Plant Molecular Biology **37:** 1069–1078, 1998.

Maize α-tubulin genes are expressed according to specific patterns of cell differentiation**

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Received 7 November 1997; accepted in revised form 23 March 1998

Key words: in situ hybridization, maize, promoter analysis, tobacco transformation, tubulin

Abstract

In the past few years many α- and β-tubulin genes of different organisms have been cloned and studied, and in most systems studied they constitute multigene families. In plants, most studies have been done in *Arabidopsis thaliana* and *Zea mays*. In this paper, the study of mRNA accumulation by *in situ* hybridization and the activity of three maize α-tubulin gene promoters (*tua1, tua2* and *tua3*) in transgenic tobacco plants are described. In maize, the expression of these three tubulin isotypes differ in the root and shoot apex and is associated with different groups of cells throughout the distinct stages of cell differentiation. In transgenic tobacco plants the promoters of the genes, fused to the *uidA* reporter gene (GUS), direct expression to the same tissues observed by *in situ* hybridization experiments. The *tua1* promoter is mainly active in cortex-producing meristematic cells and in pollen, whereas *tua3* is active in cells which are differentiating to form vascular bundles in the root and shoot apices. The accumulation of *tua2* mRNA is detected by RNA blot in a similar form as *tua1*, but at a very much low level. *In situ* hybridization indicates that the *tua2* mRNA specifically accumulates in the maize root epidermis. No GUS staining was detected in transgenic tobacco plants with the *tua2* promoter. The difference in expression of the specific genes may be linked to processes where microtubules have different functions, suggesting that in plants, as in animals, there are differences in the function of the tubulin isotypes.

Introduction

Microtubules are a component of the filamentous cytoskeleton present in eukaryotic cells and participate in many different cell processes. They play an important role in intracellular transport, are the principal component of structures such as cilia and flagella and the mitotic and meiotic spindle, and, with other cytoskeleton components, they take part in the control of cell shape. In plant cells microtubules have a number of specialized roles, including participation in division and differentiation processes. Due to the existence of the cell wall, differentiation in plants is regulated not by cell migration or changes in the shape of the cells, but by the definition of the plane of cell division and the direction of cell elongation, processes in which microtubules are involved. In this sense, microtubules are at the basis of morphogenetic processes occurring in plant cells.

Plant microtubule arrays change during the cell cycle. During interphase, cortical microtubules lie near the plasma membrane and are believed to be involved in the orientation of deposition of cellulose microfibrils during the growth of the cell wall. In elongating cells, cortical microtubules have a transverse orientation with respect to the axis of elongation, and their

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density is maintained as the cell elongates [11]. In dividing root cells different cortical microtubule densities and orientations have been established in various regions [2], showing that cells preparing for rapid elongation have a transverse orientation of cortical microtubules. The microtubules in isotropically growing cells in the root cap do not show a preferential orientation.

All microtubules are mainly composed of two polypeptides of ca. 50 kDa, α- and β-tubulin, with a sequence that has been highly conserved throughout evolution. These proteins are encoded by families of related sequences dispersed in the genome, with family sizes ranging from one or two genes in simple unicellular systems to 20 or more sequences that cross-hybridize with tubulin-coding sequences in the sea urchin and human genomes [6], although most of these are known to be pseudogenes. Complexity is increased by post-translational modifications, which produce a variable population of tubulins. These phenomena suggest that precise mechanisms control the function of these proteins. In many cases the genes of different isotypes are expressed differentially during development and in response to different stimuli.

Tubulin genes have been studied in only a few plant species. In *Arabidopsis thaliana*, nine β-tubulin genes and six α-tubulin genes are expressed [17, 26], and the expression of tubulin isoforms is heterogeneous within various tissues. One α-tubulin gene is constitutively expressed in all tissues, another is unique to pollen and the others are differentially expressed in roots, leaves and flowers [5]. In maize, three α-tubulin genes have been cloned and sequenced [20, 21], three other cDNAs have been reported [28] and at least one more has been detected by PCR [22]. It has also been reported in maize that certain α- and β-tubulin genes are preferentially expressed in rapidly dividing tissues such as root tips rather than in more mature tissues [16].

Why there are so many tubulins in an organism, with only slight sequence differences, remains an open question. Fulton and Simpson argued in 1976 [9] that each isotype was involved in a specific microtubule array or function; this was called the 'multitubulin hypothesis'. In contrast with this idea of a specific function for each tubulin, a number of results showed that different isotypes could perform the same functions both *in vitro* and *in vivo*, and were functionally interchangeable. In the past few years some differences between isotype functions have been reported in animals [13]. A study of the expression of tubulin genes coding for different isotypes is necessary in order to correlate their expression with their function.

In this study the patterns of expression of three maize α-tubulin genes, *tua1*, *tua2* and *tua3*, were analysed by *in situ* hybridization, showing that these genes are differentially expressed within young tissues. Using the promoter region of these genes fused to the *uidA* gene we also studied transient expression in tobacco protoplasts and in stable transgenic tobacco plants using *Agrobacterium*-mediated transformation. Interestingly, the β-glucuronidase (GUS) activity driven by these promoters in tobacco, reflects the pattern of expression shown by these genes in maize using *in situ* hybridization. The fact that these genes are expressed in different cells indicates that they may be associated with specific processes involving microtubule activity.

Materials and methods

Plasmid constructs

The 1076 bp *Bgl*II-*Bgl*II fragment from the genomic clone MG19/15, containing the *tua3* promoter [20] was cloned into pUC18 vector at the *Bam*HI site. After testing that the 5′ end of the promoter corresponded to the *Hin*dIII side of the polylinker, a *Hin*dIII-*Sma*I fragment of 1115 bp was introduced into the pBI101.1 plasmid [14] digested by these restriction enzymes. The result was a transcriptional fusion between the promoter of the *tua3* gene, the coding region of the *uidA* gene and the *Nos term* polyadenylation signal (3211 bp). The different 5′ deletions were made using single restriction sites in the promoter: *Cla*I, *Nae*I, *Dra*II and *Hpa*I, and by digestion with exonuclease *Bal*31 (see Figure 3A).

Similar fusions with the reporter gene *uidA* and the *Nos term* were made with the *tua1* and *tua2* promoters. The *Xho*I-*Sac*II fragment from the genomic clone MG19/6 [21], corresponding to the *tua1* promoter, and different 5′ deletions were introduced into pBI101.1 using appropriate enzymes [25]. Similarly, a transcriptional fusion between the *tua2* promoter and the *uidA* gene was made by ligating the 1754 bp *Eco*RI-*Sal*I fragment from the genomic clone MG19/6 into pBI101.1.

Transient expression assays

Leaf mesophyll protoplasts of *Nicotiana tabacum* cv. Petit Havana SR1 were isolated from sterile shoot cul-

tures as described by Paszkowski and Saul [24], and resuspended in fusion medium at a concentration of 2×10^6 pps/ml for electroporation. 20 μg of the plasmid and 30 μg of the salmon sperm DNA were added to 0.7 ml of protoplast suspension and an electric pulse of 10 ms and 750 V/cm was applied. The protoplasts were diluted immediately in 10 ml of AA medium [10] and incubated for 24 h in the dark, then collected and washed three times by low speed centrifugation in 250 mM NaCl. The remaining pellets were frozen in liquid nitrogen and stored at −80 °C until the glucuronidase activity was determined.

Tobacco stable transformation

Vectors carrying the different plasmid constructs were introduced into DH5α *Escherichia coli* K12 strain and into *Agrobacterium tumefaciens* LBA4404 strain via transformation of competent *Agrobacterium*. *Agrobacterium* strains were then used to inoculate sterile leaf pieces of *N. tabacum* cv. Petit Havana SR1. After 48 h, transformed tobacco cells were selected from shoot-inducing medium [23] supplied with 100 μg/ml kanamycin and 500 μg/ml carbenicillin. Shootlets were transferred to root-inducing medium supplied with 100 μg/ml kanamycin and 250 μg/ml carbenicillin. T1 seeds were collected and germinated on MS medium supplied with 200 μg/ml kanamycin. Genomic DNA, from the different transgenic plants, was isolated and analysed by Southern blot in order to assure the integration of the transgenic DNA, hybridizing with a probe corresponding to the 5′ *Hin*dIII-*Sna*BI fragment of *uidA* gene.

GUS assay

Histochemical localization of GUS in transgenic plants was performed as described by Jefferson *et al.* [15]. Small pieces of tissue were immersed in a histochemical reaction mixture containing 1 mg/ml X-Gluc in 50 mM sodium phosphate buffer. After a brief imbibition, the reaction was carried out in the dark at 37 °C overnight. Tissues were rinsed several times in 70% ethanol and examined by light microscopy.

'In situ' *hybridization*

The 3′-untranslated region of the tubulin genes (a 361 bp *Xho*I-*Dra*I fragment for *tua1*, a 373 bp *Xho*I-*Dra*I fragment for *tua2*, and a 458 bp *Taq*I-*Dra*I fragment for *tua3*) were subcloned into pBluescript plasmid. These probes are specific for the genes, as shown by Montoliu *et al.* [20, 21]. The plasmids were linearized to obtain sense and antisense riboprobes with T3 and T7 RNA polymerases. Root segments and young shoots from 3- to 5-day old maize seedlings were collected and fixed in absolute ethanol/glacial acetic acid 3:1, dehydrated through ethanol/tert-butyl-alcohol series and embedded in paraffin. DIG-UTP-labelled probes were prepared as described in the supplier's protocols (RNA colour kit for non-radioactive *in situ* hybridization, Amersham) and hybridized to 8 μm sections according to the procedure described by Langdale [18].

Results

mRNA accumulation of tua1, tua2 *and* tua3 *genes in maize*

Previous results obtained by northern analysis had shown that mRNAs corresponding to α1, α2 and α3 tubulin genes are preferentially accumulated in tissues rich in dividing cells, such as root and shoot meristems [16, 20, 21], but with significant differences. The mRNA of *tua1* is accumulated mainly in the root apex and pollen, but also in young shoots and embryos. The accumulation of *tua2* is nearly 100 times less than *tua1* and more specifically in root meristems and in immature cop. *tua3* mRNA is accumulated similarly to *tua1* but it is not present in pollen and is more expressed in immature embryo, in immature cop and in the vascular cylinder of the root. The pattern of mRNA accumulation studied by *in situ* hybridization techniques also produced significant differences.

mRNA accumulation corresponding to the *tua1* gene in the root was found preferentially in specific groups of meristematic cells (Figure 1A, C, E and G). Longitudinal sections showed that the expression was seen in the more apical zone of the meristem, where undifferentiated and dividing cells are more abundant, but not in the quiescent centre, where the rate of division is very slow. In more distal zones expression is concentrated in the cortical cells, preferentially in the vascular cylinder borders (Figure 1A). Hybridizations using transverse sections also showed a greater accumulation of mRNA in the meristem (Figure 1C) and in more mature zones in cells associated with the vascular bundles (Figure 1E), but always in discrete groups throughout the cortex (Figure 1G).

As for *tua1* gene, the expression of *tua3* is detected in specific areas of the root meristem. Longitudinal sections show accumulation of mRNA in cells

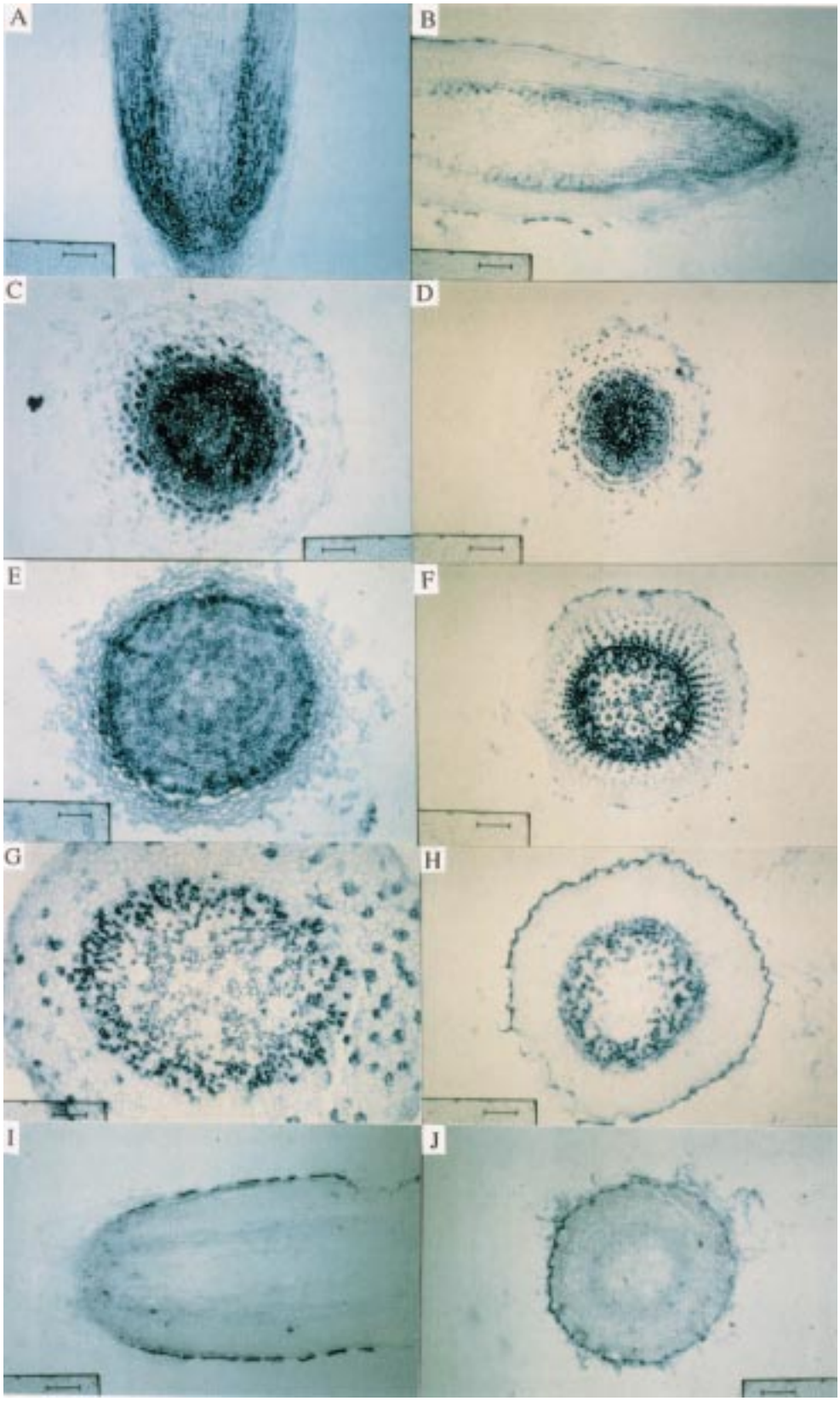
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Figure 1. In situ hybridization of 6-day old maize roots. A (70×) and B (70×) correspond to longitudinal sections hybridized with *tua1* and *tua3* probes, respectively. C (45×), E (45×) and G (70×) correspond to root cross sections hybridized with *tua1* at different distances from the root tip, close to distal. D (45×), F (45×) and H (45×) are hybridized with *tua3*, from close to distal to the tip. I (70×) and J (45×) correspond to longitudinal and cross sections hybridized with *tua2*. There is a difference in expression of all three genes in this zone of root differentiation.

associated with vascular bundles and calyptrogen (Figure 1B, 1D), but, as with *tua1*, there is no expression in the quiescent centre. Differences were observed in the pattern of mRNA accumulation of the two genes in transverse sections. Here, the *tua3* gene is strongly associated with vascular bundles, but mRNA distribution is more or less continuous within the vascular cylinder, although not in the inner cortex or in the external parenchymatic cells in the more mature cells (Figure 1F, 1H), in contrast to *tua1*. With *tua3* there is a low level of mRNA expression in younger cells in these zones. The vascular-associated expression appears to involve all the cells: protoxylematic, protophloematic and associated cells. There is also an accumulation of *tua3* mRNA in the epidermis in zones distal to the root tip (Figure 1H). The small groups of cells with *tua1* expression could be the cells which are probably still undifferentiated. The other cells have reached a later step in differentiation, which the *tua3* gene seems to indicate.

Finally, mRNA levels of *tua2* are much lower than *tua1* or *tua3* in all tissues studied [21]. *In situ* hybridization shows that these low levels are probably due to a very restricted expression of the gene. In roots, *tua2* mRNA is found only in the epidermis and, rarely, in discrete meristematic cells (Figure 1I, 1J) of the root tip. This epidermal expression seems to be in apical undifferentiated cells, while *tua3* mRNA accumulates in epidermal cells more distal to the root tip, probably at a later stage of differentiation. The *in situ* hybridization studies seem to indicate that, in the root apex of maize, the expression of these three isotypes of tubulin is different, being associated with different groups of cells and in different differentiation stages. For all these *in situ* hybridizations sense probes were used simultaneously as negative controls (data not shown).

Promoter-driven expression in transgenic tobacco plants

Results obtained by *in situ* hybridization techniques indicate that *tua1* and *tua3* genes were expressed in defined tissues and groups of cells. The putative full-length promoters of these tubulin genes (see Materials and methods) were fused to the *uidA* gene and introduced into tobacco via *Agrobacterium* transformation. Experiments using *tua1* promoter:GUS fusions in transgenic tobacco plants have shown cell- and tissue-specific expression [25].

Sixteen independent transgenic tobacco plants carrying the *tua3* promoter-*uidA* fusion were generated. The number of copies integrated and the integrity of the cassette were identified in each transgenic plant by Southern analysis. The number of integrated copies was between 1 and 3. The histochemical localization of GUS activity shows that the expression driven by the *tua3* gene promoter observed in maize is maintained in transgenic tobacco plants (Figure 2). Blue staining is detected in root and shoot meristems and, at least in root, is related to meristematic cells, other than those where the *tua1* promoter is active [25]. Thus, *tua3* is expressed in the more apical cells, equivalent to the cells in the calyptrogen, where the expression was detected in maize, and in a distal and central meristematic zone (Figure 2A). In cross sections the staining is in the form of four central spots (Figure 2C), corresponding to the four vascular bundles present in tobacco. This indicates that *tua3* is expressed in cells which are differentiating to vascular bundles.

The pattern of GUS expression driven by the *tua1* promoter is different to that shown by the *tua3* promoter. When transgenic plants containing the *tua1* promoter fused to *uidA* gene were analysed, GUS activity was also detected in the root meristem. This expression is not associated with vascular bundles but with the cortex-producing tissues. In this case a good correlation between blue staining and the presence of dividing cells (stained with haematoxylin-eosin) is observed (Figure 2B, 2D). These differences in GUS expression driven by these two promoters might be, again, a consequence of the organization of different meristematic cells and specific cellular machinery being involved in specific differentiation pathways.

The *tua3* gene promoter also drives expression in other organs, such as secondary forming roots (Figure 2E), shoot apices (Figure 2F), cotyledons (Figure 2G) and pollen grains (Figure 2H). *tua1* is also active in these tissues, except in the shoot meristem. *tua3* expression in the shoot meristem starts in very young plantlets (seedlings around 6 days old) and continues throughout the life of the plant. The localization

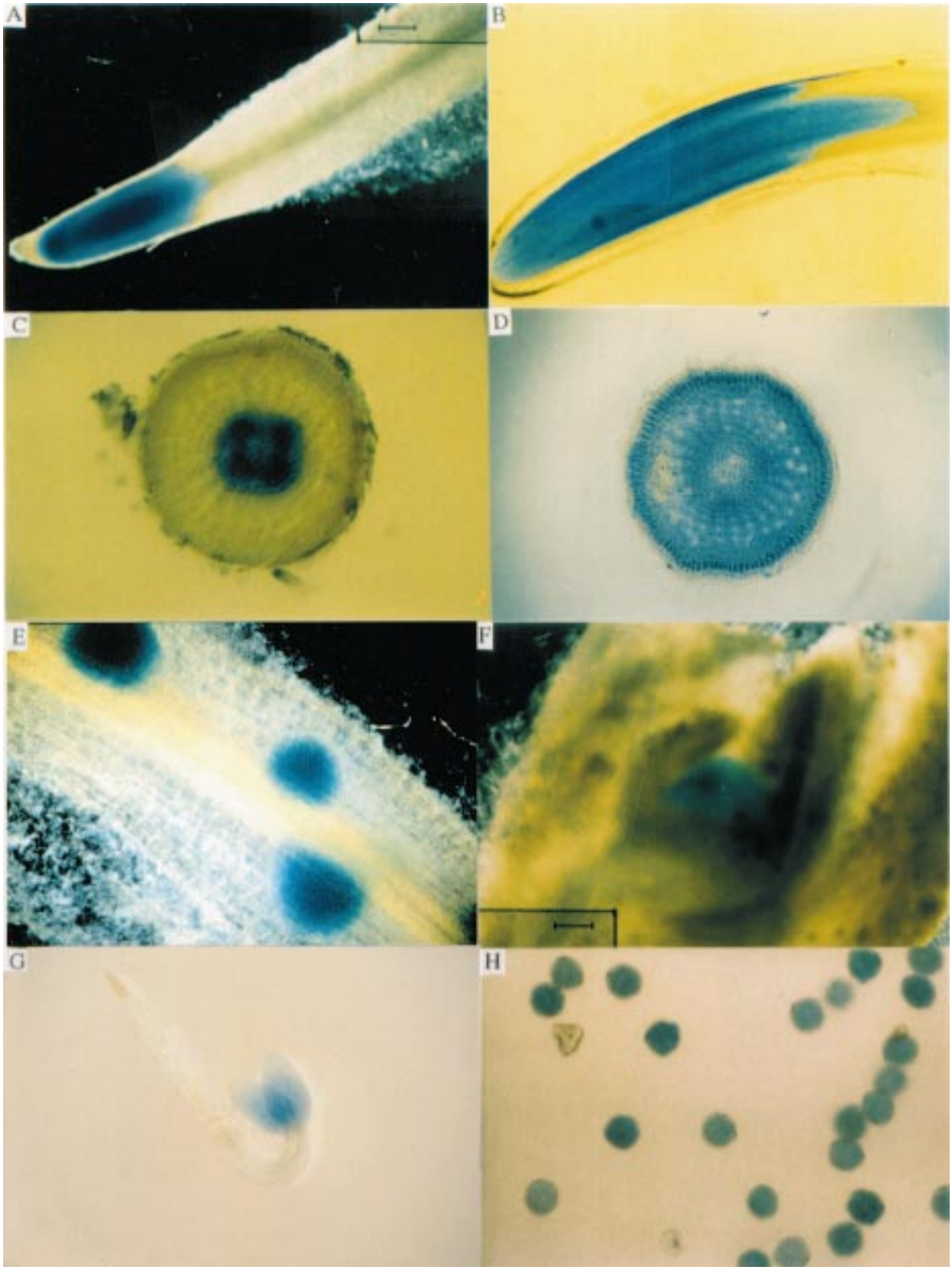
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Figure 2. A to D. Comparison of GUS activity in transgenic tobacco plants transformed with α1- and α3-tubulin promoters fusion genes. A (20×) and C (40×): expression driven by *tua3* promoter, longitudinal and cross sections, respectively. B (20×) and D (40×): expression driven by *tua1* promoter, longitudinal and cross sections, respectively. E to H. Expression induced by the *tua3* promoter in other tissues and organs of transgenic tobacco plants. Blue staining is found in new ramification sites in the root (E: 45×), in the meristem apex (F: 70×), in cotyledons (G: 10×) and in pollen grains (H: 150×).

Table 1. Histochemical analysis of transgenic plants carrying different deletions of *tua3* gene promoter fused to the *uidA* reporter gene. Fraction indicates the proportion of transgenic plants which express the construct by histochemical staining among all transgenic plants obtained.

	Root tip	Shoot meristem	Leaf	Stem	Cotyledons
−1076	8/16	8/16	0/16	0/16	8/16
−633	3/6	3/6	0/6	0/6	3/6
−486	3/10	3/10	0/10	0/10	3/10
−333	0/25	0/25	0/25	0/25	0/25
−159	0/30	0/30	0/30	0/30	0/30

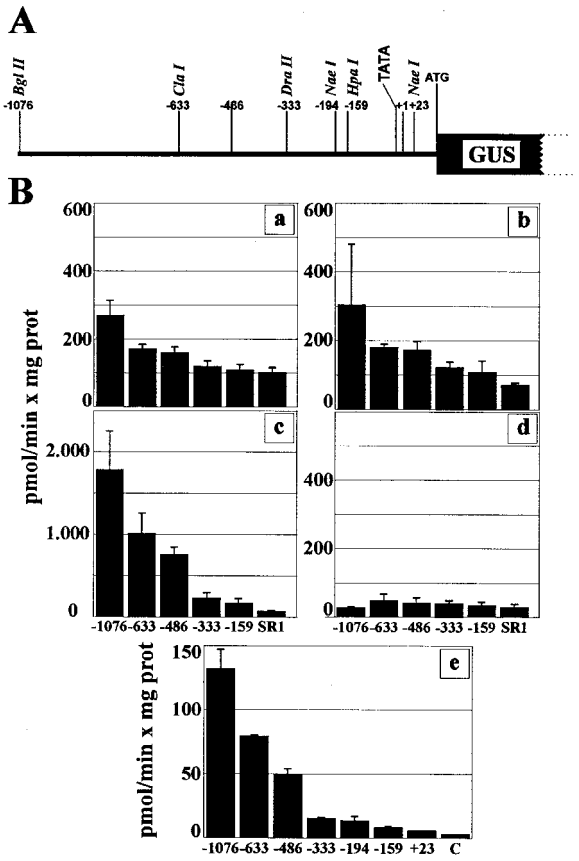

Figure 3. A. Schematic representation of *tua3* transcriptional fusions used for transient and stable transformation of tobacco, and restriction enzymes used to produce the different deletions. The numbers corresponds to the distance from the transcription start. B. Fluorometric assays of plant tissues carrying different deletions of *tua3* promoter. a, root apex; b, shoot apex; c, cotyledons from 5-day old plantlets; d, mature leaf; e, leaf protoplast suspension.

of the blue staining, central and sub-apical, suggests that expression could also be related to vascular tissues. Finally, cotyledon expression is very high around 5 days after germination (Figure 2G), and very similar to the expression of *tua1*. In pollen, there is expression in a relatively small percentage of grains, as compared with *tua1* [25].

When transgenic tobacco plants containing the *tua2* promoter fused to *uidA* gene were analysed, GUS activity was not detected in root tissues or in the other tissues examined, even though some activity is detected in pollen, by fluorometric assays, when the fusion is transiently expressed in tobacco protoplasts (data not shown). The very restricted and low expression of *tua2* gene in maize may explain this result in tobacco.

Expression of tua3 *promoter deletions*

Tobacco plants were transformed with different 5′ deletions of the *tua3* promoter fused to *uidA* gene (Figure 3A), as published with the *tua1* promoter [25]. Expression is found in root and shoot meristems, and cotyledons with the −633 and −486 constructs, as in the case of the −1076 bp (putative full promoter), but no staining is detected with the smaller constructs, −333 bp or less (Table 1).

As the histochemical assay is less sensitive than fluorometric analysis [15], the glucuronidase activity was measured by fluorometry in the different organs where the full *tua3* promoter is active. Results are summarized in Figure 3B. In all cases, higher activity is found in tissues rich in dividing cells and in plants transformed with the longer constructs, but in all cases values are higher than in untransformed plants. In shoot (Figure 3B.b) and root (Figure 3B.a) apices, where there are a high percentage of dividing cells, the largest differences are found when reducing the size of the constructs from −486 to −333 and −1076 to −633, reducing the level of expression to

30–50% of the original construct. In cotyledons these phenomena are more marked, and only 23% of the activity of the −486 construct is conserved in the −333 construct (Figure 3B.c). However, in the mature leaf (Figure 3B.d), an organ with relatively little cell division, the decrease is not significant. These results are quite similar to those observed with the *tua1* promoter deletions [25].

In the different organs analysed there is a high diversity of cell types. The values obtained are an average of the expressing and non-expressing cells. To obtain a homologous population of cells, we developed similar studies in transiently transformed tobacco leaf protoplasts with these different constructs. The results are shown in Figure 3B.e and are similar to the ones obtained with transgenic plants. In protoplasts, there is a continuous decrease of activity concomitant with a decrease in length of promoter, and this decrease in expression is greatest when the −486 construct is reduced to −333 bp, as occurred for cotyledons. In these two organs, where the expression is very high (cotyledons) or the population of non-expressing cells is minimal (protoplasts), there are two promoter control mechanisms working together, one specific between −486 and −333 and the other non-specific and present in full-length promoter. In the other organs this result may be diluted by the non-expressing cells, resulting in less clear values. When compared with the histochemical results, in both cases the major decrease of promoter activity is found when reducing the −486 bp to a −333 bp construct, indicating that the most important *cis* elements of the promoter are present in this zone. Similar results were obtained with the *tua1* promoter [25].

Discussion

The expression patterns of three maize α-tubulin genes (*tua1*, *tua2* and *tua3*) have been studied in maize by *in situ* hybridization, and by histochemical staining in transgenic tobacco plants containing constructs with the respective promoters. The results indicate that, with some exceptions, for these genes the main features of their expression are essentially equivalent in both maize and tobacco plants. Previous studies by RNA blot showed that α-tubulin genes are in general highly expressed in meristematic tissues [16, 20]. With *in situ* hybridization it is possible to distinguish, within a meristem, which cell types express the different genes. Until now, *in situ* hybridization studies have only been used with the *tua4* gene [8]. In this study, when a *tua4* cDNA was used as a probe, meristematic regions were also labelled. α-tubulins are highly conserved genes, so a cDNA probe containing the conserved region of the protein may detect not only the *tua4* transcript, but also mRNA corresponding to other α-tubulin genes. In the *in situ* experiment, using the sense probe of the *tua4* gene as a control, they detected a significant level of signal in some tissues [8]. In our case, using 3′-specific probes, no signal in the sense controls of the *in situ* experiments were found for any of the three *tua1*, *tua2* or *tua3* genes.

In the present study, using probes corresponding to the 3′-untranslated region of these three α-tubulin genes it was possible to observe that the three genes are differentially expressed within the meristematic tip of the root as deduced from the RNA blot results [16, 20]. *tua1* expression appears to be higher in the cortex-producing cells, whereas *tua3* is associated with the cells that differentiate to vascular bundles. Expression of the third gene, *tua2*, is limited to the root apex epidermis and its promoter is not active in transgenic tobacco plants although, due to the fact that GUS assays are usually more sensitive than *in situ* hybridization experiments, the inability to detect GUS activity in tobacco plants carrying the *tua2*-GUS transgene probably could result from a different pattern expression between the tobacco dicot plant and the maize monocot plant.

The cells where these α-tubulin genes appear to be expressed at the highest levels are cells from meristems that differentiate to cells with different morphological features. Vascular cells, for example, are usually very long and parallel to the root axis. This adult cell shape is the result of spatially controlled division and elongation processes [27], in which microtubules participate [19]. It seems reasonable, therefore, to suggest that this particular morphology of the adult cell is the result of elongation and division processes where the *tua3* isotype, which is preferentially taking part, is the most highly expressed. Following this idea, *tua3* would be expressed in provascular cells that undergo division preferentially in a longitudinal axis and subsequently show a concentric deposition of the cellulose microfibrils to the root axis. Therefore, it may be supposed that microtubules taking part in these processes would be composed preferentially by α3 tubulin. In contrast, *tua1* appears to be expressed in cells, such as those in the cortex, where the orientation of divisions is preferentially transversal and periclinal [27],

and its pattern of expression does not seem as strictly controlled as for *tua3*.

It has been proposed that undifferentiated meristematic cells contain all the tubulin isotypes [12, 16] and microtubule arrays [2]. When differentiating, and acquiring different characteristics, the levels of some isotypes increase [16] in order to favour processes in which specific tubulin isotypes may be taking part. Therefore, in those cells in which *tua3* is expressed at high levels, the other α-tubulin isotypes are probably also present [2, 12] but at a lower level [16], and their participation in the same processes would be lower. As the *tua3* gene is preferentially expressed in meristematic cells producing vascular bundles, the processes of differentiation of these cells probably require the participation of tubulin α3.

An interesting data that implies a clear regulation difference between *tua1* and *tua3* promoters is that in transgenic tobacco plants it is possible to induce only the tubulin α3 isotype in response to infection by mycorrhizae, while tubulin α1 is not induced [3]. Mycorrhizal infection is produced in mature root zones, where cells have a large central vacuole and the nucleus is eccentric. Infection causes the reduction of the central vacuole and the repositioning of the nucleus towards the centre of the cell, in a kind of de-differentiating process. Moreover, a restructuring of chromatin is produced, which is probably accompanied by *de novo* expression of many genes [4]. It is then possible that the *tua3* protein may be involved in processes of restructuring cortex cells in the absence of cell division, a feature that would favour a role for this protein in processes of cell differentiation occurring at later stages than those where *tua1* expression is observed.

The expression in the shoot apex is maintained for the *tua3* promoter in the transgenic tobacco plants and lost in the case of the *tua1* gene. As suggested previously, the lack of activity of the *tua1* promoter may be a result of the different structure of the meristem in the shoot apex and differentiation processes of maize and tobacco [25] but, as the *tua3* gene is expressed, and in a similar way in the two species, at least some of the processes appear to be common. This conservation of expression of *tua3* and not of *tua1* seems to indicate that *tua1* is expressed in earlier steps of differentiation, which are more variable in maize and tobacco than later stages. This hypothesis is also supported by the root results where, in longitudinal sections of both maize *in situ* hybridizations and transgenic tobacco root, the *tua1* gene seems to be more highly expressed in zones more apical than those expressing *tua3* (Figure 1).

An interesting pattern of expression, found in both *tua1* and *tua3* transgenic plants is observed in cotyledons. A similar pattern of expression has been reported for other genes [1, 7]. It has been suggested that this pattern corresponds to the expression in the maize embryo, which is not reflected in tobacco but is reproduced in cotyledons [7]. In the case of tubulins, the level of expression is probably so high due to the growth of the cotyledon cells after the first endo-reduplication [1]. This growth occurs in all orientations, and probably needs many different tubulin isotypes, producing a high level of expression of these constructs.

The study of the transgenic tobacco plants carrying different fragments of the promoter of the *tua3* gene gives an indication of the regulation of this promoter. It appears that, in all cases, the largest decrease in expression is found with the −486 to a −333 construct deletions, and this change is more pronounced in cotyledons and protoplasts than in root and shoot meristems (Figure 3). This may be due to the fact that in root and shoot apices there is only a small population of cells expressing the *uidA* reporter gene, as shown by histochemical assays. Due to the high background produced by this ratio between expressing and non-expressing cells, the expression may be diluted by the non-expressing cells, and therefore specific activity values decrease. In cotyledons, the population of non-expressing cells is smaller and the values are not diluted. Similarly, in protoplasts the population of cells expressing the genes may be more homogeneous, reducing the dispersion and increasing the values obtained over the background.

In systems where the promoter activity is high, such as protoplasts and cotyledons, using both *in situ* hybridization and histochemical assays, a larger decrease in expression, or a lack of blue staining, is observed when the length of the *tua3* construct is from −486 to −333. This decrease is maintained, to a lesser degree, in the other systems. It is therefore possible to conclude that in cotyledons and protoplasts, and probably in the other organs, the most important *cis* elements of this promoter are located in this region (see Table 1). Interestingly, in the case of the *tua1* promoter, the major loss of activity is also found between positions −449 and −352 [25], which seems to indicate that, in both tubulin promoters, the main *cis* elements would be located around −400. This effect happens in the absence of any detectable sequence

similarity. It could be possible that in genes where the coding sequence has been so highly conserved as is the case for α-tubulins, the general distribution of controlling elements has also been conserved in the different members of the multigene family.

Acknowledgements

We thank Dr Georges Freyssinet from Rhône-Poulenc Agrochimie for valuable comments and suggestions. The study was supported by a grant from Plan Nacional de Investigación Científica y Técnica (Bio 97/0729), Rhône-Poulenc Agrochimie, within the framework of the Centre de Referència of the Generalitat de Catalunya. X.U. and M.C. were recipients of fellowships from Generalitat de Catalunya. M.A.T. received support from DGICYT (Spain).

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