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Analysis by PCR of the number of homologous genomic sequences to α-tubulin in maize

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The protein sequences of α-tubulins are among the best conserved in eukaryotes. This degree of conservation also remains high at the nucleotide level. The genes encoding these proteins are organized into families of up to 30 members in animal systems. In the few plant species studied, the α-tubulin gene family shows a lower complexity. In the reported genomic sequences, for a given species the site of insertion for an intron in the α-tubulin genes occurs at specific points in the gene and this is also true for maize. In this paper, advantage has been taken of the fact that the first intron of maize α-tubulin is variable in length between the different genes reported and it is flanked by coding sequences conserved at both protein and nucleotide levels. These facts offer the possibility of estimating the number of genes coding for this protein in maize. The results and the limitations of the approach are described.

Key words: genomic organization; introns; maize; multigene family; polymerase chain reaction; tubulins

Introduction

The α-tubulins of higher eukaryotes are encoded by complex gene families occasionally containing pseudogenes. This is true in the case of vertebrate genomes where a maximum of 30 members have been reported in one species [1,2]. In spite of a lower degree of knowledge in the plant kingdom, the available data suggest the existence of gene families with a lower complexity. In the dicotyledonous plant *A. thaliana*, a maximum of five α-tubulin genes have been described, two of which have been characterized in detail [3–5]. Studies of Southern analysis in *Z. mays* indicate a similar but unknown number of α-tubulin genes [6,7].

In previous reports we have presented the characterization of three α-tubulin genes from maize, two of them organized in tandem, separated by 1.5 kb and the other one with no apparent relation to the other two [6,7]. By using specific probes corresponding to each one of the two members of the family, the expression of these genes and the number of related sequences in maize have been studied. The two families of genes coding for α-tubulin studied so far in maize have an expression in meristematic tissues of the plant but an enhanced expression of *Tubα*1 and *Tubα*2 was observed in the radicular system. It was of interest to know whether other genes from maize could account for expression in other plant organs. In fact, when probes corresponding to the most conserved part of the protein sequences were used, a higher number of homologous genomic sequences could be detected by Southern analysis, but using this method it is difficult to have a quan-

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Abbreviations: bp, base pair(s); dNTP, deoxyribonucleoside triphosphate; PCR, polymerase chain reaction; *Tubα*n genes encoding αn-tubulin proteins, where n is the number of the corresponding gene.

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titative idea of the number of genes coding for α-tubulin in maize.

The comparison of the nucleotide sequences corresponding to the three α-tubulin genes indicates the presence of three introns in each gene. Homologous introns appeared at the same nucleotide position of the gene, interrupting identical codons of the open-reading frames. The first intron (the longest in all three genes) showed a striking variability in length, ranging from 0.8 to 1.7 kb. We found several clones in a maize genomic library corresponding to a different α-tubulin genes and in the present paper we discuss the amplification of the first intron, via polymerase chain reaction (PCR), as a molecular marker for elucidating the total number of α-tubulin genes in maize.

Materials and Methods

Plant material and genomic DNA purification

The studies were carried out with *Zea mays* L. (inbred line W64A) grown under greenhouse conditions. Genomic DNA was isolated from both leaves of two-months old plants and seven-days old coleoptiles as described before [6,7].

General methods

Harvesting and preparation of λ-phage DNA (from genomic clones) were carried out according to reported protocols [8]. Probes were labelled by random-primer (Boehringer-Mannheim kit) reactions to a specific activity up to 10^8–10^9 counts/min per μg [9]. The PCR primers were prepared at 0.2 μmol scale following described methods [10]. DNA sequencing reactions were done as reported previously [6]. Protein and DNA sequences were analyzed in a PC computer using software provided by Micro-Genie (Beckman) [11].

PCR reactions, DNA amplification

The PCR analysis was carried out on genomic maize DNA, as well as on a number of phage DNAs from the isolated genomic clones of a maize λ-Ch35 genomic library [13]. The protocol used was as described in the literature [12], with minor modifications. The apparatus used was a Bio-med Thermocycler 60 from B.Braun and the oligonucleotides were synthesized in an Applied Biosystems 392 DNA synthesizer and purified by Sep-Pak cartridges (Waters).

The optimal results were achieved when 100 ng of genomic DNA (or < 10 ng of phage DNA) and primers (0.25 μM each) were subjected to 30 PCR cycles using Taq polymerase (2 I.U., Boehringer-Mannheim) in a 100-μl reaction mixture containing: 50 mM KCl, 7.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.4/25°C), 0.2 mM each dNTP and 100 μg/ml gelatin. Each PCR cycle was performed as follows: 1 min at 94°C, 1 min at 50°C and 3 min at 72°C. An extra 5-min extension step (at 72°C) was performed at the end. The samples were overlayed with 50–100 μl of paraffin oil. After amplification 100 μl of chloroform/isoamyl alcohol (24:1) were added to every sample and the upper-aqueous phase was collected. 1/10 of the sample (10 μl) were analyzed on a 1% agarose native gel in TAE buffer (TAE: 40 mM TRIS-acetate, 2 mM EDTA). Gels were blotted onto nitrocellulose or nylon membranes and hybridized with representative probes, to verify the specificity of DNA amplification, as described before [6].

Results

The isolation and characterization of three genes coding for α-tubulin in maize (*Zea mays* inbred line W64A) has allowed us to analyze the structure and expression of three members (two of them forming a tandem) of this particular gene family [6,7]. The three genes (*Tubα1*, *Tubα2* and *Tubα3*) exhibit three introns, each one at identical positions. The length of the first intron varies from 1724 bp (*Tubα2*) to 859 or 846 bp (*Tubα1* and *Tubα3*, respectively). The exonic regions flanking these introns code for one of the most conserved regions of the α-tubulin protein [2]. A Southern blot analysis using 3′ specific probes from the three genes, as well as probes from the zone coding for the conserved region of this protein, has revealed at least 3–5 copies homologous to these probes in the maize genome [6,7].

(a) Hypothesis

The situation described above led to the hypothesis that all the genes coding for α-tubulin

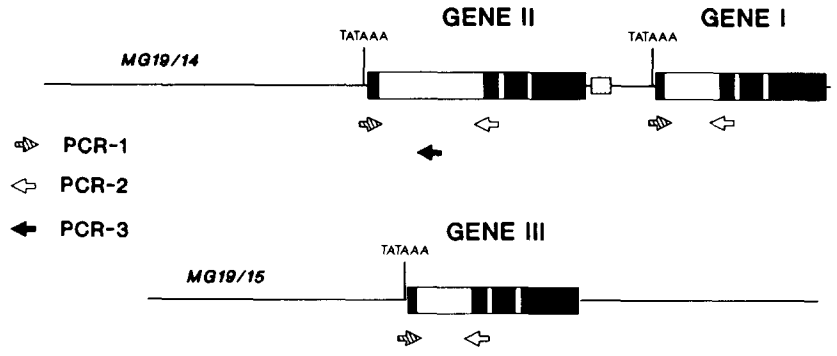

Fig. 1. Location of the three oligonucleotides PCR-1, PCR-2 and PCR-3 in relation to the sequences of α1, α2 and α3 tubulin genes. The sequence used for the design of oligonucleotides was that corresponding to the α2 gene. The PCR-3 is complementary only to the α2 sequence and it was used as a control for the specificity of the PCR reaction. Black boxes correspond to exons of the gene and white ones to the interrupting introns. The PCR primers were prepared at 0.2 μmol scale following described methods [16].

in maize might have similar structure and, therefore, all other possible genes coding for this protein will probably have introns in equivalent positions to the three already characterized. In addition, the first intron is variable in sequence and length. The amplification of the corresponding sequences by PCR (using oligonucleotides from the flanking sequences that code for a conserved region of the protein) would produce one band for each gene which exists in the genome of maize.

(b) Oligonucleotide design

In order to test the previous hypothesis and to evaluate the use of PCR in solving these questions, three 20-bp-long oligonucleotides (PCR-1, PCR-2 and PCR-3), located as indicated in Fig. 1, were synthesized. Two of them are complementary to the flanking exonic regions of the first intron of the α-tubulin genes. The third oligonucleotide corresponds to an internal region from the first intron of the *Tubα2* gene and it was used as a specificity and reaction control for PCR.

(c) Characterization of genomic clones

In addition, it has been possible to isolate different clones from a maize genomic library in λCh35 [13] using a cDNA (MR19), corresponding to the *Tubα1* gene, as a probe. The clones (called MG19/*n*, $n = 1,17$) were analyzed and grouped according to their restriction site pattern and their restriction fragments hybridizing with the MR19 probe (see Fig. 2). From these data and the sequence of the *Tubα1*, *Tubα2* and *Tubα3* genes it could be deduced that the MG19/6 and MG19/14 clones contain overlapping genomic inserts corresponding to the *Tubα1* and *Tubα2* genes in tandem and the MG19/1, /2, /3, /5, /7, /13 and /15 clones correspond to the *Tubα3* gene. Partial sequencing of the inserts from MG19/4, /12 and /16 clones indicated that they contained other genes coding for α-tubulin, still not characterized (data not shown). The similarity of these newly sequenced fragments with the equivalent ones from *Tubα1*, *Tubα2* and *Tubα3* is between 75% and 93%.

(d) PCR amplification

Figure 3 shows the results of a representative PCR amplification. Using the PCR-1/PCR-3 initiators on the genomic plant DNA (Fig. 3A) a single band (750-bp long, corresponding to the fragment of the first intron of *Tubα2* gene, used as a control for the specificity of the reaction) could be seen. Figure 3B shows the results of the amplification of genomic plant DNA, as well as several DNAs from the genomic clones, using the PCR-1/PCR-2 initiators. An amplification reac-

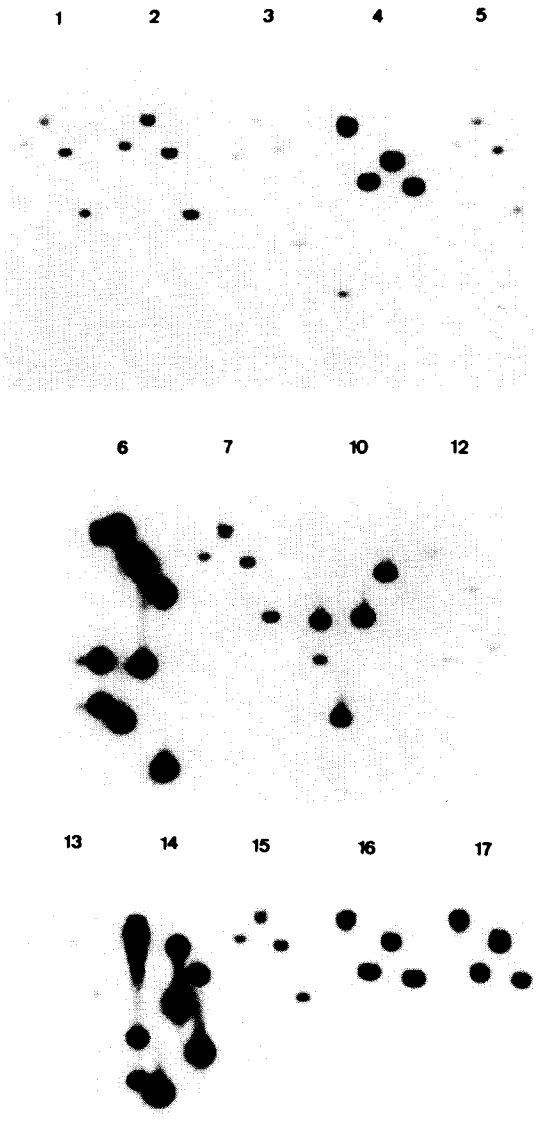

Fig. 2. Analysis of the different genomic clones isolated from a maize genomic library with the MR19 probe [6], cDNA from the *Tubα*1 gene. DNAs from the λ phages were digested with *Bam*HI, *Xho*I, *Eco*RI and *Hin*dIII (left-to-right in every case), blotted onto nitrocellulose and hybridized with the MR19 probe. Numbers correspond to the genomic clones (i.e. 1, MG19/1 and 2, MG19/2, etc.).

tion of genomic DNA with PCR-2/PCR-3 initiators (Fig. 3B, lane 3) as a negative control of the reaction is also shown. In Fig. 3B (lanes 1 and 2), corresponding to the genomic DNA, three groups of bands can be observed. There are two bands of similar size (1 kb), three bands between 500 and 700 bp and two more bands of 350 bp, approximately. In the lane corresponding to the genomic clone MG19/6 DNA (Fig. 3B, lane 4) two clear bands 950 and 1800 bp-long are observed, perfectly matching in size with those of the first intron present in the tandem genes *Tubα*1 and *Tubα*2, represented in that genomic clone. The PCR amplification of the MG19/13 genomic clone (Fig. 3B, lane 5) shows a single band corresponding to the first intron of the *Tubα*3 gene. The amplifications from the DNA of clones MG19/4, /10, /12 and /16 (Fig. 3B; lanes 8, 9, 7 and 6, respectively) shows bands which probably correspond to homologous introns of other maize α-tubulin genes, not yet characterized.

All the bands obtained from the amplification of genomic clones can also be observed in the genomic DNA lanes, except the 1800 bp of the MG 19/6 clone, which corresponds to the first intron of the *Tubα*2 gene. The efficiency of the PCR reactions decreases with the length of the target DNA to be amplified so the gels were transferred onto a nitrocellulose filter and hybridized with a DNA probe containing the first intron from the *Tubα*2 gene and exonic flanking regions (Fig. 3C, lane 1). In this case a band, approx. 2 kb long, clearly appears. By subcloning and partial sequencing of bands which gave positive hybridization from the PCR amplification (data not shown) it was observed that the single band corresponding to the MG19/10 clone contains the first intron from the *Tubα*1 gene. The MG19/16 clone shows two amplified bands, upon hybridization with a DNA probe derived from the first intron of *Tubα*2, with a larger one (not visible in the ethidium bromide stained gels) around 2 kb (Fig. 3C, lane 2).

Discussion

To evaluate the number of genes coding for a given protein is an interesting exercise when trying to understand the regulation of the expression of these genes. In maize, the α-tubulin genes described so far show a preferential expression in defined tissues of the plant. Advantage has been taken in the present article of the particular intron struc-

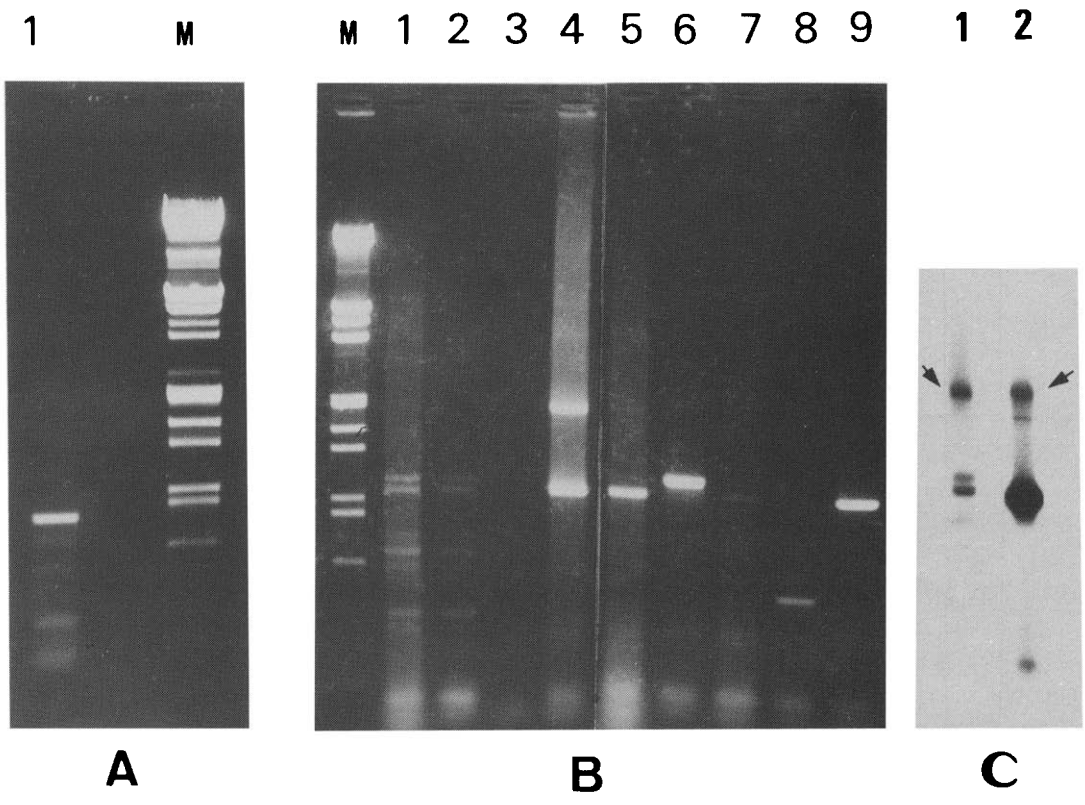

Fig. 3. Electrophoresis in agarose (1%) with 1/10 of the final PCR reaction mixture. M is a relative molecular weight marker (λcI857 digested by *Hin*dIII and *Eco*RI with length varying from 21 to 564 bp). (A) Amplification using PCR-1/PCR-3 initiators on genomic DNA of maize (100 ng). (B) Amplification using PCR-1/PCR-2 initiators, except lane 3, which is PCR-2/PCR-3 initiated and corresponds to the negative control. Lanes 1 and 2 correspond to the genomic DNA from maize. Lanes 4, 5, 6, 7, 8 and 9 correspond to DNA from MG19/6, /13, /16, /12, /4 and /10 genomic clones, respectively. (C) Hybridization of a transferred equivalent electrophoresis gel using a probe containing the first intron and flanking exons from *Tubα*2 gene. Lane 1 is an amplification using PCR-1/PCR-2 initiators on genomic DNA from maize. Lane 2 corresponds to DNA of MG19/16 genomic clone.

ture of the genes to evaluate the number of α-tubulin genes in maize by the use of PCR.

The results from the PCR amplification and the previous analyses with isolated genomic clones, allow us to conclude that the MG19/6, /14 clones contained the *Tubα*1 and *Tubα*2 genes [6], which are in tandem. The genomic clone MG19/10 contains only the *Tubα*1 gene. The MG19/15, /1, /2, /3, /5, /7 and /13 represents a set of overlapping genomic clones containing the *Tubα*3 gene [7]. The PCR reaction of the MG19/4 and /12 clones seem to amplify two other independent bands corresponding to different α-tubulin genes (that could be called *Tubα*4 and *Tubα*7, respectively), since previous partial sequences of hybridizing fragments show differences at the nucleotide level in comparison with the three well-characterized genes. The MG19/16 clone seems to contain two more α-tubulin genes, probably organized in tandem like the *Tubα*1 and *Tubα*2 genes (the *Tubα*5 and *Tubα*6 genes), based on the differences in sequence and the appearance of two amplified bands by PCR. Nothing is known about the middle-group bands (500–700 bp long, Fig. 3B, lanes 1 and 2) appearing in the genomic DNA lanes amplified by PCR. The same is true for the lowest band (350 bp long, Fig. 3B, lanes 1 and 2) also in the genomic DNA amplifications. By hybridizing these bands to a probe containing the first intron of the *Tubα*2 gene and its flanking

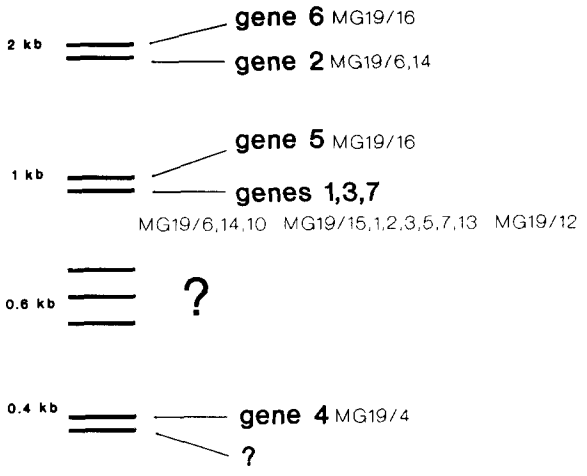

Fig. 4. Summary diagram for the bands obtained by PCR amplification of genomic DNA and genomic clones and putative correspondence with each of the α-tubulin genes present in the maize genome.

exonic sequences only very faint signals (after longer exposures) were apparent indicating a weak cross-hybridization. In one case it has been possible to subclone one of these regions and to obtain a partial sequence and it was found not to have similarity with any of the other α-tubulin sequences. It is therefore possible that these bands could represent artefacts amplified by the PCR reaction during the amplification step.

In conclusion, the number of genes coding for α-tubulins in maize seems to be higher than that indicated by previous Southern analysis [6,7]. A minimum of 7 genes have been detected by both PCR and genomic clone analyses. A schematic interpretation of the accumulated data is shown in Fig. 4. Those bands which appeared in genomic DNA and genomic clones have been assigned to different α-tubulin genes, from 1 to 7. The PCR technique has been shown to be useful in this type of analysis provided that certain features are taken into account. First, the existence of overlapping bands (as is the case for genes having introns around 1000 bp in our case), which may result in an underestimation of the complexity of this gene family. Second, the production of bands that may be the result of the amplification of DNA regions that hybridize to the oligonucleotides used as initiators, but do not correspond to homologous α-tubulin genes (as those having ~500–700 bp). And finally, the possibility of missing those genes which lack the first intron at the expected position or those which contain interrupting sequences too big to be efficiently amplified via PCR. Although we cannot rule out these facts, the data accumulated so far in the literature demonstrate the invariance of positioning of introns in the α-tubulin genes within a given species [2,5] and the relative shortness of introns in the plant genes [14,15]. This method could be useful for the evaluation of the gene copies in other gene families with defined and conserved intron positions.

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