against sea urchin tubulin and reacts with β-tubulins from plants and algae (C. Silflow, unpublished data).

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