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The Small Genome of Arabidopsis Contains at Least Six Expressed α-Tubulin Genes

Steven D. Kopczak,[a] Nancy A. Haas,[a] Patrick J. Hussey,[a,1] Carolyn D. Silflow,[a,b,c] and D. Peter Snustad [a,c,2]

[a] Department of Genetics and Cell Biology, University of Minnesota, St. Paul, Minnesota 55108-1095
[b] Department of Plant Biology, University of Minnesota, St. Paul, Minnesota 55108-1095
[c] Plant Molecular Genetics Institute, University of Minnesota, St. Paul, Minnesota 55108-1095

The goal of our investigations is to define the genetic control of microtubule-based processes in a higher plant. The available evidence suggests that we have achieved our first objective: the characterization of the complete α-tubulin and β-tubulin gene families of Arabidopsis. Four additional α-tubulin genes (*TUA2*, *TUA4*, *TUA5*, and *TUA6*) of Arabidopsis have been cloned and sequenced to complete the analysis of the gene structure for all six α-tubulin genes detectable on DNA gel blots of Arabidopsis genomic DNA hybridized with α-tubulin coding sequences. *TUA1* and *TUA3* were characterized earlier in our laboratory. Noncoding gene-specific hybridization probes have been constructed for all six α-tubulin genes and used in RNA gel blot analyses to demonstrate that all six genes are transcribed. The six genes encode four different α-tubulin isoforms; *TUA2* and *TUA4* encode a single isoform, as do *TUA3* and *TUA5*. Two-dimensional protein gel immunoblot analyses have resolved at least four α-tubulin isoforms from plant tissues, suggesting that all of the predicted *TUA* gene products are synthesized in vivo.

INTRODUCTION

Microtubules play central roles in several basic processes of eukaryotic cells: cell division, cell motility, intracellular transport, and the control of cell shape. In plants, specific arrays of microtubules appear to be especially important in morphogenesis. The rigid walls of plant cells largely preclude cell migrations, which are very important in the morphogenesis of animals. As a result, morphogenesis in plants is controlled primarily by the processes that establish the planes of cell divisions and determine the axes of cell elongations. Three arrays of microtubules unique to plant cells, namely, preprophase bands, phragmoplasts, and cortical microtubules, are known to be involved in these two morphogenetic processes (reviewed by Hepler and Palevitz, 1974; Gunning and Hardham, 1982; Tiwari et al., 1984; Lloyd, 1987; Fosket, 1989). Yet, little is known about the genetic control of microtubule function in any higher plant.

In algae, fungi, and animals, numerous studies of the genes encoding α-tubulins and β-tubulins, the conserved structural proteins of microtubules, have demonstrated the presence of α-tubulin and β-tubulin gene families of two to more than 20 members (reviewed by Raff, 1984; Cleveland and Sullivan, 1985; Silflow et al., 1987). However, in mammals, most of the tubulin gene family members are pseudogenes (Lee et al., 1983; Lewis et al., 1987), with only five α-tubulin genes and six β-tubulin genes being functional in the mouse (Villasante et al., 1986; Wang et al., 1986). Before our study with Arabidopsis, the seven expressed β-tubulin genes in chicken (Monteiro and Cleveland, 1988) represented the largest family of functional α-tubulin or β-tubulin genes known in any species.

In plants, multiple tubulin isoforms have been identified in a few species (reviewed by Fosket, 1989; Hussey et al., 1991), with four electrophoretically separable α-tubulin isoforms reported in maize (Joyce et al., 1992) and six separable β-tubulin isoforms identified in both carrot (Hussey et al., 1988) and maize (Joyce et al., 1992). Although the results of genomic DNA gel blot hybridization studies have suggested the existence of multiple α-tubulin (*TUA*) genes in Arabidopsis and maize (Ludwig et al., 1987; Montoliu et al., 1989; Villemur et al., 1992) and multiple β-tubulin genes in several plant species (Guiltinan et al., 1987; Marks et al., 1987; Oppenheimer et al., 1988; Hussey et al., 1990), only a few plant tubulin genes have been characterized to date. These include two α-tubulin genes (Ludwig et al., 1987, 1988) and two β-tubulin genes (Marks et al., 1987; Oppenheimer et al., 1988) of Arabidopsis, two β-tubulin genes of soybean (Guiltinan et al., 1987), one β-tubulin gene and a distinct β-tubulin cDNA of maize (Hussey et al., 1990), and three α-tubulin genes of maize (Montoliu et al., 1989, 1990). In addition, a detailed analysis of 81 α-tubulin cDNA clones has shown that the maize genome contains at least six expressed α-tubulin genes, and genomic DNA gel blot data indicate that maize contains a minimum of seven α-tubulin genes and/or pseudogenes (Villemur et al., 1992). However, an entire α-tubulin or β-tubulin gene family of a higher plant had not been characterized before this study.

[1] Current address: Department of Biochemistry, Royal Holloway and Bedford New College, University of London, Egham Hill, Egham, Surrey TW20 OEX, U.K.
[2] To whom correspondence should be addressed.

The long-term goal of our investigations is to characterize the genetic control of microtubule function in a higher plant. Arabidopsis was selected as the system of choice for two major reasons: (1) it has many advantages for genetic and molecular studies (reviewed by Rédei, 1970, 1975; Meyerowitz and Pruitt, 1985; Somerville et al., 1985), and (2) its gene families tend to be smaller than those of other flowering plants (reviewed by Meyerowitz, 1987). The initial objectives of this study were to clone and sequence all the tubulin genes of Arabidopsis and to construct gene-specific hybridization probes with which to analyze the expression of the individual genes. We believe that these initial objectives have now been achieved. In this study and in Snustad et al. (1992), we report the cloning and characterization of 11 additional tubulin genes (four encoding α-tubulins and seven encoding β-tubulins) of Arabidopsis. Gene-specific hybridization probes were constructed for all 11 tubulin genes, and RNA gel blot analyses performed with the gene-specific probes showed that all 11 genes were expressed. In total, 15 tubulin genes (six encoding α-tubulins and nine encoding β-tubulins) of Arabidopsis have now been characterized. In Carpenter et al. (1992), we present the results of studies with a chimeric β-glucuronidase reporter gene in which its expression in transgenic Arabidopsis plants shows that the *TUA1* gene (encoding α1-tubulin) of Arabidopsis is preferentially expressed in pollen.

RESULTS

The *TUA* Gene Family in Arabidopsis

The first two Arabidopsis *TUA* genes identified (*TUA1* and *TUA3*) were isolated (Ludwig et al., 1987, 1988) from a genomic library in λ *sep6 lac5* (Pruitt and Meyerowitz, 1986). Clones of three additional *TUA* genes, designated *TUA2*, *TUA4*, and *TUA5*, were isolated from an Arabidopsis genomic library constructed in the cosmid vector pOCA18 (Olszewski et al., 1988). Because the original λ *sep6 lac5* library was constructed from a partial EcoRI genomic digest, we have used EcoRI-digested DNA gel blots to compare our *TUA* clones with the total genomic complement of *TUA* genes. Such comparisons showed that one genomic EcoRI fragment (approximately 2.9 kb) that cross-hybridized with *TUA* coding sequences was not represented in our collection of *TUA* clones. A clone of this 2.9-kb fragment was subsequently isolated from a library prepared from size-fractionated genomic restriction fragments (see Methods) and shown to contain all of the *TUA6* gene except for 105 bp of 3′ coding sequence plus the 3′ noncoding sequence. This distal portion of *TUA6* was cloned by using the polymerase chain reaction (PCR) to amplify the 3′ end of the gene from size-fractionated HindIII fragments ligated into the HindIII site of pUC119 (see Methods).

Figure 1 shows a DNA gel blot comparison of the *TUA* clones in our collection and genomic EcoRI fragments containing DNA sequences that hybridize to *TUA* coding sequence probes. The DNA gel blot was probed with a mixture of labeled subclones spanning the complete coding sequences of *TUA1*, *TUA2*, and *TUA3*. The results demonstrate that all detectable *TUA* genes of Arabidopsis are represented in our collection of clones. Genes *TUA1* and *TUA2* are each located on a single EcoRI fragment. *TUA3*, *TUA5*, and *TUA6* are each located on two EcoRI fragments, and *TUA4* is present on three EcoRI fragments. The *TUA* gene-containing fragments not documented in Figure 1 are those carrying the short 3′ ends of *TUA3*, *TUA5*, and *TUA6*. Because of their relatively short tubulin coding sequences, which include the segments encoding the variable C-terminal regions of the tubulins, these fragments hybridize weakly on genomic DNA gel blots. These three fragments have been identified on gel blots in which genomic DNA was hybridized to short 3′ noncoding sequence probes (data not shown). The genomic fragment containing the short 5′ end of *TUA4* was not always detected; it is barely visible on the gel blot shown in Figure 1 (dashed arrow).

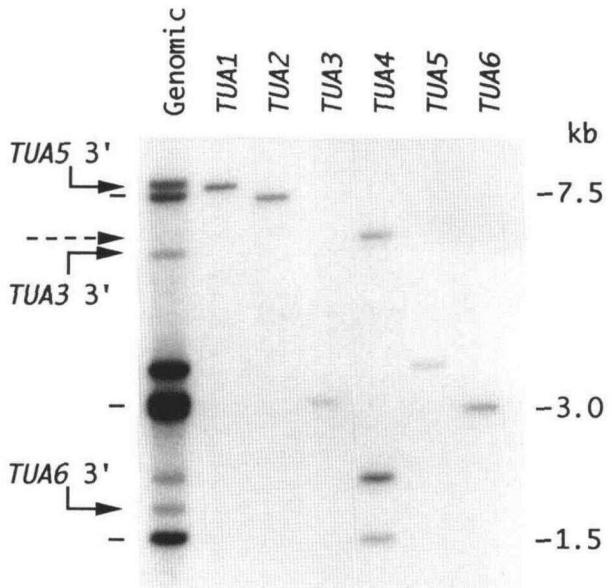

Figure 1. DNA Gel Blot Comparison of Cloned and Genomic *TUA* Genes of Arabidopsis.

All DNAs analyzed were digested with EcoRI. The DNA restriction fragments characterized on the gel blot are as follows: genomic DNA, 7.5 μg of EcoRI-digested total DNA; and *TUA1* through *TUA6*, agarose gel-purified EcoRI fragment inserts of their respective clones with quantities of DNA loaded being adjusted based on insert 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 with a mixture of four restriction fragments collectively harboring the complete coding sequences of the *TUA1*, *TUA2*, and *TUA3* genes (see Methods). The genomic fragment containing the short 5′ end of *TUA4* (dashed arrow) is just barely visible on this autoradiogram. The genomic fragments carrying the short 3′ ends of *TUA3*, *TUA5*, and *TUA6* (solid arrows) were identified by hybridizing the DNA on the gel blot with 3′ noncoding subclones of their respective genes.

TUA3 and *TUA5* Are Linked in Reverse Orientation

One of the clones isolated from the pOCA18 library contained both the *TUA3* gene (Ludwig et al., 1987) and the *TUA5* gene on the same cosmid clone. Figure 2 presents a restriction map showing the arrangement of these two genes on the genomic clone being studied. In the following section, *TUA3* and *TUA5* are shown to encode the same tubulin isoform.

The Six *TUA* Genes Predict Four α-Tubulin Isoforms

The EcoRI restriction fragments containing genes *TUA2*, *TUA4*, *TUA5*, and *TUA6* 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 (Sanger et al., 1977). The complete nucleotide sequences of all six *TUA* genes were submitted to EMBL, GenBank, and DDBJ; the accession numbers are given in Methods. The nucleotide sequences of the *TUA* coding regions range in similarity from 74% nucleotide identity (*TUA1* versus *TUA2*) to 98% nucleotide identity (*TUA3* versus *TUA5*). All six genes specify proteins 450 amino acids in length; the predicted molecular weights and isoelectric points of the primary translation products of the *TUA* genes are 49,803 and 4.68 (*TUA1*), 49,544 and 4.69 (*TUA2/4*), 49,657 and 4.72 (*TUA3/5*), and 49,650 and 4.69 (*TUA6*), respectively. All the predicted proteins contain a C-terminal tyrosine residue, indicating that they may be subject to the post-translational detyrosination/tyrosination cycle described in animal systems (Raybin and Flavin, 1977). The predicted amino acid sequences of the α2-tubulin, α4-tubulin, α5-tubulin, and α6-tubulin are presented in Figure 3, along with those of the α1-tubulin and α3-tubulin (Ludwig et al., 1987, 1988), to show that the six *TUA* genes of Arabidopsis encode four different α-tubulin isoforms.

Comparisons of DNA sequence data showed that the Arabidopsis *TUA* gene family contains two subsets of related genes. As is shown in Figure 3, *TUA2* and *TUA4* encode the same protein, although the coding sequences of these two genes differ by 46 nucleotide pair substitutions (data not

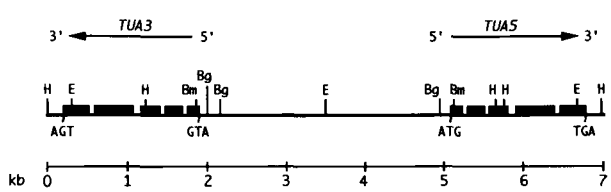

Figure 2. Restriction Map of the Closely Linked *TUA3* and *TUA5* Genes.

This abbreviated restriction map of the 7-kb region spanning the *TUA3* and *TUA5* genes shows their relative positions and their reverse orientation. The arrows indicate the direction of transcription of the two genes; the ATG and TGA translation initiation and translation termination codons also are shown. Restriction sites shown are as follows: H, HindIII; E, EcoRI; Bm, BamHI; and Bg, BglII.

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                 10                  30                  40                  50
TUA2  MRECISIHIG QAGIQVGNAC WELYCLEHGI QPDGQMPSDK TVGGGDDAFN
TUA4  .......... .......... .......... .......... ..........
TUA6  .......... .......... .......... .......G.. ..........
TUA3  ...I...... ........S. .......... ....M....T ...VAH....
TUA5  ...I...... ........S. .......... ....M....T ...VAH....
TUA1  ...I...... ........S. .......... ....T....S ...ACH....

                 60                  70                  80                  90                 100
TUA2  TFFSETGAGK HVPRAVFVDL EPTVIDEVRT GTYRQLFHPE QLISGKEDAA
TUA4  .......... .......... .......... .......... ..........
TUA6  .......... .......... .......... .......... ..........
TUA3  .......... .......... .......... .......... ..........
TUA5  .......... .......... .......... .......... ..........
TUA1  ......SS.Q .......L.. .......... .......... ..........

                 120                 130                 140                 150
TUA2  NNFARGHYTI GKEIVDLCLD RIRKLADNCT GLQGFLVFNA VGGGTGSGLG
TUA4  .......... .......... .......... .......... ..........
TUA6  .......... .......... .......... .......... ..........
TUA3  .........V .......... .V........ .......... ..........
TUA5  .........V .......... .V........ .......... ..........
TUA1  .........V .R....T..E .L........ .......... ..........

                 160                 170                 190                 200
TUA2  SLLLERLSVD YGKKSKLGFT VYPSPQVSTS VVEPYNSVLS THSLLEHTDV
TUA4  .......... .......... .......... .......... ..........
TUA6  .......... .......... .......... .......... ..........
TUA3  .......... .......... I........A .......... ..........
TUA5  .......... .......... I........A .......... ..........
TUA1  .......... .F........ I........A .......... ..........

                 210                 220                 230                 260
TUA2  SILDNEAIY DICRRSLSIE RPTYTNLNRL VSQVISSLTA SLRFDGALNV
TUA4  .......... .......... .......... .......... ..........
TUA6  .......... .......N.. .......... .......... ..........
TUA3  AV........ .......D.. .......... I..I.....T .......I..
TUA5  AV........ .......D.. .......... I..I.....T .......I..
TUA1  VV........ .......D.. ....S..... I..T.....T .......I..

                 260                 270                 300
TUA2  DVTEFQTNLV PYPRIHFMLS SYAPVISAEK AFHEQLSVAE ITNSAFEPAS
TUA4  .......... .......... .......... .......... ..........
TUA6  .......... .......... .......... .......... ..........
TUA3  .I........ .......... ........A. .Y......P. ...AV.....
TUA5  .I........ .......... ........A. .Y......P. ...AV.....
TUA1  .I........ .......... .......SA. .Y...F..P. ..T.V...SN

                 310                 320                 330                 340                 350
TUA2  MMAKCDPRHG KYMACCLMYR GDVVPKDVNA AVGTIKTKRT IQFVDWCPTG
TUA4  .......... .......... .......... .......... ..........
TUA6  .......... .......... .......... .......... ..........
TUA3  .......... .......... .......... .......... V.........
TUA5  .......... .......... .......... .......... V.........
TUA1  .......... .......... .........T ..AA..A... ..........

                 360                 370                 380                 390                 400
TUA2  FKCGINYQPP TVVPGGDLAK VQRAVCMISN STSVAEVFSR IDHKFDLMYA
TUA4  .......... .......... .......... .......... ..........
TUA6  .......... .......... .......... .......... ..........
TUA3  .......... .......... .......... N.A....... ..........
TUA5  .......... .......... .......... N.A....... ..........
TUA1  .......... S......... .......... N.A....... .........S

                 410                 420                 430                 440                 450
TUA2  KRAFVHWYVG EGMEEGEFSE AREDLAALEK DYEEVGAEGG DDEDDEGEEY•
TUA4  .......... .......... .......... .......... ..........•
TUA6  .......... .......... .......... .......... ..........•
TUA3  .......... .......... .......... .......... ...E....D.•
TUA5  .......... .......... .......... .......... ...E....D.•
TUA1  .......... .......... .......... ......G..A E.D.E..D..•
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Figure 3. Comparison of the Predicted Amino Acid Sequences of the Six α-Tubulins of Arabidopsis.

The complete predicted amino acid sequence of the *TUA2* gene product (α2-tubulin) is given on the top line. The predicted amino acid sequences of the other α-tubulins are given on the lines below the α2-tubulin sequence and show only those amino acids that differ from the corresponding amino acids in the α2 sequence. The dots indicate identity of amino acids with those shown on the top line for the α2 sequence; they are included as an aid in the alignment of sequences. The translation termination codons are represented by bold periods. The arrows above the α2 sequence denote amino acid residues where the α1 and α3/5 isoforms both differ from the α2/4 and α6 isoforms by the same amino acid substitutions. The sequences shown for *TUA3* and *TUA1* were obtained from Ludwig et al. (1987, 1988, respectively).

shown). The sequence comparisons in Figure 3 also show the close relationship between the *TUA2/TUA4* gene pair and a third gene, *TUA6*, which encodes a protein differing from the *TUA2/TUA4* gene product at only two amino acid residues. In the coding region, *TUA6* differs from *TUA2* and *TUA4* by 66 and 58 nucleotide pair substitutions, respectively (data not shown). The 5′ noncoding regions of these three genes also are quite closely related. For the 200 bp upstream from the ATG, *TUA6* exhibits 87 and 78% nucleotide sequence identity with *TUA2* and *TUA4*, respectively, whereas the latter share only 74% identity.

The closely linked *TUA3* and *TUA5* genes also encode a single α-tubulin isoform and represent a second subset of closely related genes. The coding sequences of these two genes differ by only 29 nucleotide pair substitutions (data not shown). Moreover, even the intron sequences of *TUA3* and *TUA5* are highly conserved, indicating that these two genes are the result of a fairly recent (on the evolutionary scale) gene duplication. Both genes contain 4 introns at precisely conserved positions, and the sizes of the introns are identical except for a single nucleotide pair addition or deletion in the first intron, as shown in Figure 4. Only 15 nucleotide pair substitutions have occurred at 361 positions within the 4 introns of this gene pair (data not shown). The 3′ noncoding sequences of *TUA3* and *TUA5* also are highly conserved (85% identity);

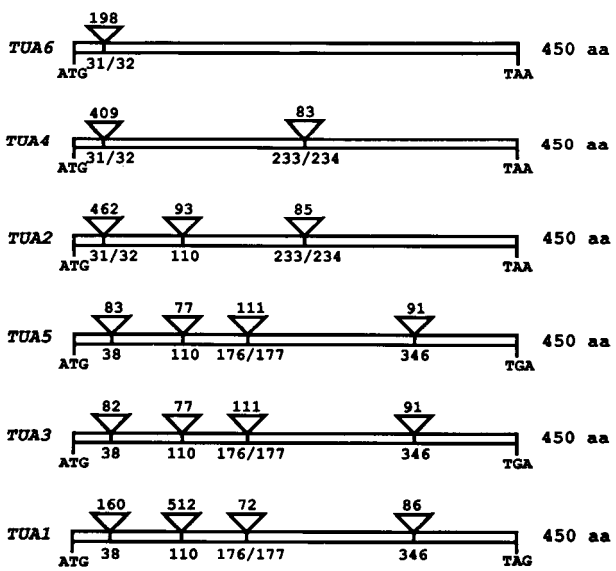

Figure 4. Positions and Sizes of Introns in the *TUA* Genes of Arabidopsis.

Diagram of the six *TUA* genes of Arabidopsis showing the sizes (numbers above the triangles indicate numbers of nucleotide pairs) and positions (numbers below the triangles indicate codon positions interrupted by the respective introns) of their introns. The translation initiation and translation termination codons also are shown. aa, amino acids.

only the 5′ regions of these two genes exhibit extensive divergence (66% identity; data not shown).

Although the *TUA1* gene is markedly divergent from the other *TUA* genes, the data shown in Figure 3 suggest that *TUA1* is more closely related to the *TUA3/TUA5* gene pair than to the *TUA2/TUA4/TUA6* subset. For example, there are 17 amino acid residues (marked by arrows in Figure 3) where the α1-tubulin and α3/5-tubulin isoforms both differ from the α2/4-tubulin and α6-tubulin isoforms by exactly the same amino acid substitution. The absence of a lysine residue at position 40 in the isoforms encoded by the *TUA1*, *TUA3*, and *TUA5* genes and the presence of this lysine in the products of *TUA2*, *TUA4*, and *TUA6* are of possible functional significance. This lysine is a substrate for acetylation, which may affect microtubule stability, in animal and algal systems (LeDizet and Piperno, 1987).

Intron Positions Are Not Conserved in the *TUA* Gene Family

The six *TUA* genes contain 1 to 4 introns that define six distinct intron sites in the Arabidopsis *TUA* genes. The locations and sizes of these introns are shown in Figure 4; note that no intron position is conserved in all six genes. The distribution of introns in these six genes provides rather striking supporting evidence for the two *TUA* gene subfamilies described above, with the three members of each subfamily having evolved from a common progenitor. *TUA1*, *TUA3*, and *TUA5* all contain 4 introns at precisely conserved sites, suggesting that they evolved from a common ancestor. Similarly, *TUA2*, *TUA4*, and *TUA6* all contain an intron at a conserved location, between the nucleotide sequences corresponding to codons 31 and 32. Whereas *TUA6* contains only this 1 intron, *TUA4* and *TUA2* have a second intron at a position corresponding to the junction of codons 233 and 234, and *TUA2* contains a third intron that interrupts the nucleotide triplet specifying codon 110; this is the only position where an intron is present in genes of both *TUA* gene subfamilies.

All Six *TUA* Genes Are Expressed

Because the coding sequences of the tubulin genes are highly conserved, 5′ and 3′ noncoding sequences were used to construct gene-specific hybridization probes to study the expression of individual tubulin genes. To determine whether the individual tubulin genes are expressed and to begin to obtain a picture of their expression patterns, we isolated total RNA from whole roots, leaves plus petioles, and whole flowers excised from 21-day-old plants and examined transcript accumulation by means of hybridization of RNA on gel blots to gene-specific probes. Figure 5 gives a summary of the RNA gel blot results obtained using a *TUA* coding sequence probe and each of the *TUA* gene-specific probes. These results show that all six *TUA* genes are transcribed in one or more of the

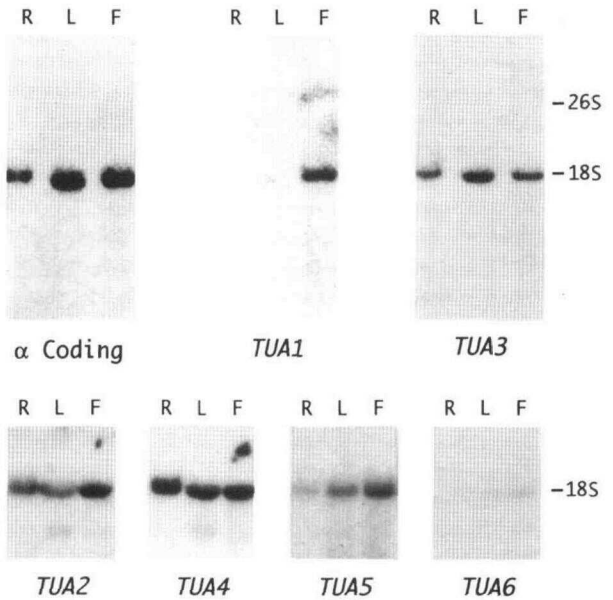

Figure 5. RNA Gel Blot Analysis of *TUA* Gene Transcript Accumulation in Roots, Leaves, and Flowers.

Total RNAs (10-μg samples) isolated from whole roots (R), leaves plus petioles (L), and whole flowers (F) at all developmental stages of 21-day-old plants were analyzed by hybridizations of the RNAs on the gel blots with a *TUA3* coding sequence probe and with noncoding sequence probes specific for each of the six *TUA* genes (see Methods). Two RNA gel blots were used to obtain the data shown. In each case, the RNAs on the gel blots were hybridized sequentially, first with the coding sequence probe and then with the gene-specific probes. The positions of the Arabidopsis 18S and 26S rRNAs are indicated at the right to provide approximate size markers.

organs examined. Based on this very limited analysis of RNAs from different organs, only *TUA1* appears to be expressed differentially in roots, leaves, and flowers (Carpenter et al., 1992).

Given that all six *TUA* genes are transcribed, the next question was whether the transcripts are translated. To examine this question, total proteins were isolated from various excised organs of plants and separated by two-dimensional gel electrophoresis, as described by Hussey et al. (1988). The separated proteins were transferred to nitrocellulose and probed with a monoclonal antibody, B-5-1-2, specific for all α-tubulin isoforms (LeDizet and Piperno, 1987). Figure 6 shows a protein gel immunoblot of the α-tubulin isoforms present in stem tissues. At least four different isoforms are evident on the gel immunoblot of proteins from stems, and the results suggest the possible presence of two additional minor isoforms. Given that the six sequenced *TUA* genes predict only four different isoforms, these results suggest, but do not prove, that all four of the predicted tubulin isoforms are synthesized in vivo. In addition, the two minor spots observed on the protein gel immunoblots (Figure 6) hint that post-translational modifications may contribute to isoform diversity in Arabidopsis.

DISCUSSION

Evidence for Six Expressed *TUA* Genes in Arabidopsis

In this study, we have documented the presence of six expressed *TUA* genes in Arabidopsis. Although the possibility of additional *TUA* genes on genomic DNA fragments in our collection of clones has been excluded, we cannot rule out the possibility of additional genes on other co-migrating restriction fragments. In addition, we cannot exclude the possibility of divergent *TUA* genes that cross-hybridize with the cloned *TUA* genes too weakly to be detected on our genomic DNA gel blots. Nevertheless, our results to date are consistent with the possibility that the Arabidopsis genome contains only six *TUA* genes. If this is correct, we have achieved our goal of characterizing the entire α-tubulin gene family of a higher plant. In the absence of evidence to the contrary, this provides a reasonable working hypothesis on which to base future investigations of the genetic control of the many microtubule-dependent processes essential to growth and development in higher plants.

The results of RNA gel blot hybridizations performed using gene-specific probes demonstrated that all six of the cloned *TUA* genes are transcribed. Moreover, two-dimensional protein gel blots probed with an α-tubulin-specific monoclonal antibody have resolved at least four α-tubulin isoforms. Because the six *TUA* genes predict only four α-tubulin isoforms, the protein immunoblot results are consistent with the possibility that all six *TUA* gene products are synthesized in vivo.

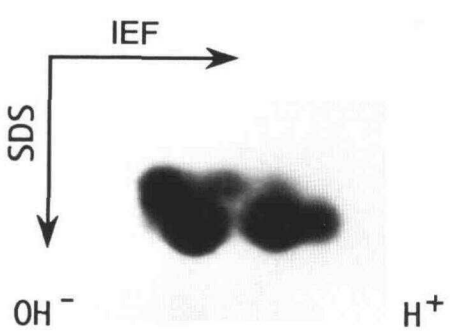

Figure 6. Two-Dimensional Protein Gel Immunoblot Analysis of the α-Tubulins in Stem Tissues of Arabidopsis.

An enlargement of the region of the two-dimensional immunoblot containing the α-tubulins isolated from 21-day-old plants is shown. The anti-α-tubulin monoclonal antibody B-5-1-2 was used. The arrows indicate the directions of SDS-PAGE (SDS) and isoelectric focusing (IEF).

The Six *TUA* Genes Have Evolved from Two Ancestral Genes

Comparisons of the nucleotide sequences of the six *TUA* genes suggest that a number of gene duplication events have occurred during the evolution of this gene family. Two pairs of sister genes (*TUA2*/*TUA4* and *TUA3*/*TUA5*) are present, with the two members of each pair encoding a single α-tubulin isoform. These gene pairs almost certainly resulted from fairly recent (on the evolutionary scale) gene duplications. Additional information about the evolutionary relationships of the Arabidopsis *TUA* genes was obtained by comparing the intron positions in the six *TUA* genes. Whereas all nine of the sequenced β-tubulin (*TUB*) genes of Arabidopsis contain 2 introns at precisely conserved positions (Marks et al., 1987; Oppenheimer et al., 1988; Snustad et al., 1992), the *TUA* genes contain 1 to 4 introns and no intron position is conserved in all six genes. Comparisons of both sequence similarities and intron locations of the *TUA* genes indicate that the six genes have evolved from two ancestral genes, one giving rise to *TUA2*, *TUA4*, and *TUA6*, and the other giving rise to *TUA1*, *TUA3*, and *TUA5*.

Comparisons of the coding sequences of the α-tubulin genes of Arabidopsis and maize (Montoliu et al., 1989, 1990; Villemur et al., 1992) provide evidence for two ancestral α-tubulin genes that predated the monocot–dicot split. The six α-tubulin coding sequences of maize define three gene subfamilies (Villemur et al., 1992), and the Arabidopsis *TUA2*, *TUA4*, and *TUA6* genes are closely related to the genes in maize subfamily I, whereas the Arabidopsis *TUA3* and *TUA5* genes are very similar to the maize subfamily II genes (for details, see Villemur et al., 1992). The intron patterns in these genes reinforce the relationships deduced from sequence comparisons. The three introns in *TUA2* and in the maize subfamily I genes *TUBα1* and *TUBα2* occur at precisely conserved positions in all three genes.

The sequenced plant α-tubulin genes provide little support for the hypothesis of Marchionni and Gilbert (1986) that introns were added to ancestral genes shortly after the emergence of eukaryotes, well before the divergence of plants and animals. The *TUA1*, *TUA3*, and *TUA5* genes of Arabidopsis, the α2-tubulin gene of Drosophila (Theurkauf et al., 1986), and an α-tubulin gene (*altB*) of *Physarum polycephalum* (Monteiro and Cox, 1987) all contain an intron that separates the nucleotide pair triplets specifying amino acids number 176 and 177 in the respective α-tubulins. Thus, this intron may predate the evolutionary divergence of plants and animals as proposed for the conserved introns in the triosephosphate isomerase genes of maize and chicken by Marchionni and Gilbert (1986). The other 5 *TUA* introns occur at positions unique to plant α-tubulin genes.

Biological Significance of the Multiple *TUA* Genes of Arabidopsis

Given that our characterization of the *TUA* and *TUB* gene families of Arabidopsis is now nearly complete, current research is focusing on the use of gene-specific hybridization probes and isoform-specific antibodies to investigate the patterns of expression of individual genes and the functions of individual isoforms. Currently, there is no evidence for functionally distinct tubulin isoforms (Fulton and Simpson, 1976) in plants as there is in animals (Asai and Remolona, 1989; Joshi and Cleveland, 1989; Savage et al., 1989; Hoyle and Raff, 1990; Matthews et al., 1990). However, if functionally divergent tubulin isoforms exist in plants, the structurally divergent α1-tubulin isoform of Arabidopsis, which is preferentially expressed in male reproductive tissues (Ludwig et al., 1988; Carpenter et al., 1992), would seem to be a likely candidate.

Whether functionally divergent tubulin isoforms exist in plants or not, the multiple tubulin genes clearly do provide for differential regulation of tubulin synthesis (Raff, 1984) during plant development. Developmentally regulated patterns of tubulin gene expression have been documented in Arabidopsis (Ludwig et al., 1988; Oppenheimer et al., 1988), soybean (Han et al., 1991), and maize (Montoliu et al., 1989, 1990; Hussey et al., 1990; Joyce et al., 1992). In Carpenter et al. (1992), we document the developmentally regulated expression of the Arabidopsis *TUA1* gene. Thus, the multiple tubulin genes of plants clearly facilitate, at least in part, the regulatory fine tuning needed to meet the differential requirements for α-tubulins and β-tubulins during plant development.

METHODS

Plant Material

All plants used in this study were of the *Arabidopsis thaliana* Columbia ecotype (Rédei, 1970) and were derived from seeds obtained from E. M. Meyerowitz, Division of Biology, California Institute of Technology, Pasadena. Plants were grown at 25°C under 16 hr of illumination and 75% relative humidity on a mixture of sterile soil, peat moss, and sand (approximately 3:2:2 per volume).

Isolation of Genomic Clones

An Arabidopsis Columbia genomic DNA library (Olszewski et al., 1988), constructed using cosmid vector pOCA18, was generously provided by N. E. Olszewski, Department of Plant Biology, University of Minnesota, St. Paul. This library was prepared 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 by Sambrook et al. (1989). Hybridization conditions were as described by Oppenheimer et al. (1988). The hybridization probe was a 1.2-kb HindIII fragment harboring the 3′ two-thirds of the *TUA3* gene (Ludwig et al., 1987) labeled by the random primer procedure of Feinberg and Vogelstein (1983).

The *TUA5* gene isolated from the pOCA18 library contained a single nucleotide pair deletion (relative to a normal α-tubulin coding sequence) that predicted a protein with a C terminus unrelated to α-tubulin. We thus recloned the *TUA5* gene from a library prepared from size-fractionated restriction fragments of genomic DNA isolated from our own Arabidopsis plants. This library was prepared by digesting genomic DNA with EcoRI, separating the restriction fragments by electrophoresis through 0.8% agarose gels, extracting DNA from selected gel

slices, ligating the size-selected fragments into the EcoRI-cleaved, phosphatase-treated pUC119 vector (Vieira and Messing, 1987) DNA, and transforming *Escherichia coli* MV1190 cells by the high-frequency transformation protocol of Hanahan (1983). The library was screened by in situ colony hybridization as described above, except using the homologous *TUA5* coding sequence probe (containing the frameshift mutation) previously isolated from the pOCA18 library. The *TUA5* gene from our plants contained a normal α-tubulin gene without the frameshift mutation.

A genomic clone of the 2.9-kb EcoRI fragment containing most of the *TUA6* gene was isolated from a similar library of size-selected restriction fragments prepared in vector λ ZAPII (Stratagene) and screened by in situ plaque hybridization (Benton and Davis, 1977) using a mixture of *TUA1*, *TUA2*, and *TUA3* coding sequences as probes.

A clone containing the 3′ end of the *TUA6* gene was isolated by using polymerase chain reaction (PCR) to amplify this portion of *TUA6* from size-selected genomic HindIII restriction fragments ligated into the HindIII site of pUC119. Because no sequence 3′ to *TUA6* was available, the ligation products were used for the PCR reaction, and an oligonucleotide corresponding to pUC119 sequence adjacent to the polycloning site was used as the second primer. The ligation products were subjected to PCR amplification using an oligonucleotide (20-mer, TCCGTTTCGATGAACCTGTT) corresponding to a *TUA6* intron sequence divergent from any sequence in the closely related *TUA2* and *TUA4* genes to prime sense strand synthesis and the 24-mer (−47) M13 sequencing primer (New England BioLabs, Beverly, MA) to prime antisense strand synthesis. Thirty cycles of PCR amplification were performed with a Perkin-Elmer Cetus (Norwalk, CT) GeneAmp PCR System 9600; each cycle consisted of denaturation for 30 sec at 94°C, annealing for 30 sec at 60°C, and extension for 2.5 min at 72°C. PCR reactions were performed using AmpliTaq DNA polymerase and a GeneAmp PCR Reagent Kit (Perkin-Elmer Cetus) according to the specifications of the supplier. DNA gel blot analysis of the PCR products showed that DNA sequences of 2.1 kb cross-hybridized with the genomic EcoRI restriction fragment containing the 3′ end of *TUA4*. The 2.1-kb DNA fragments were agarose gel purified, digested with EcoRI, and ligated into EcoRI-cleaved, phosphatase-treated pUC119 vector DNA. Transformation of *E. coli* MV1190 cells with the ligation products yielded clones of the 3′ terminal region of *TUA6*, which were characterized as described below.

Subcloning and DNA Sequencing

All subclonings and template preparations were done using the phagemid vectors pUC118 and pUC119 (Vieira and Messing, 1987) by standard methods (Sambrook et al., 1989). Nested subclones for sequencing were constructed by the T4 DNA polymerase unidirectional deletion method of Dale et al. (1985). Single-stranded template DNAs were prepared as described by McMullen et al. (1986) and were sequenced by the dideoxynucleotide chain termination method of Sanger et al. (1977). DNA sequence data were analyzed with the IntelliGenetics software on a Sun Microsystems (Mountain View, CA) 2/120 computer made available by the University of Minnesota Molecular Biology Computer Facility. The GenBank accession numbers for the *TUA* genes are M21414 (*TUA1*), M84696 (*TUA2*), M17189 (*TUA3*), M84697 (*TUA4*), M84698 (*TUA5*), and M84699 (*TUA6*).

DNA Gel Blot Analysis

Plant DNA was isolated and DNA gel blots were prepared and analyzed as described by Oppenheimer et al. (1988). The DNA gel blot shown in Figure 1 was hybridized with a mixture of four gel-purified restriction fragments carrying the complete coding sequences of *TUA1*, *TUA2*, and *TUA3*. The four probe fragments are as follows: *TUA1*, a 2.7-kb EcoRI fragment extending from 190 bp 5′ of the ATG to about 300 bp 3′ of the TAG (subclone terminus); *TUA2*, a 2.24-kb EcoRI fragment extending from 243 bp 5′ of the ATG to 7 bp 3′ of the TAA (subclone terminus); and *TUA3*, two fragments, one a 726-bp EcoRI-HindIII fragment spanning from 51 bp 5′ of the ATG (subclone terminus) to the internal HindIII site, and the second a 1235-bp HindIII fragment from the internal HindIII site to the HindIII site 177 bp 3′ of the TGA. The probe fragments were ^{32}P-labeled by the random-primer protocol of Feinberg and Vogelstein (1983). Hybridizations performed to detect all *TUA* sequences were carried out in a solution containing 50% formamide at 37°C, whereas hybridizations done to test the specificity of noncoding sequence probes were carried out in a 50% formamide solution at 42°C.

RNA Gel Blot Analysis

Total RNA was prepared from Arabidopsis leaves plus petioles, whole roots, and whole flowers containing the entire range of developmental stages by the method of Berry et al. (1985). RNA samples (10 μg) were fractionated on 1.2% agarose-formaldehyde gels, as described by Ausubel et al. (1987). RNA gel blots were prepared, probed, and stripped of radioactive probes, as described by Oppenheimer et al. (1988).

All probes except the *TUA6* 3′ gene-specific probe were templates in pUC119 and were labeled by primer extension (Hu and Messing, 1982). The *TUA6* gene-specific probe was synthesized by PCR, agarose gel purified, and labeled by the random-primer protocol of Feinberg and Vogelstein (1983). The α-tubulin coding sequence probe was a 1.2-kb HindIII fragment containing most of the *TUA3* gene (Ludwig et al., 1987). All gene-specific probes except the *TUA6* probe were subclones containing almost exclusively the noncoding sequences and are as follows: *TUA1*, a 150-bp fragment from 70 bp 3′ to 219 bp 3′ of the TAG codon; *TUA2*, a 204-bp fragment from 2 bp 5′ to 199 bp 3′ of the TAA codon; *TUA3*, a 108-bp fragment from 74 bp 3′ to 181 bp 3′ of the TGA codon; *TUA4*, a 215-bp fragment from 20 bp 5′ to 192 bp 3′ of the TAA codon; and *TUA5*, a 454-bp fragment from 460 bp 5′ to 7 bp 5′ of the ATG codon. The *TUA6* gene-specific probe was a 162-bp fragment extending from 18 bp to 179 bp 3′ of the TAA codon synthesized by PCR. The specificity of each probe was tested by DNA gel blot analysis. If a probe hybridized with a single restriction fragment in each of three enzyme (EcoRI, BamHI, and HindIII) digests of genomic DNA as determined by the presence of a single dark band in each lane on the resulting autoradiograph, the probe was considered to be gene specific.

Two-Dimensional Protein Gel Immunoblot Analysis

Proteins were extracted, separated by two-dimensional polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and reacted with the anti-α-tubulin monoclonal antibody B-5-1-2 (LeDizet and Piperno, 1987) as described by Hussey et al. (1988).

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