A tandem of α-tubulin genes preferentially expressed in radicular tissues from *Zea mays*

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Abstract

The identification of a cDNA (MR19) corresponding to a maize α-tubulin and homologous genomic clones (MG19/6 and MG19/14) is described. The cDNA has been isolated by differential screening of a cDNA maize root library. We have found two α-tubulin genes in a tandem arrangement in the genomic clones, separated by approximately 1.5 kbp. One of the genes (gene I) contains an identical nucleotide sequence which corresponds to the cDNA clone. The two deduced proteins from DNA sequences are very similar (only two conservative replacements in 451 amino acids) and they share a high homology as compared with the published α-tubulin sequences from other systems and in particular with the *Arabidopsis thaliana* and *Chlamydomonas reinhardtii* sequences reported. The structure of both genes is also very similar; it includes two introns, of 1.7 kbp and 0.8 kbp respectively, in each gene and only one intron placed at a homologous position in relation to *Arabidopsis thaliana* genes. By using specific 3′ probes it appears that both genes are preferentially expressed in the radicular system of the plant. The α-tubulin gene family of *Zea mays* seems to be represented by at least 3 or 4 members.

Introduction

Microtubules are the basic components of the cytoskeleton. They play an essential role in eukaryotic cells in many processes such as cell division, internal transport, motility and morphogenesis. As compared with animal systems plant cells present particular features that both restrict and give additional functions to microtubules. In fact, plant microtubules have specialized roles in mitosis controlling the plane of cell division and consequently the direction of cell elongation. In this sense microtubules are involved in determin-

ing plant cell morphogenesis [16]. A particular set of plant microtubules, the cortical array, has been proposed to take part in the orientation of cellulose fibrils in the wall [27].

The major component of microtubules is tubulin, a heterodimer protein formed by two different subunits, alpha and beta, of approximately 50 kDa each. Both subunits share 40% of homology. In addition, α- and β-tubulins have been extremely conserved through evolution. The majority of published sequences can be placed below 15% of divergence [26]. Tubulins (α and β) are coded in vertebrates by complex multigene

families. In these systems, members of the family are organized in a dispersed way. In man more than 20 α-tubulin genes have been described and approximately 15 genes have been characterized in mouse or rat [7]. Most of these are supposed to be pseudogenes [25].

In addition, several α-tubulin sequences have been published in lower eukaryotic systems. More than 15 α-tubulin genes have been described in *Trypanosoma* forming clusters, organized into tandem repeats of alternating α and β gene units 3.7 kbp long [24].

α-tubulin genes have also been characterized from various photosynthetic organisms such as *Chlamydomonas reinhardtii* [41] and *Volvox carterii* [30]. These two species present two unlinked genes in each case. However, in comparison with the abundant information available for genes coding for α-tubulin in animal systems, very few data exist from the plant kingdom [42]. Tubulins have been purified from plant cells by using their property of *in vitro* assembly into microtubules [32]. Protein data have indicated the existence of multiple isotypes in tissues of the higher plant *Phaseolus vulgaris* [21], and monocotyledon endosperm cells [35]. Only the α-tubulin gene family from *Arabidopsis thaliana* has been extensively characterized so far in higher plants [28]. In this species four genes coding for this protein have been detected organized in a dispersed form in the genome. A possible cluster of alternating α/β tubulin genes has been proposed from mung bean [38], but no sequence data from cloning studies are yet available.

The existence of different tubulin genes in a given organism may correspond to the need of a differential expression in defined tissues or developmental stages. Data from the literature indicate that in some cases different genes are expressed in distinct tissues or developmental stages. In rat the presence of a specific cerebellum isoform of α-tubulin has been described [14] while in mouse two specific genes are expressed in testis [49] and an unusually highly divergent testicular isoform of α-tubulin, not found in brain, has been described [20]. Constitutive and tissue-specific isoforms of α-tubulin have been observed in *Drosophila*

melanogaster [48]. In contrast with the hypothesis of different α-tubulin genes present in functionally different microtubules, data from two yeast species studied, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, show only two genes for α-tubulin in their genome, only one of which seems to be essentially required whereas the other one is dispensable [43, 1].

As has been shown in animal organisms, plant tubulin genes may be interesting markers to study developmental processes occurring in plants. More complete information available for a plant system comes from *Arabidopsis thaliana*, where one of the genes seems to be specifically expressed in flowers as compared with leaves and roots [29]. Recent reports based on protein data indicate the existence of specific patterns of tubulin polypeptides in different organs of carrot [22]. A developmental control of the expression of tubulin proteins during somatic embryogenesis in cultured carrot cells has been described [9].

In the present paper we show that α-tubulin genes are cloned in *Zea mays* by a family of genes that includes a subgroup of two genes forming a tandem array. Both genes have a preferential expression in the radicular system. The structure of the proteins is compared with other known systems showing a high level of homology. The novel organization of α-tubulin genes in this plant species is discussed, as well as in different varieties of the maize group.

Materials and methods

Plant material

All our studies were done with *Zea mays* L. (inbred line W64A) grown under greenhouse conditions. Plantlets were obtained by germinating dry seeds through imbibition in water at 25 °C in the dark for the indicated period of time.

General methods

DNA ligation, bacterial transformation (*Escherichia coli* DH5αF' strain) and cloning procedures

followed published methods [17]. Harvesting and preparation of lambda-phage DNA were carried out according to described protocols [10] as well as preparation of plasmid DNA, restriction enzyme analysis, agarose gel electrophoresis of nucleic acids and blotting [31]. Vectors used in subcloning and sequencing procedures were pUC18 and M13mp18/19 [50].

Enzymes were purchased from Boehringer (Mannheim) unless stated otherwise, labelled nucleotides were from Amersham.

RNA isolation and poly(A)$^+$ RNA selection

20–40 g of plant material were frozen in liquid nitrogen and ground to a fine powder in a mortar. For the construction of cDNA libraries the guanidinium isothiocyanate method was used [31]. For preparing single–strand cDNA probes and Northern blot analysis the RNA was extracted according to described methods [11, 45]. Poly(A)$^+$ RNA was selected on oligo(dT)-cellulose chromatography.

cDNA library

A λgt10 cDNA library was prepared from poly(A)$^+$ RNA of *Zea mays* roots two months old, essentially according to described protocols [15], with modifications.

1) For first-strand cDNA synthesis 6 μg of poly(A)$^+$ RNA was denatured by incubation at 70 °C for 5 min and the reaction carried out in a volume of 55 μL containing 50 mM Tris-HCl pH 8.3 (at 42 °C), 10 mM $MgCl_2$, 10 mM DTT, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM dTTP, 120 mM KCl, 25 u of RNasin (Stehelin), 5.5 μg of oligo-d(T)$^{12-18}$ and 20 u/μg RNA of Super Reverse Transcriptase (Stehelin). The mix was incubated at 42 °C for 1 hour.

2) For second-strand cDNA synthesis up to 1 μg of single-stranded cDNA (*i.e.* 2 μg of hybrid) was processed in 200 μl of 20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$; 10 mM $(NH_4)_2SO_4$, 100 mM KCl 150 μM NAD, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 200 μM dTTP, 50 μg/ml BSA, 2.7 u RNase-H (Stehelin), 50 u

DNA Polymerase I (Stehelin), and 5 u *E. coli* DNA ligase (Biolabs). Incubations were sequentially 2 h at 15 °C and 1 h at 22 °C.

3) The cDNA was methylated with *Eco* RI methylase (Biolabs), the ends repaired with T4-DNA-Polymerase I (Klenow, Biolabs), and the cDNA ligated to synthetic *Eco* RI linkers (10-mer, Biolabs). The cDNA attached to the linkers was cleaved with *Eco* RI and fractionated by agarose gel electrophoresis according to length. cDNAs between 500 bp and 3.5 kbp were inserted into the unique *Eco* RI site in the cI gene of the λgt10 [23]. The DNA was packeged *in vitro* [31] and plated onto C600 Hfl *E. coli* K 12 cells. 2 μg of poly(A)$^+$ RNA yielded 1.2 $\times$ 10^6 independent clones of λcI recombinants.

Differential screening of the cDNA library

Single-strand cDNA probes were obtained with poly(A)$^+$ RNA from 2-month-old roots and leaves of maize by a modification of the method described previously [46]. The reactions were carried out in a volume of 25 μl containing 1 μg of poly(A)$^+$ RNA of each organ separately; 1.4 μg of oligo d(T)$^{12-18}$ (both prewarmed 3 min at 65 °C and cooled on ice); 50 mM Tris-HCl pH 8.3 (42 °C), 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dTTP, 0.03 mM dGTP, 50 μCi ^{32}P-dGTP (3000 Ci/mmol, Amersham), 30 u RNasin (Genofit) and 10 u of Reverse Transcriptase (Genofit). The mixes were incubated at 42 °C for 45 min and unincorporated ^{32}P-dGTP separated by chromatography on a 0.7 cm $\times$ 10 cm Sephadex G-50 column. Three plaque filter replicas of 5 plates of the cDNA library (3000 pfu/plate) were prepared. Replicas were obtained by transferring each one during 2, 4 and 8 min onto nitrocellulose paper. The first and third filters were hybridized with single- strand cDNA probes from roots, and the second one with corresponding probes from leaves at a specific activity of ca. 1.5 $\times$ 10^7 cpm/μg. Hybridization was performed at Tm-25 with 0.6 – 1 $\times$ 10^6 cpm/ml solution for 24–36 hours, in the presence of 50% formamide, and final washes

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(30 min each) were done in 0.2 × SSC, 0.1% SDS at 65 °C.

Northern blot analysis

10 μg of total RNA and 0.5 μg of poly(A)$^+$ RNA were fractioned in 1.5% agarose-formaldehyde gels [3], transferred to either nitrocellulose (BA85, Schleicher & Schuell, 0.45 μm) or nylon membranes (Hybond-N, Amersham) and hybridized according to protocols suggested by the suppliers. Probes were labelled by nick-translation [39] or random-primed (Boehringer–Mannheim kit) reactions [12] to a specific activity up to 10^9 cpm/μg.

Southern blot analysis

Isolation of DNA was performed following procedures described previously [4] with variations. 20 g of plant material were frozen in liquid nitrogen and reduced to powder in a mortar. 4 ml/g of extraction buffer (0.35 M NaCl, 10 mM Tris-HCl pH 8, 0.1 M EDTA, 1% Sarcosyl, 7 M urea and 5% phenol) was added. The mixture was incubated for 2 h at 37 °C under gentle shaking. After phenol/chloroform extraction, DNA was ethanol-precipitated and purified through a CsCl gradient.

10 μg of genomic DNA digested with restriction enzymes were fractioned in 0.8% agarose gels and blotted onto nylon membranes (ZetaProbe, BioRad). Hybridizations were done in: 1.5 × SSPE (1 × SSPE is 180 mM NaCl, 10 mM NaH_2PO_4 pH 7.7, 1 mM EDTA), 1% SDS, 0.5% powered skimmed milk (Molico, Nestlé) and 0.5 mg/ml of denatured salmon sperm DNA at 65 °C for 16–20 h. Washes were performed also at 65 °C up to 0.1 × SSC, 1% SDS.

DNA sequence analysis

Reactions were carried out using the M13-dideoxy nucleotide method according to de-scribed protocols [40] with modifications in order to use ^{35}S-labeled nucleotides [2]. Klenow DNA polymerase (New England Biolabs) and the T7-Sequencing Kit (Pharmacia) were used. The products obtained were separated in 6% acrylamide 8 M urea 02–0.45 mm wedge gels. Sequence alignment and analysis was done by using software from the CITI2 database (Paris) and Micro-Genie-(Beckman) [37].

Screening of the genomic library

The screening of a λCh35 genomic library of maize [13] was carried out with cDNA clones labelled by random priming (Boehringer–Mannheim kit). Hybridization conditions of nitrocellulose replicas were: 10^6 cpm/ml, 5 × SSC, 5 × Denhardt's solution, 50% formamide, 5 mM EDTA, 25 mM sodium phosphate, 0.1% SDS and 250 μg/ml of denatured salmon sperm DNA, at 42 °C for 24 h. The final two washes were done in 0.2 × SSC, 0.1% SDS at 65 °C for 15 min each.

Results

cDNA cloning and sequencing

A group of cDNA clones corresponding to maize α-tubulin was found in the course of a differential screening of a maize root cDNA library using labelled single-stranded cDNA probes synthesized from root and leaf poly(A)$^+$ RNA. The library was contructed in λgt10 and 15 000 recombinant clones were screened. From those giving a preferential signal with root RNA, a number were selected to confirm their predominant expression in the radicular tissues by means of a northern analysis. One of these clones (MR19), giving a 7-fold increase in root DNA as compared to leaf (see Fig. 1A), was one of those chosen for further analysis. The estimated size of the mRNA was approximately 1750 nucleotides.

When northern analysis was carried out with RNA extracted from different parts of the maize plant the preferential expression of MR19 in the
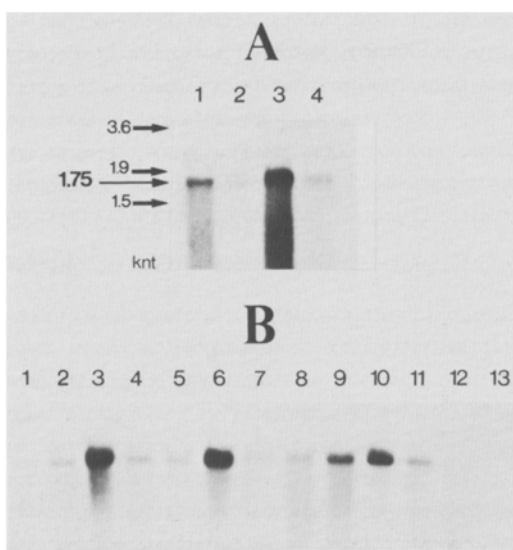
A

3.6→

1.75 —— 1.9→
1.5→

knt

B

1 2 3 4 5 6 7 8 9 10 11 12 13

Fig. 1. A. Northern blot analysis of maize adult root and leaf RNA. Total RNAs (10 μg) isolated from adult (60 days after germination) roots (1) and leaves (2) and corresponding poly (A)+ RNAs (0.5 μg) fraction from roots (3) and leaves (4) were probed with the insert of MR19 clone (cDNA). The mobilities of 25S, 18S and 16S ribosomal RNAs are indicated. The estimated length of the transcript (in kilonucleotides) is shown. B. Northern blot analysis of MR19 α-tubulin in maize in different organs and several stages of development. The following RNAs were probed with the insert of MR19 (cDNA) clone. Polysomal RNAs, membrane–bound (2 μg) purified from endosperm 20 days after pollination (1); total RNAs (10 μg) extracted from embryo 20 days after pollination (2); young roots two (3), four (4) and seven (5) days old; root tip from seven-day-old young roots (6); adult root (7); coleoptiles two (8), four (9) and seven (10) days old; upper part of coleoptile (including node) (11) and seven-day-old shoots (12) and adult leaves (13).

radicular tissues was confirmed. The northern analysis of RNA extracted from endosperm and embryo 20 days after pollination, adult (two months old) roots and leaves, and different parts of different ages of the young roots and coleoptiles is presented in Fig. 1B. It appears that, while RNA hybridizing with this cDNA is poorly represented in leaves and shoots, the highest amount of mRNA is found in the meristematic part of the young roots, but it is also found in adult roots. In general, it appears that the mRNA is especially abundant in zones of the young plant rich in meristematic regions, such as young roots and coleoptiles, with a special increase in the radicular

tissues. Although there is a good hybridization signal in coleoptiles, a clear decrease of hybridization in adult leaves is observed as compared with adult roots. Expression is also detected at a lower level in embryo and endosperm.

Sequencing of the insert was carried out with the dideoxy method. The nucleotide sequence and the protein sequence deduced from it are shown in Fig. 2 (between asterisks at positions 6530 and 8210, excluding introns), together with the MG19/6 and MG19/14 genomic clones (described below) in an overlapping form. Searching in the EMBL databank revealed a strong homology with *Chlamydomonas renhardtii* α-tubulin [41], and subsequently with all the other published sequences coding for α-tubulins. As could be expected from the size of the cDNA insert (1483 bp), as compared with the estimated mRNA length, the protein sequence deduced lacks the initial part of the N-terminus of maize α-tubulin. The non-translated 3′ end of the mRNA is 280 nucleotides long and has two possible polyadenylation signals at 27 and 104 nucleotides before the beginning of the poly(A) tail (at position 8210) as shown in Fig. 2. Both sequences (AATAAT and AGTAAA) depart from a single nucleotide in the consensus sequence (AATAAA).

Genomic cloning and sequencing

The cDNA insert of the MR19 clone was used to screen a genomic library contructed in λCh35 by partial digestion with Mbo I of endosperm DNA from the maize inbred line W64A [13]. Eleven clones were found positive, and two having a strong hybridization signal (MG19/6 and MG19/14) were used for further analysis. Clones MG19/6 and MG19/14 were found to overlap, MG19/14 being longer at its 3′ end and therefore containing the whole cDNA sequence (see Fig. 3). The sequence of the overlapping fragments showed that they corresponded to identical genomic sequences.

From the restriction analysis and partial sequence (8.5 kbp) of the insert of the genomic

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   1 gtcgacgccccggtacgaagtgggtgggggcaggcgagaggggtgcaccatgggccacccagtgcgtggtccggttttgatccatgtaactctatataaatctctatttaattcggtat

 121 aaaatagttaagatgaaagagaggaataaaaatttagtagatttgacagtcatataaaatttctagatcgacccctgtggtgggtgcgtcgaaatttctagaccgccctaggccggggatga

 241 cacatggaaccgtgtatgcacaaagctgctgcattataattgtagagattaattatgttatttaggaaataaaagtttaggaaatagtatataaaaacaaggattgagctccagatatataa

 361 taggccgagtcctgtaattttgtgacatttttttgaaaccaggattgagctgcagtttttagtgttagagtccagcgtctcacaggagtggtccaattcaaattcgaaaatgtatcacg

 481 ctgaagcgaaaaatatcataaattcataacgaaccaaccgaccgttgcacggacgagaactcgacgagaccgaaccgtgaaaacaaccgaaagcacaggggctcaaacgaaacaatcccg

 601 cccacacttccttcgccggctcatggtggccactggccaggctgtcatcctgttctataaagcgagccgagggggaagagccggaaccctagcccagcaccgcagaggcgcgagagacaggc

 721 gtcttcgtactcgcctatctccgcgactcaaagcttcttccatttcctaccgccgccgctgcagctccacccattccgtcgacaccATGAGGGAGTGCATCTCGATCCACATCGGGCAA
   1                                                                                          M  R  E  C  I  S  I  H  I  G  Q

 841 GCCGGTATCCAGGTCGGAAACGCGTGCTGGGAGCTCTACTGCCTCGAGCATGGCATTCAGgtaacgagctgtccaattcctcttccattctcgcactgttaggttagatgtgtaatcggt
  12 A  G  I  Q  V  G  N  A  C  W  E  L  Y  C  L  E  H  G  I  Q

 961 atgggacttccgtcgctgatgcgtgctgtattcggtgggactaaatcgggggaggaaatggcattcaaatcggtagatctgtaggggatctggctgtatgtgatggtggctgcccagatc

1081 cgcgtttgtcaggtctgatccgtatatatggtgagatggtgtctgatcgaggttcggttgtttttatctggattttggatagaaaactcgaatggcgattagccgaaattgcgcattat

1201 tttccatgatccaatgaatttagtagttatgtatcatatttgttgttaatttatgcctatacgtgtgttaggtgtgagtgccctgaaatcgtttaaatgcacaggcattcacgatctgta

1321 aattttcataggccaagttcctattgcatgcctataccttcgattcgatgtacggttacagagttagtatacttaatgttaattgatgtacaatacatctattcgtactgttagagcatc

1441 tccaacaaggagctctaaaatagagccctaaaagttaaaataggaccgtatttaaagtgtgttaggattactccaacagttaggccttaaataattctatttaggaaataaatagttgt

1561 agactaagaggaaaggaggatagggctccaacggtgtactatatattaggacccgagagaccgagcccctataatctttgatgattttttataaaataggggctactgttgggagagcgtttt

1681 ataatcccacaccctatatttcaaaatacagcccttgtttagactatctccagcgtgtagtgttaaataggagacgcggaactgtagactgtttgacactgtttgcatagtgaaatttgaa

1801 ttttgaaatagtggatgtgataggagatgaattggggatctgctggagatagcctagggccccttttggagatgctcttagatctgcaaaaaatatgaatacgcatcagcattagacg

1921 ccttgacccaattgcctttgctgtttagatctgttagaaagattatatatacaggaatgctgttatactaaattgcttttgtcctagttgttattagctttatgtaggactaaatgctg

2041 ttcacatatccgaagtgtttgcaatcatggatttaatcatggctccttaggacaattgaaatttcgaattgtgtttaggggtggtaatggactctagattttcacactaaaatttaaggat

2161 cgggctctaatcctattcattttgaattaaaaattatttaagggccctaactttttatgaagaagcgtttggatcgtgatccattaccacccctaattgtgtgtcatggtttgaaatatg

2281 tccattcaggcatgtgttacctaagattatgcgtctgtctggtgtactagtgtaggtggcatgcaatttttttttacacagactgcttatgttctcctttgtaagggcctgtttggaag

2401 ctcccagttttaagaaactggtttatagaattagaggtggttccaaacataccagtttatgtctcagtttatagaaattgattgtcagttccttaaaaaccaataagctatcaagctag

2521 tctctcctagctaaaagataaaaaccagtttcttaaaaactggattgcttccaaacaggacctaattggcataaataacactgctacatttgtattttgttcagGCCGATGGTCAGATGC
  32                                                                                                          A  D  G  Q  M

2641 CCGGTGACAAGACCATTGGGGGTGGTGATGATGCTTTCAACACCTTCTTCAGTGAGACTGGTGCTGGGAAGCACGTCCCTCGTGCTGTTTTTGTTGACCTTGAACCAACTGTCATCGATG
  37 P  G  D  K  T  I  G  G  G  D  D  A  F  N  T  F  F  S  E  T  G  A  G  K  H  V  P  R  A  V  F  V  D  L  E  P  T  V  I  D

2761 AGGTGAGGACTGGCACCTACCGGCAGCTCTTCCACCCTGAGCAGCTTATCAGTGGCAAGGAGGATGCAGCCAACAACTTCGCCCGTGGTCACTACACCAgtaagtaacctgatctgactg
  77 E  V  R  T  G  T  Y  R  Q  L  F  H  P  E  Q  L  I  S  G  K  E  D  A  A  N  N  F  A  R  G  H  Y  T

2881 tcagtttacaatgctcctggatatttgctttgtaatgaacatcttatttaaacttgattgcttgtcttgcagTTGGCAAGGAGATTGTTGACCTGTGCCTTGATCGCATCAGGAAGCTTG
 110                                                                        I  G  K  E  I  V  D  L  C  L  D  R  I  R  K  L

3001 CCGACAACTGCACTGGTCTCCAGGGCTTCCTCGTCTTCAACGCTGTTGGCGGAGGAACCAGGCTCTGGCCTTGGTTCCCTCCTCCTGGACGTCTGTCTGTTGACTACGGCAAGAAGTCCA
 126 A  D  N  C  T  G  L  Q  G  F  L  V  F  N  A  V  G  G  G  T  G  S  G  L  G  S  L  L  L  E  R  L  S  V  D  Y  G  K  K  S

3121 AGCTTGGTTTCACTGTGTACCCATCCCCCCAAGTTTCGACCTCAGTGGTTGAGCCATCAACAGTGTCCTGTGCCACCCACTCCCTCCTCGAGCACACTGATGTGGCTATCCTGCTGGACA
 166 K  L  G  F  T  V  Y  P  S  P  Q  V  S  T  S  V  V  E  P  Y  N  S  V  L  S  T  H  S  L  L  E  H  T  D  V  A  I  L  L  D

3241 ACGAGGCCATCTATGACATCTGCCGGCGGTTCCCTTGACATTGAGCGGCCCAACCTACACCAACCTCAACAGGCTTGTGTCCCAGgtactgtcttggtttacagcctaagcattaagtaaat
 206 N  E  A  I  Y  D  I  C  R  R  S  L  D  I  E  R  P  T  Y  T  N  L  N  R  L  V  S  Q

3361 ggtctgtaatctgggcttttctctttaaccatcagttgttgctgtgtcgttgtacagGTCATCTCATCCCTGACTGCCTCCCTGAGGTTCGATGGTGCTCTTAACGTGGATGTGAATGAGTT
 234                                                          V  I  S  S  L  T  A  S  L  R  F  D  G  A  L  N  V  D  V  N  E  F

3481 CCAGACCAACTTGGTGCCCTACCCGGAGGATCCCACTTCATGCTTTCCTGCTATGCTCCAGTCATCTCTGCTGAGAAGGCCTACCACGAGCAGCTGTCTGTGGGCGAGATCACCAACAGCGC
 256 Q  T  N  L  V  P  Y  P  R  I  H  F  M  L  S  S  Y  A  P  V  I  S  A  E  K  A  Y  H  E  Q  L  S  V  A  E  I  T  N  S  A

3601 CTTCGAGCCCATGCTCCATGATGGGCCAAGTGCCGATCCCCGCCATGGTAAGTACATGGCCTGCCTCATGTACCGTGGTGATGTGGTCCCCAAGGATGTGAATGCTGCTGTGGCCACCAT
 296 F  E  P  S  S  M  M  A  K  C  D  P  R  H  G  K  Y  M  A  C  C  L  M  Y  R  G  D  V  V  P  K  D  V  N  A  A  V  A  T  I

3721 CAAGACCAAGCGCACCATCCAGTTTGTGGACTGGTGCCCCAACTGGCTTTAAGTGCCGGCATTAACTACCAGCCTCCAAGTGTGGTGCCTGGTGGTGACCTGGCCAAGGTGCAGCGCGCTGT
 336 K  T  K  R  T  I  Q  F  V  D  W  C  P  T  G  F  K  C  G  I  N  Y  Q  P  P  S  V  V  P  G  G  D  L  A  K  V  Q  R  A  V

3841 GTGCATGATCTCCAACTCCACCAGTGTTGTGGAGGTGTTCTCCCGCATCGACCACAAGTTCGACCTCATGTACGCCAAGCGTGCCTTCGTGCACTGGTATGTCGGTGAGGGTATGGAGGA
 376 C  M  I  S  N  S  T  S  V  V  E  V  F  S  R  I  D  H  K  F  D  L  M  Y  A  K  R  A  F  V  H  W  Y  V  G  E  G  M  E  E

3961 GGGCGAGTTCTCTGAGGCCCGTGAGGATCTGGCAGCGCTCGAGAAGGACTACGAGGAGGTTGGTGCTGAGTTTGATGAGGGTGAGGAAGGTGATGATGGTGATGAGTACtagaagtatcc
 416 G  E  F  S  E  A  R  E  D  L  A  A  L  E  K  D  Y  E  E  V  G  A  E  F  D  E  G  E  E  G  D  D  G  D  E  Y

4081 tgatgcggtcatcgtcaggcttgtgtgctgctcttgtccccgttgtggtttgcaacacctgatgttgtaagactttctggttatgtccgccccgctgtgccactggttattaagaacgt

4201 cgttatggatggttgtctacactacattattgcttctcgatattggaaaactgttatgcgcctcggtggattgtgttgttgtcgtaatgtcatcactcatacgccgctgggaattttgag

4321 gcctgtcaagcatcaggattgcgttatgagttaaatgcttcagcgacgtttaaacttgtctaaaggtgccatctagatcatgaacttgtcaagggttgccacttagatcatgaacttcgta

4441 aatatgtttttggatccaaaatatgtttttttatccttaagggtgtgtttgtgtgtttggttgaatgtataagaaggggatgaaagagggaatgtcataatttctatagtgtttggttgagag

4561 acaagtgaggacgagataaaataccctaagaagggatgaaagagggaatgccacaattctatagtgtttggttcagagacaagtgacaattctatagtgtttggttgagagacaagtgagg

4681 gcgagtaaataccgcaataattttttggtggcaccaaattttgtgaagttgtatacatttggacaccaatagaaaatagaattaaaaaaatataaaaactggtgtcatttaaatcagtg

4801 tcacgttattaaaatttaaaactatcaactaaaattgtctaatggattatttatgtggtttttgtaaagttgtgggagattaaacaaccagtttgaagataagtaagtgaaatagtcaaat
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```
4921  agaccgtactaaaggttaagaatttaggtacacttacgactagtttagatgccgcaaaatgggttaaattttcttcttattcaaaattaaataataaggtgaatttaactactctaatt
5041  tcctctgttttttaactcccaaactatcccttattcgtaataataggaagcggtgacagtttggtggtgagaactcaggtatcaacaaaaagaaatgtattttgaaatattttgctcg
5161  taatgccctgcaaggtttcgatttccgtagccagtacatgtccgctcttgacccaggtactgtgacacgaaccaaccgaccgttgaacggacgtggagcacgaaccattaaaacaatcaa
5281  aatctcaggggctcaaacgaaaaaacaccgcccccttccctcgcttggctggcactccatcgtgggctcgtggccaggctgtcgttctgttctataaagcgagacgagtgggagcaggcg
5401  taaccctaattgagcatcgcagagataggcgtcttcgtactcgcctacctccgcggctcaaacctttcccccttctcccaattccttccgccggccgccgctccacccgtacgacgacac
5521  cATGAGGGAGTGCATTTCGATCCACATCGGCCAGGCCGGTATCCAGGTCGGAAACGCGTGCTGGGAGCTGTACTGCCTTGAGCATGGCATTCAGgtaacgatccgtcattccctcccccg
      1 M  R  E  C  I  S  I  H  I  G  Q  A  G  I  Q  V  G  N  A  C  W  E  L  Y  C  L  E  H  G  I  Q
5641  ttctccctctgttgagggtagatctataatcggtatgggggtttcatcgctaatatttgctggattttttggtggggctgaatccgccggaggaaatgacgtttcaaatcggtagatctgta
5761  cggatctggatgcatgtaatggtggctaccggatccgggtttgctaggtctgatccggagaactggtgtctgagcgagattgcaattgttttgaactggaatttgatacgtagaattt
5881  gaatagggatcacccgaaacccacgtgcgtggtagatctgagtgcccagaattcgtttaaatgtactgtcagtttggatctttgaattttcataggctaagttccgtattgcctgttgat
6001  atgtgagccttgatgtacaaatacagactatagtatgtttagtgttaatttgatgtgagccttgatgaatttcacaggctaagttccatgcccacaatgcatccattcatactgttatt
6121  aattctgtaaaaaaaacgaatacacgtcactcttaggcaccttaacccaatttgcattttctgtttaaatctgttagaaatgggatagagactggcatgttgttatgtagtcctagctg
6241  ttattagctttatctaggactaaatgctgttcacatatttcaagtgtttgcaatcggagattcaatcatggctccctaggacatttgaatcgtgtgtcatagtttgaaatatgagattca
6361  ggcctgtgtttaccttaagatcatgcatctgtctggtctactatgtagatggcatgtattttacatagactccttaagtcgcatattaacactactacattgtgtttgttcagGCTGACG
      32                                                                                                               A  D
6481  GTCAGATGCCCGGTGACAAGACCATTGGGGGAGGTGATGATGCTTTCAACACCTTCTTCAGTGAGACTGGTGCTGGGAAGCACGTGCCCCCGTGCTGTTTTTGTTGACCTTGAACCCACTG
      34 G  Q  M  P  G  D  K  T  I  G  G  D  D  A  F  N  T  F  F  S  E  T  G  A  G  K  H  V  P  R  A  V  F  V  D  L  E  P  T
6601  TCATCGATGAGGTGAGAACTGGCACCTACCGCCAGCTCTTCCACCCTGAGCAGCTCATCAGTGGGAAGGAGGATGCAGCCAACAACTTTGCCCGTGGTCACTACACCCAgtaagtaatctg
      74 V  I  D  E  V  R  T  G  T  Y  R  Q  L  F  H  P  E  Q  L  I  S  G  K  E  D  A  A  N  N  F  A  R  G  H  Y  T
6721  atctgagcgtcagttttcactgctccttgatatttgctctgtaatgaacatctcatttaaacctgattccttgttttgcagTTGGCAAGGAGATTGTTGACCTGTGCCTTGATCGCATCA
      110                                                                                I  G  K  E  I  V  D  L  C  L  D  R  I
6841  GGAAGCTTGCCGACAACTGCACTGGTCTCCAGGGCTTCCTCGTCTTCAACGCTGTTGGTGGAGGAACGGGCTCTGGCCTTGGTTCCCTCCTCCTAGAGCGCCTGTCTGTTGACTACGGCA
      123 R  K  L  A  D  N  C  T  G  L  Q  G  F  L  V  F  N  A  V  G  G  G  T  G  S  G  L  G  S  L  L  L  E  R  L  S  V  D  Y  G
6961  AGAAATCCAAGCTTGGGTTCACTGTGTACCCTTCCCCCCAAGTTTCTACTTCGGTAGTTGAGCCATACAACAGTGTACTGTCCACCCACTCCCTCCTCGAGCACACTGATGTGGCTATAC
      163 K  K  S  K  L  G  F  T  V  Y  P  S  P  Q  V  S  T  S  V  V  E  P  Y  N  S  V  L  S  T  H  S  L  L  E  H  T  D  V  A  I
7081  TGCTGGACAATGAGGCCATCTACGACATCTGCCGCCGATCCCTTGACATTGAGCGCCCAACCTACACCAACCTCAACAGGCTTGTGTCCCAGgtactgacttactgtcttggtctactgc
      203 L  L  D  N  E  A  I  Y  D  I  C  R  R  S  L  D  I  E  R  P  T  Y  T  N  L  N  R  L  V  S  Q
7201  tttcgcattttgcctggtaaactggttcaaatactggctgttgtctgtaacaactacctgttactgatgttgtacagGTCATCTCATCCCTTACTGCCTCCCTGAGGTTCGATGGTGCTC
      234                                                                            V  I  S  S  L  T  A  S  L  R  F  D  G  A
7321  TGAACGTGGATGTTAACGAGTTCCAGACCAACCTGGTACCCTACCCGAGGATCCACTTCATGCTTTCTTCGTACGCTCCAGTCATCTCTGCTGAGAAGGCCTACCACGAGCAGCTCTCTG
      248 L  N  V  D  V  N  E  F  Q  T  N  L  V  P  Y  P  R  I  H  F  M  L  S  S  Y  A  P  V  I  S  A  E  K  A  Y  H  E  Q  L  S
7441  TGGCTGAGATCACCAACAGTGCCTTCGAGCCCATCTTCCATGATGGCCAAGTGCGACCCACCGCCATGGCAAGTACATGGCCTGCTGCCTCATGTACCGTGGTGATGTGGTCCCCAAGGATG
      288 V  A  E  I  T  N  S  A  F  E  P  S  S  M  M  A  K  C  D  P  R  H  G  K  Y  M  A  C  C  L  M  Y  R  G  D  V  V  P  K  D
7561  TGAATGCTGCTGTGGCCACCATCAAGACCAAACGCACCATCCAGTTCGTGGATTGGTGCCCGACTGGCTTCAAGTGCGGCATCAACTACCAGCCTCCTAGCGTTGTGCCTGGTGGCGACC
      328 V  N  A  A  V  A  T  I  K  T  K  R  T  I  Q  F  V  D  W  C  P  T  G  F  K  C  G  I  N  Y  Q  P  P  S  V  V  P  G  G  D
7681  TGGCCAAGGTGCAGCGTGCCGTGTGCATGATCTCCAACTCCACCAGTGTTGTGGAGGTGTTCTCCCGCATCGACCACAAGTTCGACCTCATGTACGCCAAGCGTGCCTTCGTGCACTGGT
      368 L  A  K  V  Q  R  A  V  C  M  I  S  N  S  T  S  V  V  E  V  F  S  R  I  D  H  K  F  D  L  M  Y  A  K  R  A  F  V  H  W
7801  ACGTCGGTGAGGGTATGGAGGAAGGTGAGTTCTCTGAGGCCCGTGAGGACCTGGCCGCGCTCGAGAAGGACTACGAGGAGGTCGGTGCTGAGTTCGATGAGGGCGAGGATGGCGACGAGG
      408 Y  V  G  E  G  M  E  E  G  E  F  S  E  A  R  E  D  L  A  A  L  E  K  D  Y  E  E  V  G  A  E  F  D  E  G  E  D  G  D  E
7921  GTGACGAGTACtagagaagtttgctgatgacgcagcatcaggccagtgtgctgcccttatcccgtgatctgccgagagttgctcctgctatcgtgttatgtgtgtctgttctgaagtatt
      448 G  D  E  Y
                                                                                  ++++++
8041  gtgtggtttacaacacctgatgttgtaagagttgttaattcccctgcattgctaccgagttattgagaataaa tatggctgtttacgttgttgcttcatcccggagatgctttacattta
                                        ++++++                           *
8161  ccaccttgttcatcttttgtcatcagtaaaatcgagtcggcggatgattttcttgcgtcttgtcatgagtattgagtagtatggattgtttgatgctttatatcacattgcagaagatag
8281  tacgatc
```
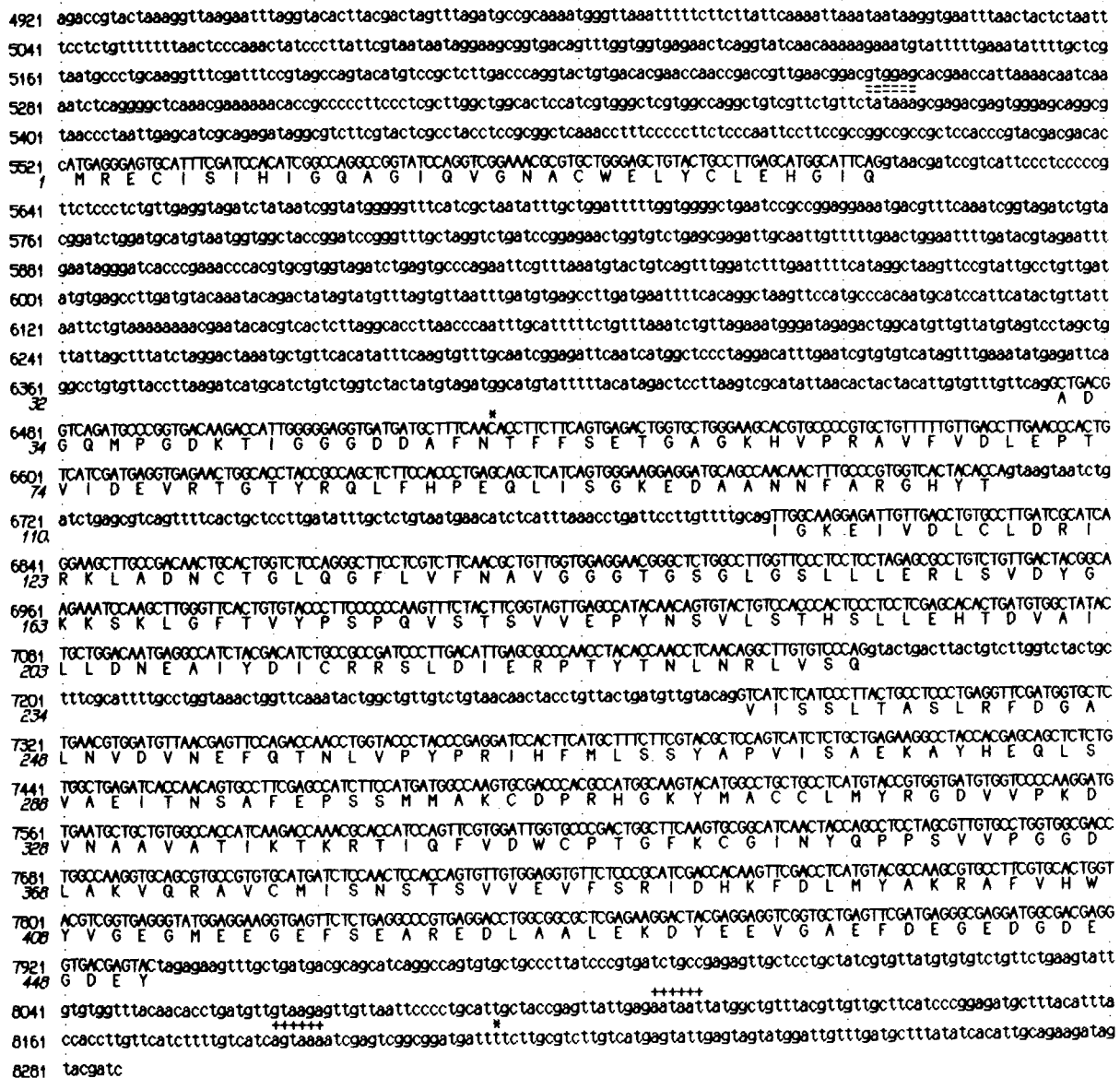
Fig. 2. Nucleotide sequence of the tandem α-tubulin genes in Zea mays. The sequence (8287 nt long) corresponds to MG19/6 and MG19/14 overlapped genomic clones as showed in Fig. 3. The sequence is numbered beginning from a Sal I site marked with an asterisk in Fig. 3. Sequence of the MR19 cDNA clone is indicated between asterisks above positions 6530 and 8210, excluding introns. The deduced coding sequences are presented in upper-case letters. The predicted amino acid sequence of α-tubulin I and II (single-letter code) is shown below the corresponding nucleotide triplets. The putative TATA boxes (=) and polyadenylation signals (+) are marked above the indicated nucleotides. The intergenic repetitive sequence shown in Fig. 4 lies between positions 4373 and 4693 denoted with points above nucleotides.

clones it appeared that the genomic fragment contained two regions homologous to the MR19 cDNA probe. Sequence data indicated that two different units coding for α-tubulin, genes I and II, were present. The restriction map of the genomic insert and the location of the coding sequences and introns are shown in Fig. 3. The 3' region (gene I) contains a nucleotide sequence identical to the cDNA, except for the presence of three insertions flanked by the consensus sequences of
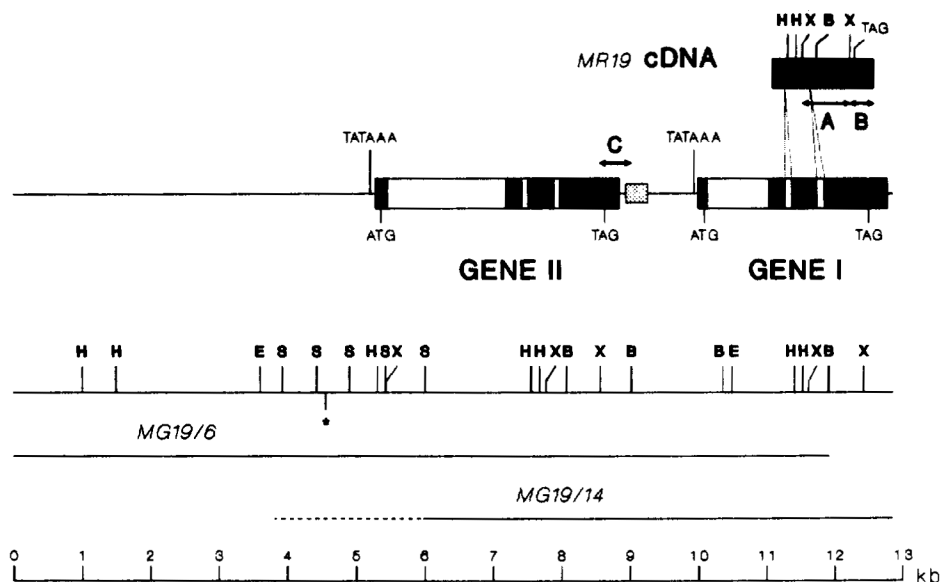
Fig. 3. Restriction maps of genomic clones MG19/6 and MG19/14 showing two α-tubulin units, genes I and II. In both genes white boxes represent introns and black ones the corresponding exon coding segments. The putative TATA boxes, ATG and stop signals are indicated. The map of the MR19 cDNA clone is also shown. The dotted box located between genes represent a zone rich in repetitive sequences (see Fig. 4). The sites presented here are marked as follows: H (*Hin*d III), E (*Eco* RI), S (*Sac* I), X (*Xho* I) and B (*Bam* HI). Arrowed lines marked with A, B and C show probes used in experiments described in Fig. 5 and 6. The nucleotide sequence presented in Fig. 2 lies between a *Sal* I site (marked with an asterisk) and the 3′ end of the MG19/14 genomic clone.

introns. The central region of the genomic clone, hybridizing with the MR19 probe, resulted in another gene (gene II) coding for an α-tubulin protein of an identical protein sequence as compared with the other one, except for two conservative replacements (Glu/Asp, Asp/Glu) in the hypervariable acidic C-terminus of the protein (see Fig. 7).

The nucleotide sequence of the two genes is very similar but clearly not identical. They show 95% homology when only the translated sequences are considered. Among 1353 translated nucleotides in both genes we have found 72 nucleotide replacements while only 2 (as mentioned before) result in amino acid changes.

The sequence of the unit of tandem genes, resulting from MG19/6, MG19/14 and MR19 clones, is presented in Fig. 2. Genes I and II are interrupted by three introns. The first and third introns are located between amino acid positions 31/32 and 233/234, respectively, whereas the second one is found between the first and second

base of triplet coding for amino acid 110 of the protein, the same position of the second intron of *A. thaliana* α-tubulin genes [42]. In particular, although their position is identical in the two genes, the length and the sequence of the introns are quite different. The first intron is 859 bp long in gene I and 1724 bp in gene II, the second one is 93 bp long in both cases, and the third one is 105 bp long in gene I and 92 bp long in gene II. The six introns present consensus 5′ (GT –) and 3′ (– AG) recognition signals as reported in the literature [18].

Both genes have consensus TATA boxes at approximately the same distance from the initial ATG, which is – 149 and – 152 for gene I and II respectively. The nucleotide positions surrounding the putative TATA boxes have been conserved showing a high degree of homology. When the 5′ end of the coding region is compared with the α3-tubulin *A. Thaliana* sequence [28], good homology is found between the transcriptional starting point for gene α3 of *Arabidopsis* and the
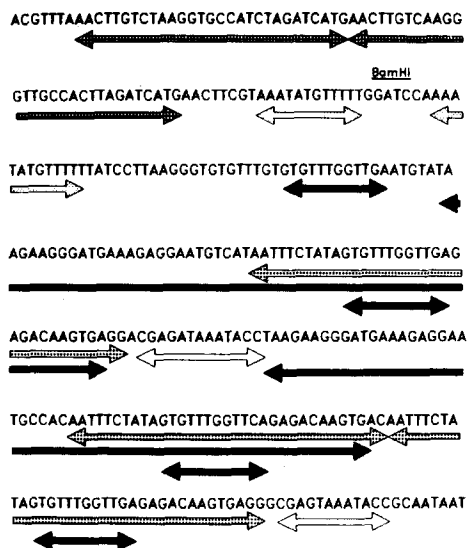
ACGTTTAAACTTGTCTAAGGTGCCATCTAGATCATGAACTTGTCAAGG

BamHI

GTTGCCACTTAGATCATGAACTTCGTAAATATGTTTTTGGATCCAAAA

TATGTTTTTTATCCTTAAGGGTGTGTTTGTGTGTTGGTTGAATGTATA

AGAAGGGATGAAAGAGGAATGTCATAATTTCTATAGTGTTTGGTTGAG

AGACAAGTGAGGACGAGATAAATACCTAAGAAGGGATGAAAGAGGAA

TGCCACAATTTCTATAGTGTTTGGTTCAGAGACAAGTGACAATTTCTA

TAGTGTTTGGTTGAGAGACAAGTGAGGGCGAGTAAATACCGCAATAAT

Fig. 4. Repetitive sequences found in the intergenic region of the MG19/6 genomic clone. Equal patterns of duplication are shown with homologous filled arrows. The Bam HI site is presented to locate the region in the genomic map (see Fig. 3).

maize genes. This point is situated at 34 bp (gene II), and 35 bp (gene I), downstream from the putative TATA boxes. Both genes have TAG as stop codon and present possible polyadenyla-

tion signals (shown in Fig. 2) placed, as mentioned before, in gene I and a single one at 120 nucleotides from the stop codon in gene II.

The intergenic region is 1452 bp long, from the stop codon of gene II to the ATG of gene I. In this zone homologies between the two genes are found in the transcribed 3′ and 5′ ends and within an approximately 200 bp region surrounding the TATA box. An especially interesting sequence is found in a zone (marked with a dotted box in Fig. 3 and denoted with dots in Fig. 2) that seems to separate the two genic regions. A number of repetitions not observed in other parts of the sequenced fragment is found. Repeats from 12 up to 60 bp long can be observed in a variable number up to 4 times. This sequence is shown in Fig. 4 with the indicated repetitions.

Gene structure and differential expression

The proteins encoded by gene I and II slightly differ in their C-terminus. the 3′ untranslated region has also marked differences between the two genes. As has been done in other cases using
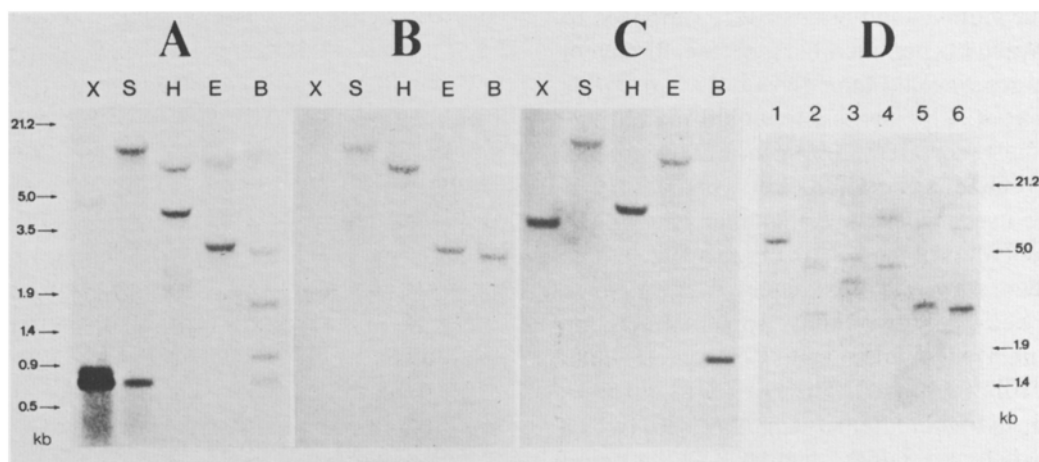
Fig. 5. Southern analysis of genomic α-tubulin sequences homologous to the MR19 cDNA clone of *Zea mays*. Southern blots were prepared from *Zea mays* genomic DNA digested with *Xho* I (X), *Sac* I (S), Hind III (H), *Eco* RI (E) and *Bam* HI (B) and hybridized with A, B and C probes respectively (see Fig. 3). Probe A corresponds to the most conserved part of α-tubulin sequences. Probes B and C correspond to the hypervariable and specific carboxyl-terminus protein sequences and the 3′ ends of genes I and II. The same filter was reused in order to allow a better comparison of the observed signals. D. Southern blot analysis of α-tubulin genes in several related cereals. DNAs were extracted from *Sorghum bicolor* (1), *Zea diploperennis* (teosinte) (2), an F1 from *Zea diploperennis* x *Zea mays* (Palomero Toluqueño) (3), *Zea mays* inbred line A188 (4), *Zea mays* inbred line W64A (5) and *Zea mays* Black Mexican Sweet (6). All these DNAs were diggested with *Eco* RI and hybridized with probe A (see Fig. 3).

3' specific probes, it could be possible to study both the genomic structure as seen in Southern analysis and the expression of the two genes by northern blots. The Southern blot using a probe that contains the nucleotide region coding for the most conserved domain of the protein (probe A in Fig. 3) gives the pattern shown in Fig. 5A.

At this level of stringency two types of bands of different intensity may be observed (see, for instance, the Hind III lane). Using 3' specific probes (probes B and C in Fig. 3) a pattern giving rise to single Southern bands is obtained (Fig. 5B and C). By comparing the three blots it is possible to interpret the pattern of intense bands obtained with the probe of the invariable zone and, with the help of the two other probes, to attribute them to either gene I or gene II (see Fig. 3).

The bands observed in the Southern blots using 3' probes correspond exactly to the genomic restriction fragments of the cloned segments as presented in Fig. 3, except where sites outside the clones are involved. The Xho I lane shows three bands when probe A is used. The lowest and most intense one corresponds to both gene I and gene II (see restriction map in Fig. 3), giving a higher intensity of hybridization. The bands having a lower mobility and intensity are supposed to correspond to other members of the family of α-tubulin genes. Sac I lane gives only two bands using probe A. The larger one would match perfectly to both corresponding genomic fragments of genes I and II, as can be seen when probes B and C are used, whereas the smaller one will be the sequence homologous to other gene(s). The Eco RI lane shows the simplest pattern since using probe A only two bands appear. Each one can be directly related to gene I or gene II when specific probes are used. The Hind III lane gives two bands of higher intensity together with two or three smaller and fainter bands. The first and second bands account for the corresponding genes (I or II, observing the signal when specific probes are used). The rest of the bands correspond to the other less homologous members of the family. A similar behaviour can be observed in Bam HI lanes.

The genomic structure of the corresponding

genes of α-tubulin in different varieties of maize and related species has also been studied. In Fig. 5D the Southern blot of DNA from Sorghum bicolor, Zea diploperennis (teosinte), and F1 generation resulting from Zea diploperennis x Zea mays (Palomero toluqueño) and three inbred lines of Zea mays (A188, W64A and Black Mexican Sweet) is shown. Although polymorphisms are observed at the genomic level, the number of genes homologous to those here studied appear to be similar in all these plants.

The same probes used for the Southern analysis were used to measure the expression of the two genes in different parts of the plant. The pattern of distribution of the mRNA using the probe for the conserved region (Fig. 6A) or the 3' probe of gene I (Fig. 6B) is quite similar. When the 3' probe of gene II is used (Fig. 6C) some dif-
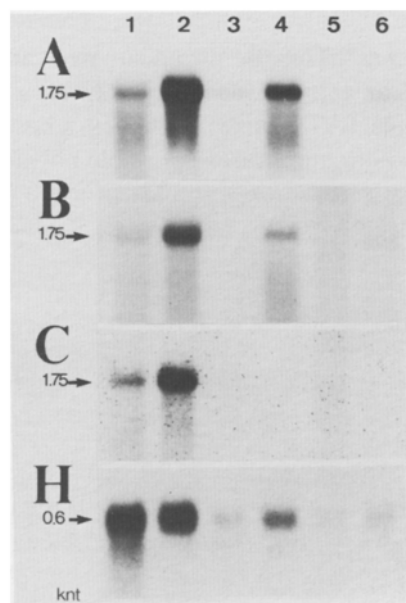

Fig. 6. Analysis of mRNA accumulation of α-tubulin genes I and II. Northern blots were prepared with total RNAs extracted from, two-day-old coleoptiles (1) and young roots (2); poly(A)+ RNA (0.5 μg) from adult leaves (3) and roots (4); total RNAs (10 μg) from adult leaves (5) and roots (6). The filters were hybridized with probes A, B and C, respectively (see Fig. 3) and with histone H3 maize probe (H). Filters were washed and reprobed for a better comparison of results. In each case the same time of exposition (7 days) was used.

ferences can be observed. In particular, the expression of this gene seems to be only confined to young plantlets with preferential expression in young roots.

Parallel experiments were carried out with maize histone H3 [6] (shown in Fig. 6) and H4 [34] (data not shown), as probes for detecting expression in organs rich in dividing cells. The results indicated that a very similar pattern of mRNA accumulation of these two histone genes is obtained in organs rich in meristematic regions, such as young roots and coleoptiles, while a different pattern is observed when probe A, B or C are used. Autoradiographs of northern blots were scanned in a densitometer to obtain relative quantification of bands in the different lanes. When the signal of histone H3 is taken as a reference, the expression of gene I in radicular tissues is 9 times more intense than in the coleoptile, and there is a 6-fold increase in gene II indicating a preferential expression of gene I (and II) in the radicular system of *Zea. mays*.

Discussion

A tandem repeat of α-tubulin genes has been cloned in maize as a result of a differential screening of a root cDNA library. The two genes code for two almost identical proteins with only two conservative (Asp/Glu and Glu/Asp) replacements in the hypervariable C-terminus of the protein. The sequences are more variable at the nucleotide level but keep 95% homology between the two genes.

When the protein sequence of maize α-tubulin is compared with that of other known α-tubulin sequences, a high degree of similarity is found. In Fig. 7 the two maize sequences are compared with the two α-tubulin sequences known from *A. thaliana*, α1 [29] and α3 [28], with the unicellular algal sequence α1-tubulin form *Chlamydomonas reinhardtii* [41], and with the kα1-tubulin from man [8]. The homology between two organisms as diverged as *Homo sapiens* and *Zea mays* yields a remarkable level of homology (83.4%).

Comparison of the protein sequences demonstrate that the maize sequences α1- and α2-tubulin corresponding to genes I and II, respectively, share an 99.6% homology. In addition, the highest similarity is found between the *Chlamydomonas* protein and α1-maize (91.8%) or α2-maize (91.6%), a degree of homology exceeding that found with α3 protein from *Arabidopsis* (91.2% for α1-maize and 90.7% for α2-maize). Homology

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                              30                        60                        90                       120
Z. mays α1    MRECISIHIGQAGIQVGNACWELYCLEHGIQADGQHPGDKTIGGDDAFNTFFSETGAGKHVPRAVFVDLEPTVIDEVRTGTYRQLFHPEQLISGKEDAANNFARGHYTIGKEIVDLCLD
Z. mays α2
A. thaliana α1    I                S               P  T  S S V ACH              SS Q        L                                                        V R    T E
A. thaliana α3    I                S               P  H  S T V VAH                           L                                                        V
C. reinhardtii    V                                P     S                             CI L       V                                                              A
H. sapiens kα1         V   V I                     P     S              S                                                          T          Y              I V

                              150                       180                       210                       240
Z. mays α1    RIRKLADNCTGLQGFLVFNAVGGGTGSGLGSLLLERLSVDYGKKSKLGFTVYPSPQVSTSVVEPYNSVLSTHSLLEHTDVAILLDNEAIYDICRRSLDIERPTYTNLNRLVSQVISSLTA
Z. mays α2
A. thaliana α1    L                              F          I           A            W                             S     | T   T
A. thaliana α3    V                                          I           A            V                             |  |    T
C. reinhardtii                                                           A            VM                                   IA
H. sapiens kα1     Q  R        HSF       FT  M         E SI  A       A        I T TT   SC FMV              N             | IV I

                              270                       300                       330                       360
Z. mays α1    SLRFDGALNVDVNEFQTNLVPYPRIHFMLSSYAPVISAEKAYHEQLSVAEITNSAFEPSSMMAKCDPRHGKYMACCLMYRGDVVPKDVNAAVATIKTKRTIQFVDWCPTGFKCGINYQPP
Z. mays α2
A. thaliana α1    I   IT            IV        SA      F  P  T V    N                         T  A  A
A. thaliana α3    I   MT                       A       P    AV   A                              G       V
C. reinhardtii        IT            I                        A    A  V                       S
H. sapiens kα1        LT        P AT                  AC    ANQ V   G          L                    |              V

                              390                       420                       450
Z. mays α1    SVVPGGDLAKVQRAVCMISNSTSVVEVFSRIDHKFDLMYAKRAFVHWYVGEGMEEGEFSEAREDLAALEKDYEEVGAEFDEG-EDG-DEGDEY
Z. mays α2                                                                               -E -D
A. thaliana α1       N A A           S                                    G  -GA- DDE
A. thaliana α3    T  N A A                                                -G -D EE  ED
C. reinhardtii    T  AIG I  L                                      F      -SAEGA EG E
H. sapiens kα1    T  L T AIA AWA L                               M          VD-SVEGE EE E
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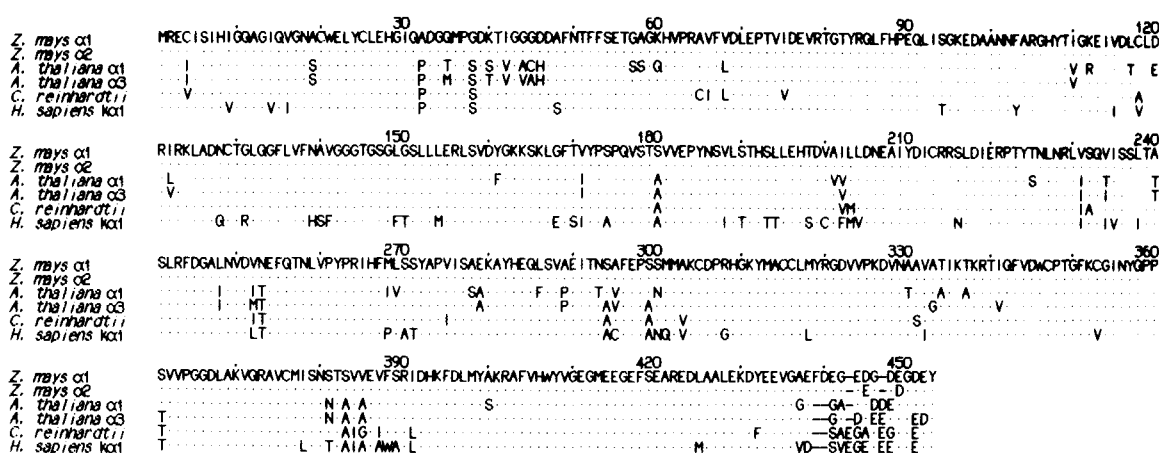
Fig. 7. Comparison of α-tubulin protein sequences between different organisms. The predicted amino acid sequence of the α1-tubulin of *Zea mays* (coded by gene I) is presented at the top line of single-letter code. The predicted amino acid sequences of the α2-tubulin of *Zea mays* (coded by gene II) and other α-tubulin are shown below respecting an optimal alignment. Only those amino acids that change from α1-tubulin of *Zea mays* are indicated. A dash in the sequence means a gap in the amino acid sequence introduced in order to maintain a good alignment. Sequences from *Chlamydomonas reinhardtii* α1-tubulin, *Arabidopsis thaliana* α1 and α3 tubulin, and *Homo sapiens* Kα1-tubulin are those published in refs. [41], [29], [28] and [8], respectively.

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between α1 and α3 proteins of *Arabidopsis* is lower (91.2%) than between the two maize tubulins. These data would suggest that the duplication observed in maize is relatively recent and, in any case, more recent than the duplication observed in *Arabidopsis*. On the other hand, we observe by Southern analysis a similar pattern of genomic structure in different varieties of *Zea mays* and related species (Fig. 5D), indicating that the existence of these two related genes may be a general characteristic of the group.

Both deduced proteins α1 and α2 from maize present a Tyr as a C-terminal end. The same has been reported in all other plant or algal α-tubulin sequences published showing that the phenomenon of absence of this ending amino acid of the variable 3' terminus may be restricted to the animal kingdom [36]. There is also a high degree of conservation in the sequence postulated to be involved in GTP binding in the α-tubulin sequences (amino acids 143–149) [44].

The introns and the flanking regions show a much larger divergence between the two genes than the coding sequence. The similarity between homologous introns of both gene I and II are 88.2% in the second intron and 61.9% in the third one. A much lower similarity in the first intron is observed. It presents homology only at defined stretches. This intron seems to have evolved incorporating different sequences in gene II which are not present at all in the corresponding region of gene I. This first intron is unusually long (859 bp for gene I, 1724 bp for gene II) for plant introns reported until now [19]. Likewise, the location of introns differs from that observed in other species. Only the second intron is placed at the same amino acid position (110) as those observed in the two α-tubulin genes of *Arabidopsis thaliana* [28, 29], while the two other ones are different in position and length. Although the position is the same in the second intron their lengths are quite different in both plants (512 bp for the α1 gene, and 76 bp for the α3 gene of *A. thaliana*; 93 bp in both genes of *Z. mays*). Low homologies are observed when the second introns of both organisms are compared, but short segments (up to 30 nt) of the sequence appear to be extremely

similar. The finding of the second intron conserved at the same place in both plants, *A. thaliana* and *Z. mays*, may be a result of the obvious evolutionary relationships between both organisms, while the homology observed when the coding region is compared puts the described maize α-tubulin genes nearer to the corresponding ones from *C. reinhardtii* (91.7% on the average) than to the other ones from *A. thaliana* (88.2% on the average). These results may reflect the existence of two different rates of evolution, one related with intron positions and the other one corresponding to the coding sequence itself.

In the flanking regions homology is found between the two genes in the region surrounding the TATA boxes up to −250 from the ATG. When more upstream regions are compared defined homologous segments can be observed in both genes separated by divergent sequences. An interesting sequence is found in the 5' end of the intergenic region. This is the only part of the 8.5 kbp sequence where a high degree of repetitions is found. In fact, a complex structure of duplications may be observed at this point (see Fig. 4). The functional meaning of these duplications either as a part of the promoter or as a hinge region in the gene duplication will be object of future experiments.

The cDNA clone presented has been identified after a differential screening of a root cDNA library searching for clones corresponding to a mRNA preferentially expressed in the radicular tissues. The northern analysis presented indicate that the α-tubulin genes described here are expressed in the meristematic parts of the plant, for example in the coleoptile or in the root tip, and therefore their expression may be correlated with cell division. In order to investigate this correlation we decided to use maize histone probes [6, 34] as a reference for division activity, since H3 and H4 genes are particularly expressed where DNA replication is present. In addition, it has been proposed that histone and tubulin genes have a cell-cycle regulation, giving the major peaks of accumulation simultaneously at the late G2 phase, as has been demonstrated in *Physarum* [5]. However, when the mRNA level of α-tubulin

is compared with that of histone genes a relatively high expression is observed in the radicular tissues, indicating that what is observed is not only the result of a concentration of dividing cells (as could result from the differential screening approach) but also an organ-specific control superimposed on these one. The two genes show similar patterns of expression with gene II mRNA accumulation being restricted to the meristematic parts of the young plantlet.

Tubulin genes expressed in defined tissues have been observed in animals and data are available from human, mouse, rat, chicken and other verte-brate and invertebrate organisms [47]. In verte-brates, the variable acidic C-terminus amino acid positions of the protein would act as a specific domain related to the particular expression of the gene. No evidence in that direction has been detected in plant tubulin sequences, probably due to the lower number of sequences and situations reported. In plants little is known about this question. Most data come from *A. thaliana* where a gene, the α1-tubulin [29], is diferentially expressed in flowers and through differents phases of flowering, whereas the α3-tubulin [28] is expressed contitutively in all organs examined. Also a β-tubulin (the β1 gene from *A. thaliana*) has been described presenting a preferential accumu-lation in the root system of the plant as we have shown for the α1 and α2 maize tubulin genes. In addition, a possible organization in a tandem array has been proposed for other β-tubulin genes of *A. thaliana*, but no sequence data are available up to now [33].

In summary, a group of α-tubulin genes from maize have been cloned whose members (genes I and II) are highly homologous. These two genes are organized in a tandem arrangement. This kind of organization for the α-tubulin genes has not been reported before in the genome of higher eukaryotic organisms, except for some clusters of α- and β-tubulin genes present in sea urchin [7].

When specific probes for gene I (probe B) and gene II (probe C) are used, a single-gene pattern can be deduced since in all five lanes (in both genes) a single band is observed (Fig. 5). If the bands observed when using those specific probes would correspond to more than one gene it would imply an extremely high conservation of se-quences, even of those located downstream and upstream of the coding regions of the α-tubulin genes. That would be unlikely in maize, specially when polymorphisms are present in most of the restriction sites used, located within the coding region of these two highly conserved genes. Southern analysis also shows that other α-tubulin sequences, having a lower degree of homology, exist in the genome of the plant. Our results and analysis of different genomic clones obtained (data not shown) would indicate a small number of genes (probably three or four) coding for the whole α-tubulin family of *Zea mays*.

The expression of both α-tubulin genes could be divided into two aspects which may interact at different levels of regulation of gene expression. First, it is clear that a divisional control exists, since both histone (H3/H4) and α-tubulin genes accumulate in the same meristematic organs such as root tips and coleoptiles. In addition, a second level of control appears indicating a preferential expression in radicular tissues when the signal of histone H3 probe is taken as a reference.

It would be very interesting to focus future experiments on analyzing the meaning of this kind of organ-specific expression, in order to locate the α1- and α2-tubulin proteins in particular sets of cells. In this way, genes I and II and their protein products may be useful markers of development in the root system of maize.

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References

1. Adachi Y, Toda T, Niwa O, Yanagida M. Differential expressions of essential and nonessential α-tubulin genes in *Schizosaccaromyces pombe*. Mol Cell Biol 6: 2168–2178 (1986).
2. Biggin MD, Gibson TJ, Hong GF. Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination. Proc Natl Acad Sci USA 80: 3963–3965 (1983).
3. Boedtker H. Conformation independent molecular weight determinations of RNA by gel electrophoresis. Biochim Biophys Acta 240: 448–453 (1971).
4. Burr B, Burr FA. Controlling element events at the shrunken locus in maize. Genetics 98: 143–156 (1981).
5. Carrino JJ, Laffter TG. Transcription of α-tubulin and histone H4 begins at the same point in the *Physarum* cell cycle. J Cell Biol 102: 1666–1670 (1986).
6. Chaubet N, Philipps G, Chaboute ME, Ehling M, Gigot C. Nucleotide sequence of two corn histone H3 genes. Genomic organization of the corn histone H3 and H4 genes. Plant Mol biol 6: 253–263 (1986).
7. Cleveland DW, Sullivan KF. Molecular biology and genetics of tubulin. Ann Rev Biochem 54: 331–365 (1985).
8. Cowan NJ, Dobner PR, Fuchs EV, Cleveland DW. Expression of human alpha-tubulin genes: interspecies conservation of 3' untranslated regions. Mol Cell Biol 3: 1738–1745 (1983).
9. Cyr RJ, Bustos MM, Guiltinan MJ, Fosket DE. Developmental modulation of tubulin protein and mRNA levels during somatic embryogenesis in cultured carrot cells. Planta 171: 365–376 (1987).
10. Davis RW, Bolstein D, Roth RJ. A Manual for Genetic Engineering. Advanced Bacterial Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1980).
11. Dean C, Van den Elzen P, Tanaki S, Dunsmuir P, Bedbrook J. Differential expression of the eight genes of the petunia rubulose bisphosphate carboxylase small subunit multi-gene family. EMBO J 4: 3055–3061 (1985).
12. Feinberg AP, Vogelstein B. A technique for radio-labelling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132: 6–13 (1983).
13. Gallardo D, Reina M, Rigau J, Boronat A, Palau J. Genomic organization of the 28 kDa glutelin-2 gene from maize. Plant Sci 54: 211–218 (1988).
14. Ginzburg I, Teichman A, Griffin WST, Littauer UZ. Differential expression of α-tubulin mRNA in rat cerebellum as revealed by *in situ* hybridization. FEBS Lett 194: 161–164 (1986).
15. Gubler U, Hoffman BJ. A simple and very efficient method for generating cDNA libraries. Gene 25: 263–269 (1983).
16. Gunning BES, Hardham AR. Microtubules. Ann Rev Plant Physiol 33: 651–698 (1982).
17. Hanahan D. Techniques for transformation of *E. coli*. In: Glover DM (ed) DNA Cloning – A Practical Approach, pp. 109–137. IRL press, Oxford (1985).
18. Hanley BA, Schuler MA. Plant intron sequences: evidence for distinct groups of introns. Nucleic Acids Res 16: 7159–7175 (1988).
19. Hawkins JD. A survey on intron and exon lenghts. Nucleic Acids Res 16: 9893–9908 (1988).
20. Hecht NB, Distel RJ, Yelick PC, Tanhauser SM, Driscoll CE, Goldberg E, Tung KSK. Localization of a highly divergent mammalian testicular α-tubulin that is not detectable in brain. Mol Cell Biol 8: 996–1000 (1988).
21. Hussey PJ, Gull K. Multiple isotypes of α- and β-tubulin in the plant *Phaseolus vulgaris*. FEBS Lett 181: 113–118 (1985).
22. Hussey PJ, Lloyd CW, Gull K. Differenital and developmental expression of β-tubulins in a higher plant. J Biol Chem 263: 5474–5479 (1988).
23. Huynh TU, Young RA, Davis RW. Constructing and screening cDNA libraries in lambda-gt10 and lambda-gt11. In/ Glover DM (ed) DNA cloning – A Practical Approach, pp. 49–78. IRL Press Oxford (1985).
24. Kimmel BE, Samson S, Wu J, Hirschberg R, Yarrough LR. Tubulin genes of the African trypanosome *Trypanosoma brucei rhodesiense*: nucleotide sequence of a 3.7 kbp fragment containing genes for alpha and beta tubulins. Gene 35: 237–248 (1985).
25. Lemischka I, Sharp PA. The sequences of an expressed rat α-tubulin gene and a pseudogene with an inserted repetitive element. Nature 300: 330–335 (1982).
26. Little M, Seehaus T. Comparative analysis of tubulin sequences. Comp Biochem Physiol 90: 655–670 (1988).
27. Lloyd CW. The Plant Citoskeleton: The impact of fluorescence microscopy. Ann Rev Plant Physiol 38: 119–139 (1987).
28. Ludwig SR, Oppenheimer DG, Silflow CD, Snustad DP. Characterization of the α-tubulin gene family of *Arabidopsis thaliana*. Proc Natl Acad Sci USA 84: 5833–5837 (1987).
29. Ludwig SR, Oppenheimer DG, Silflow CD, Snustad DP. The α1-tubulin gene of *Arabidopsis thaliana*: primary structure and preferential expression in flowers. Plant Mol Biol 10: 311–321 (1988).
30. Mages W, Salbaum JM, Harper JF, Schmilt R. Organization and structure of *Volvox* α-tubulin genes. Mol Gen Genet 213: 449–458 (1988).
31. Maniatis T, Fritsch EF, Sambrook J. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).
32. Morejohn LC, Fosket DE. Higher plant tubulin identified by self-assembly into microtubules *in vitro*. Nature 297: 426–428 (1982).
33. Oppenheimer DG, Haas N, Silflow CD, Snustad DP. The β-tubulin gene family of *Arabidopsis thaliana*: preferential accumulation of the β1 transcript in roots. Gene 63: 87–102 (1988).
34. Philipps G, Chabet N, Chaboute ME, Ehling M, Gigot C. Genomic organization and nucleotide sequences of two corn histone H4 genes. Gene 42: 225–229 (1986).

35. Picquot P, Lambert AM. Tubulin from monocotyledon endosperm cells: Particular biochemical and immuno-logical properties. J Plant Physiol 132: 561–568 (1988).

36. Pratt LF, Okamura S, Cleveland DW. A divergent testis-specific α-tubulin isotype that does not contain a dosed C-terminal tyrosine. Mol Cell Biol 7: 552–555 (1987).

37. Queen C, Korn LJ. A comprehensive sequence analysis program for the IBM personal computer. Nucleic Acids Res 12: 581–599 (1984).

38. Raha D, Sen K, Biswas BB. cDNA cloning of β-tubulin gene and organisation of tubulin genes in *Vigna radiata* (mung bean) genome. Plant Mol Biol 9: 565–571 (1987).

39. Rigby PWJ, Diekmann M, Rhodes C, Berg P. Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J Mol Biol 113: 237–251 (1977).

40. Sanger F, Nicklen S, Coulsen AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463–5467 (1977).

41. Silfow CD, Chisholm RL, Conner TW, Ranum LPW. The two alpha-tubulin genes of *Clamydomonas renhardtii* code for slightly different proteins. Mol Cell Biol 5: 2389–2398 (1985).

42. Silfow CD, Oppenheimer DG, Kopczak SD, Ploense SE, Ludwig SR, Haas N, Snustad PD. Plant tubulin genes: structure and differential expression during devel-opment. Dev Genet 8: 435–460 (1987).

43. Schatz PJ, Solomon F, Botstein D. Genetically essential and nonessential α-tubulin genes specify functionally interchangeable proteins. Mol Cell Biol 6: 3772–3733 (1986).

44. Sternlicht H, Yaffe MB, Farr GW. A model of the nucle-otide-binding site in tubulin. FEBS Lett 214: 226–235 (1987).

45. Stiefel V, Perez-Grau Ll, Albericio F, Giralt E, Ruiz-Avila L, Ludevid MD, Puigdomenech P. Molecular cloning of cDNAs encoding a putative cell wall protein from *Zea mays* and immunological identification of related polypeptides. Plant Mol Biol 11: 483–493 (1988).

46. St John TP, Davis RW. Isolation of galactose inducible DNA sequences from *Saccharomyces cerevisiae* by dif-ferential plaque filter hybridization. Cell 16: 443–452 (1979).

47. Sullivan KF. Structure and utilization of tubulin iso-types. Ann Rev Cell Biol 4: 687–716 (1988).

48. Theurkauf WE, Baum H, Bo J, Wensink PC. Tissue-specific and constitutive α-tubulin genes of *Drosophila melanogaster*. Proc Natl Acad Sci USA 83: 8477–8481 (1986).

49. Villasante A, Wang D, Dobner P, Dolph P, lewis SA, Cowan NJ. Six mouse α-tubulin mRNAs encode five distinct isotypes: testis-specific expression of two sister genes. Mol Cell Biol 6: 2409–2419 (1986).

50. Yanisch-Pervon C, Vieira J, Messing J. Improved M13 phage cloning and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33: 103–119 (1985).