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SHORT COMMUNICATION

Analysis of a maize α-tubulin gene promoter by transient expression and in transgenic tobacco plants

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Summary

The pattern of expression directed by the promoter of the maize *Tubα1* gene was investigated by analysis of chloramphenicol acetyl transferase (CAT) and β-glucuronidase (GUS) activities in transient expression experiments of maize and tobacco protoplasts. The same promoter was also investigated by histochemical GUS analysis in transgenic tobacco plants containing promoter gene fusions. As determined by histochemical tests, the *Tubα1* promoter gene preferentially directs GUS expression in regenerating root tip meristems and pollen. This pattern corresponds to the distinctive features of natural expression of the gene in maize as determined by Northern analysis. However, no expression is observed in other meristematic tissues of the transgenic tobacco plants, as in shoot apex or in coleoptiles, which is weakly detected in maize. Analysis of the regulatory properties of 5' promoter deletions showed that the proximal region of the promoter, from positions –1410 or –449 to 15 bp upstream of the ATG, is sufficient to establish the qualitative pattern of expression in transgenic tobacco plants. Deletions to positions –352 or –117 abolished the expression in roots, but not in pollen, suggesting that upstream of these positions there are elements responsible for the pattern in root. Further deletions abolished all the promoter activity, suggesting that this promoter region contains the elements essential for expression in pollen. The different patterns and levels of transient and stable expression are discussed.

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Introduction

Microtubules are present in all eukaryotic cells as a major component of the cytoskeleton. They have vital roles in plant growth, in cellular morphogenesis, and in the organization of the cytoplasm. They are indirectly responsible for the morphology of plant cells. Many dividing plant cells show five different microtubule arrays: the cortical array, the pre-prophase band, the mitotic spindle, the phragmoplast, and the radial array (Fosket and Morejohn, 1992). Each of these microtubular systems appears during a particular phase of the cell cycle and is succeeded by a different array as the cycle progresses (Lloyd, 1987; Wick, 1991). Microtubules are mainly composed of the heterodimeric protein tubulin, formed by the non-covalent interaction of α- and β-tubulin subunit polypeptides (Gunning and Hardham, 1982). In fact, tubulin subunits are encoded in eukaryotes by complex families of genes. Up to 21 charge variants of brain tubulin have been observed by isoelectric focusing (Field *et al.*, 1984). Angiosperm tubulins are also heterogeneous (Dawson and Lloyd, 1985; Hussey *et al.*, 1988; Kerr and Carter, 1990).

Tubulin heterogeneity is the result of two mechanisms. First, tubulins are encoded by multigene families in most organisms, including the angiosperms (Fosket, 1989; Silflow *et al.*, 1987). Second, both α- and β-tubulins can be modified by one or more post-translational mechanisms (Cleveland and Sullivan, 1985; Field *et al.*, 1984). Tubulin genes generally exist as 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 in the sea urchin and human genomes (Cleveland and Sullivan, 1985). In mammalian systems some of them are pseudogenes (Lewis *et al.*, 1987). Vertebrates have at least seven expressed α-tubulin genes which encode six different α-tubulin isotypes (Lewis and Cowan, 1988; Pratt and Cleveland, 1988).

α- and β-tubulins have been studied in a few plant species such as maize (Montoliu *et al.*, 1989, 1990; Villemur *et al.*, 1992), *Arabidopsis* (Kopczak *et al.*, 1992), soybean (Han *et al.*, 1991), pea (Liaud *et al.*, 1992) and carrot (Hussey *et al.*, 1988). In these cases multiple α- and β-tubulin genes are found, which are differentially expressed during development. In *Arabidopsis* a thorough analysis of the genes coding tubulins has been

carried out, showing that 15 genes (six α-tubulin genes and nine β-tubulin genes) code for these proteins (Kopczak *et al.*, 1992; Snustad *et al.*, 1992). In maize three α-tubulin genes have been cloned and sequenced (Montoliu *et al.*, 1989, 1990). Recently, three other distinct cDNAs have been reported (Villemur *et al.*, 1992) and at least one more gene can be detected by PCR (Montoliu *et al.*, 1992).

Tubα1 and *Tubα2* from maize form a tandem of genes separated by less than 2 kb and both are expressed in the meristematic tissues of maize, but preferentially in the radicular system (Montoliu *et al.*, 1989). *Tubα1* gene is expressed at a higher level than *Tubα2* and it is also highly expressed in pollen (Montoliu *et al.*, 1990). In *Arabidopsis* a gene has been shown to be preferentially expressed in pollen (Carpenter *et al.*, 1992). None of the genes described in *Arabidopsis* has a pattern of expression similar to the *Tubα1* and *Tubα2* genes of maize. The other *Tubα3* gene described from maize is expressed in all the organs of the plant that are rich in dividing tissues and in particular in the immature embryo (Montoliu *et al.*, 1990).

In the present report, the activity of the promoter of *Tubα1* gene is studied by transient expression, in tobacco and maize (Black Mexican Sweet) protoplasts, and by *Agrobacterium*-mediated stable transformation of tobacco plants. The results indicate that the preferential expression of the *Tubα1* promoter in the radicular system and in pollen is conserved in tobacco. The expression observed in transgenic tobacco plants and in maize by *in situ* hybridization seems to indicate that this gene is expressed in the course of activation of meristem from quiescent centre. The scope of the present report was to analyze the promoter of this gene in an attempt to identify the sequences responsible for the different features of its expression by transient expression in protoplasts and by analysis of GUS activity in transgenic tobacco plants.

Results

Transient expression of the Tubα1 *promoter in protoplasts*

Tubα1 is part of a tandem formed by two genes in maize. The *Tubα1* gene is situated 1.4 kb downstream from another α-tubulin gene, the *Tubα2* gene (Montoliu *et al.*, 1989). Both genes code for almost identical proteins and have a similar genomic structure, although they differ strongly in the sequence and length of their three introns. The two genes are expressed in a similar way, but the level of *Tubα1* mRNA is two orders of magnitude higher than *Tubα2* mRNA. There are only 1449 bp between the stop codon of the *Tubα2* gene and the ATG of the *Tubα1* gene. In this region, a stretch that contains many internally repeated sequences can be found between positions −1034 and −714 relative to the transcription start point of the *Tubα1* gene. A general description of this region and the DNA constructs used in this report are presented in Figure 1.

An initial approach in the study of the promoter of the *Tubα1* gene is to explore its activity in transient expression by protoplast transformation. Such experiments were carried out in tobacco and in maize protoplasts from the Black Mexican Sweet (BMS) variety. Initially, constructs of the intergenic region between the *Tubα1* and *Tubα2* genes and the CAT coding sequence were analyzed by electroporation of tobacco leaf protoplasts and by polyethylene glycol treatment of maize protoplasts, in order to ascertain the promoter strengths in the two species. To build chimeric constructs, advantage was taken of the presence of a *Sac*II restriction site between the site of transcription initiation and the ATG 10 bp upstream from this point (see Figure 1). The constructs were done by isolation of the promoter restriction fragments and ligation to the vector containing the CAT gene (−1410). A series of 5' deletions of the promoter sequence were also subcloned (−956, −449, −352, −297, −252, −184, −117, −64, and −3). The result of the CAT activity measurements of these three constructs in tobacco and maize protoplasts is shown in Figure 2(a). The promoter constructs appeared to be functional in both tobacco and maize protoplasts, but the level of expression was higher in the homologous species. A control with a construct having the 35S promoter with CAT revealed that this promoter was 1.5 times more active than the *Tubα1* promoter in maize protoplasts (not shown). The level of CAT activity was similar when the

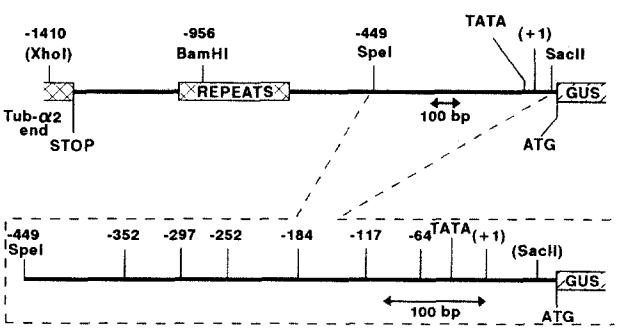

Figure 1. Schematic representation of the chimeric *Tubα1* promoter-GUS constructs.
The numbers are given relative to the transcription start site. The upper part of the figure corresponds to the intergenic region between the *Tubα2* and *Tubα1* genes and the 5' deletions were made by restriction in the appropriate sites. The lower part of the figure corresponds to the −449 fragment where deletions were made by Exonuclease III digestion at different times. The *Sac*II site (+48) corresponds to the transcriptional fusion point between the promoter and the pBI101.1 plasmid. (+1) Corresponds to the transcription start site of the *Tubα1* gene.

−1410 and −956 constructs were used and decreased significantly for the −449 construct.

A more detailed study was carried out in tobacco protoplasts using a GUS assay (Figure 2b). A construct that contained the full intergenic region between genes *Tubα1* and *Tubα2* showed the highest activity and this was steadily reduced when the promoter length was gradually shortened (−956 or −449 constructs). The expression in tobacco protoplasts was drastically reduced in subsequent deletions reaching its lowest value at −252, then a slight increase in expression was observed at −184 and −117 and a basal level appeared when approaching the TATA box. These results indicated that, although some quantitative elements could be present between positions −1410 and −449, most of the promoter signals are probably present between the −449 position and the TATA box.

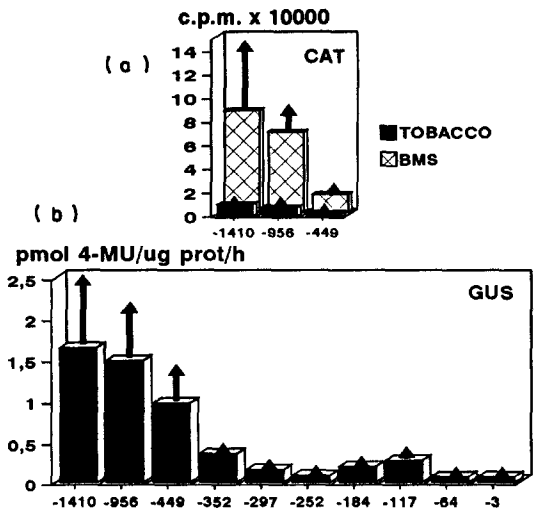

Figure 2. CAT and GUS activity induced by the chimeric constructs in BMS (maize) and tobacco protoplasts.
CAT activity is measured in c.p.m. and GUS activity in piromoles of 4-methyl-umbeliferone per microgram of protein per hour.
(a) CAT activity. Black boxes correspond to the levels obtained in tobacco protoplasts, and crossed boxes to those obtained in BMS protoplasts.
(b) GUS activity in tobacco protoplasts measured fluorometrically. Individual bars represent the average of three independent experiments and the arrows indicate the standard deviation.

Expression in transgenic tobacco plants

The transient expression results indicated that the maize *Tubα1* promoter was active in tobacco protoplasts. It was important to check the qualitative features of its expression in the plant. To this end tobacco plants were transformed with different constructs using Ti plasmid transformation via *Agrobacterium tumefaciens*. The expression of a construct with the GUS reporter gene having a promoter fragment from position −1410 to the *Sac*II site (see Figure 1), shows a pattern of expression with most of the qualitative features observed naturally in maize (Montoliu *et al.*, 1989). Twenty independently transformed plants resistant to kanamycin were obtained and 12 of these showed GUS activity. The pattern of expression was, in general, the same in all the plants transformed with identical constructs (see Table 1). Table 1 shows the results of the histological GUS assay from a number of independent transgenic plants containing the different constructs and tissues analyzed. All the transgenic plants showing expression had at least one copy of the GUS gene, which was transmitted in a Mendelian way to the F_1 progeny. In general, the level of GUS activity was correlated to the number of copies of the gene as measured by Southern blots (result not shown).

In Figure 3 the patterns of GUS expression in transgenic tobacco plants using the maize *Tubα1* promoter are presented. The promoter is active in the root tip of the transgenic plants showing a clear tissue specificity. For instance, in the root cap no expression was detected (Figure 3a and b). Weak activity was detected in the primary roots of the germinating seed. Roots from *in vitro* tobacco cultured transformed plants were sectioned (ca. 0.5 cm long) and the root apex was eliminated to induce root regeneration. The chimeric gene was then highly expressed in these new formed roots and only in radicular

Table 1. Histochemical analysis of the transgenic plants

	Tissue				
Construct	Root tip	Shoot tip	Leaf	Stem	Pollen
−1410	+ (12/12)	− (12/12)	− (12/12)	− (12/12)	+ (12/12)
−956	+ (6/6)	− (6/6)	− (6/6)	− (6/6)	+ (6/6)
−449	+ (8/8)	− (8/8)	− (8/8)	− (8/8)	+ (8/8)
−352	− (12/13)	− (13/13)	− (13/13)	− (13/13)	+ (6/10)
−117	− (9/9)	− (9/9)	− (9/9)	− (9/9)	+ (4/9)
−64	− (11/11)	− (11/11)	− (11/11)	− (11/11)	− (11/11)

Numerals represent the number of plants with (+) or without expression (−) (blue stained), out of the total plants analyzed. Data are shown for each construct in different parts of the plant.

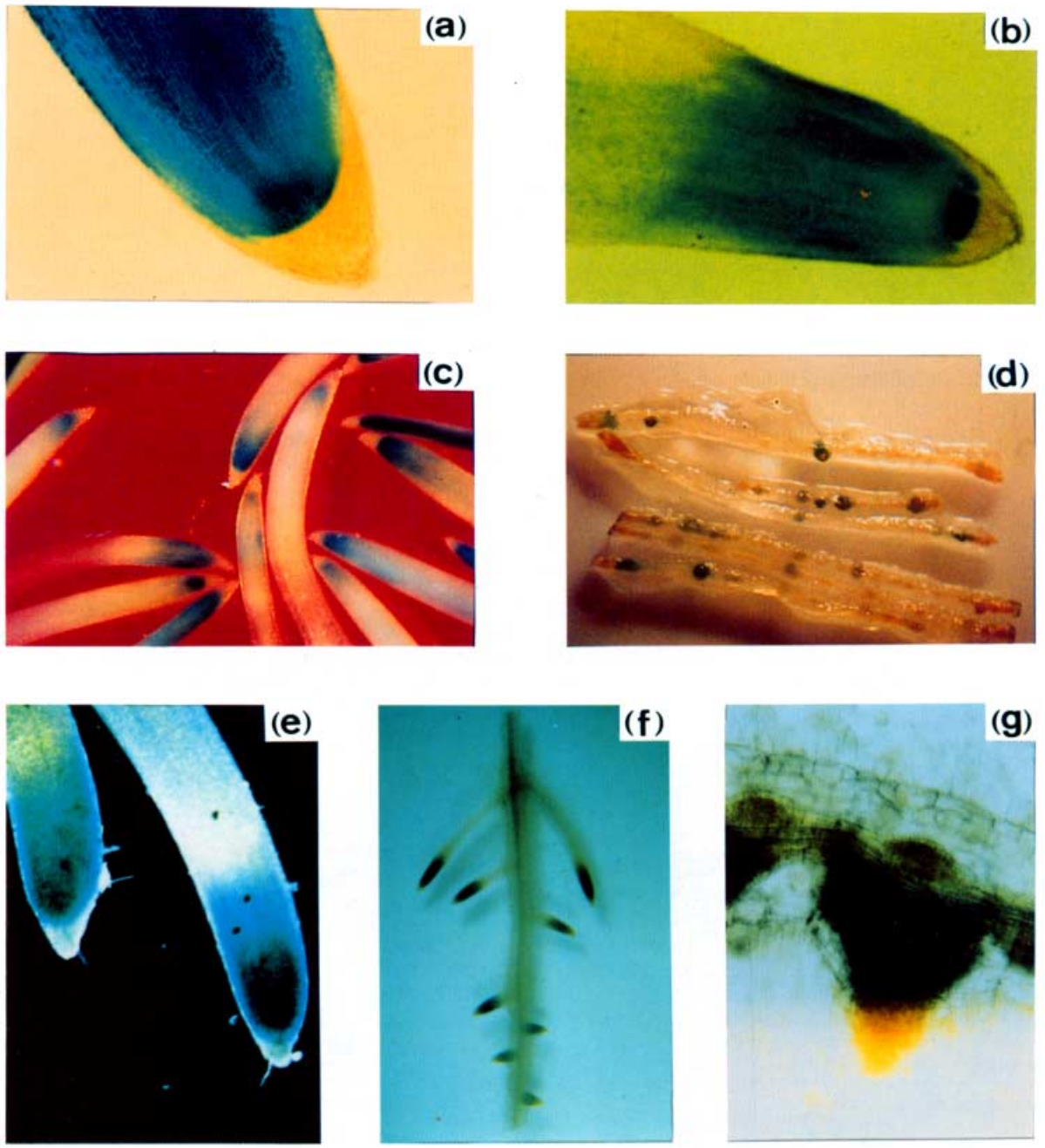

Figure 3. Histochemical localization of GUS activity obtained by transcriptional fusions with the *Tubα1* promoter in tobacco root tip (a: 35×, b: 35×). The localization of GUS is the same for the −1410, −956 and −449 constructs. Root tips of regenerating roots (c: 6×). Auxin-induced regenerating secondary roots after cutting the tip (d: 10×, f: 5×, g: 35×).

regions where meristematic cells are abundant. In these new formed roots the activity is present in all cases (Figure 3c and f) and the expression in the meristematic regions is very clear when the quiescent center is induced in these roots (Feldman, 1984). Isolated roots from the transgenic plants were induced to lateral root formation by culture with auxin. In this case the appearance of the buds can easily be visualized by the initiation of the GUS activity (Figure 3d and g).

The *Tubα1* gene shows a high level of expression in the pollen of maize. This feature is also found in transgenic tobacco plants. GUS activity can be observed in pollen grains of plants containing the −1410 construct (Figure 4a). No activity is found in the anther tissue

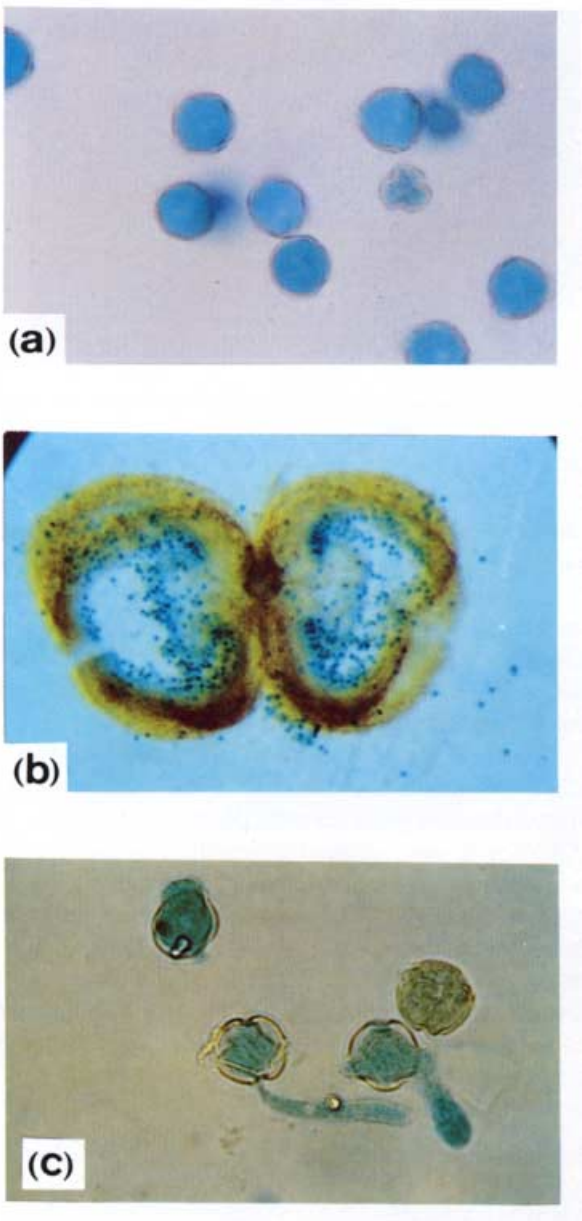

Figure 4. Histochemical localization of GUS activity in pollen (a: 170×) and anthers (b: 30×) of tobacco transgenic plants transformed with the −1410 construct of *Tubα1* promoter.
The same was obtained by the other positive pollen constructs (from −1410 to −117). Expression in a pollen tube is also shown (c: 170×).

(Figure 4b). Between the −1410 and the −449 deletions, the root tip pattern is maintained (see Table 1). No plant shows expression in roots in construct −352, except for a single plant that has seven or eight copies incorporated in the genome. In a large proportion of the transformed plants the expression in pollen is observed up to the −117 deletion. In the −64 deletion no GUS activity is found in any of the tissues analyzed.

The GUS activity found in the transgenic plants using the *Tubα1* promoter correlates in general terms with the expression of this gene in maize. In maize the gene is preferentially expressed in the radicular meristems (Montoliu *et al.*, 1989) and in pollen (Montoliu *et al.*, 1989). In order to study this expression, *in situ* hybridization experiments were carried out in maize root tips using a specific *Tubα1* probe (Montoliu *et al.*, 1989). It appears that this gene is not expressed in the quiescent center of maize (Figure 5a and b), and it is highly expressed in the cells surrounding this organ in the central root meristem (Figure 5a). No expression of any of the constructs with the *Tubα1* promoter was observed in other meristematic regions than in roots, although a weak general expression in meristems was observed in maize.

Discussion

The *Tubα1* gene in maize is expressed in meristematic tissues with a preferential accumulation of its mRNA in root tips and pollen (Montoliu *et al.*, 1989). In maize it shows high levels of transcription in the root tip, mainly around the quiescent center (Figure 5a). In the present report the promoter of the maize *Tubα1* gene is studied by transient expression in tobacco and maize (BMS) protoplasts, and in stable *Agrobacterium*-mediated transformed tobacco plants.

Transcriptional fusions of the *Tubα1* promoter and the GUS reporter gene show transient expression activity in transfection experiments of protoplasts from tobacco leaves and from maize suspension cells (BMS), although the activity is always higher (around six times) in the homologous system than in the heterologous system. The level of activity in tobacco protoplasts of the different 5' deletions of the promoter decreases in 5' shorter promoter fragments indicating a loss of different enhancing elements of the promoter. A reproducible low activity appears in deletion −252, which could correspond to the presence of a specific silencer between −252 and −184. Between the −252 and −64 deletions there is a moderate increase in expression.

Tobacco transgenic plants stably transformed with these constructs show that the pattern of expression observed for this gene in tobacco is the same for all the constructs from deletions −1410 to −449. These constructs are the same as those that have a higher level of expression in protoplasts. The transgenic plants with these constructs show blue stained root tip and pollen in histochemical analysis (Figures 3 and 4). This specific pattern is maintained in 5' promoter deletions until −449 and in one case probably by overexpression due to the high copy number of the chimeric gene containing the −352 deletion. At this stage there is only activity in pollen, showing that *cis*-acting sequence(s) important for root expression are located upstream of deletion −352. With the −352 and −117 constructs around 50% of the trans-

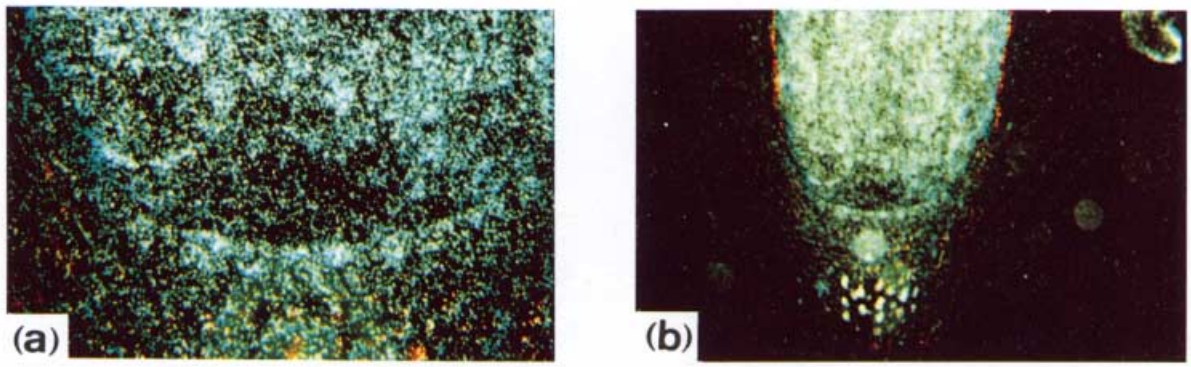

Figure 5. *In situ* hybridization of the maize root tips, using a specific ^{35}S-labelled probe corresponding to the 3'-untranslated region of the *Tubα1* gene (b: 35×).
(a: 120×) An enlargement of the area around the quiescent center zone of root tip.

genic plants have a pollen-positive GUS pattern, indicating that the root and pollen expression can be separated, but that the control of the expression by this promoter is not strong in this heterologous system. Only with the –64 deletion is it possible to eliminate the pollen pattern.

If anthers from different stages of maturity are observed, the beginning of GUS activity can be approximately correlated with the stages reached by the pollen cells at the end of mitotic division, and the activity can be observed even during the formation of the pollen tube (Figure 4c). Microspore mitosis appears to be a critical point in commitment to the gametophytic pathway. The major developmental switch in gene expression occurs at this time (Bedinger and Edgerton, 1990; Mandaron *et al.*, 1990). Studies of pollen-specific gene expression are consistent with this hypothesis, in that most of such genes appear to become actively transcribed only after microspore mitosis (McCormick, 1991; Stinson *et al.*, 1987).

The root expression pattern is mainly found in regenerating roots, and it is not observed, or is very weak, in the new roots formed from seeds. This indicates that this gene is highly expressed when root formation is induced, as when the root tip is cut. That is, the *Tubα1* gene promoter is expressed in roots during the activation of the quiescent centre (Feldman, 1984). In maize, activation is not necessary for the observation of *Tubα1* transcription by Northern analysis, although in tobacco strong activation is required. Another difference exists between the expression found in maize and tobacco. This difference appears mainly in the expression of the *Tubα1* gene in meristems other than the radicular ones in maize, a feature not found in transgenic tobacco plants. The difference may be due either to the fact that the activity is too low to be detected or to the loss of this feature of *Tubα1* expression in a heterologous system. This loss may be either because specific *cis*-acting sequences are not recognized by the protein factors in the heterologous system or because the cells, where these factors are present, have a different developmental stage or role in the formation of the meristems. Indeed, the distribution of cell types in the developing root is very different in maize and tobacco. The transgenic plants obtained with these constructs may be useful in the analysis of these aspects.

These results also confirm the hypothesis that α-tubulins in plants are encoded by different genes with specific transcriptional controls (Fosket *et al.*, 1992). The *Tubα1* gene contains specific regulatory sequences in its promoter that direct the expression of this α-tubulin to specific cell types. In some cases the factors controlling these sequences are ubiquitous in plants, as seems to be the case in the recognition of maize sequences by tobacco factors during the induction of the quiescent center and in root specificity. Analysis of these promoters may be useful in the study of these phenomena.

Experimental procedures

Plasmid constructs

The 1588 bp *Xho*I–*Alu*I DNA promoter fragment from the MG19/6 genomic clone (Montoliu *et al.*, 1989) of the *Tubα1* gene was inserted into the pUC18 plasmid. From this, the 1474 bp *Hin*dIII-filled/*Sac*II DNA fragment was inserted into the *Hin*dIII and *Sma*I digested pBI101.1 plasmid (Jefferson *et al.*, 1987), resulting in a transcriptional fusion of the *Tubα1* promoter with the encoding region of the GUS gene and the *Nos term* polyadenylation signal (–1410 construct). Likewise, the same fragment was inserted into the *Hin*dIII-filled/*Bam*HI sites of the pRPA-BL-504 plasmid (plasmid similar to pBI101.1, except that the CAT reporter gene replaced the GUS gene, which was kindly provided by Dr Bernard Leroux, Rhône-Poulenc), resulting in a transcriptional fusion of the *Tubα1* promoter with the coding region of the CAT gene and the *Nos term* polyadenylation signal. Two deletions were effected by cutting with *Bam*HI (–956) and *Spe*I (–449) and recircularization. Seven more deletions (–352, –297, –252, –184, –117, –64, –3) were obtained after different times of digestion with ExonucleaseIII (see Figure 1).

Transient expression assays

Tobacco protoplasts. Leaf mesophyll protoplasts (pps) of *Nicotiana tabacum* cv. Petit Havana SR1 were isolated from sterile shoot cultures as described by Paszkowski and Saul (1986) and resuspended in fusion medium at a concentration of 2×10^6 p.p.s. ml^{-1} for electroporation. Fifteen micrograms of the plasmid and 35 μg of salmon sperm DNA were added to 0.7 ml protoplast suspension and an electric pulse of 10 msec and 750 V cm^{-1} was applied. After electroporation the protoplasts were diluted immediately in 10 ml of 'To' medium and incubated for 24 h in the dark. This time was determined previously to be the optimal for the level of expression of these constructs in protoplasts. Protoplasts were collected and washed twice in 250 mM NaCl to eliminate the remaining enzymes. The pellet was frozen in liquid nitrogen and stored at –80°C. The fluorometric GUS assay was performed following the protocol described by Jefferson (1987), modified by adding methanol to the assay buffer as suggested by Kosugi *et al.* (1990). Measurements were carried out with a TKO 100 MINI-Fluorometer (Hoefer Scientific Instruments, San Francisco, CA).

Maize protoplasts. Maize protoplasts were obtained from a *Zea mays* var. Black Mexican Sweet (BMS) cell suspension maintained continuously in MSE medium consisting of MS salts and vitamins (Murashige and Skoog, 1962), 3% sucrose, 1 mg l^{-1} 2,4-D, 0.02 mg l^{-1} BAP, pH adjusted to 6.3 before autoclaving. Cells were grown in 250 ml erlenmeyer flasks at 130 r.p.m. and 28°C in the dark. Medium was changed every 3 days and diluted (1:3) after 6 days. For protoplasting, cells were diluted (1:2) 2 days before the transformation. Protoplasts were isolated from cell suspension cultures and transformed by polyethylene glycol following the method described by Amstrong *et al.* (1990). After transformation, protoplasts were collected by centrifugation at 100 ***g***, and the supernatant was removed, resuspended in 8 ml N6 medium (Chu *et al.,* 1975) and incubated for 48 h. Protoplasts were then collected and washed twice in 250 mM NaCl. The protoplasts were frozen in liquid nitrogen and stored at –20°C. Two hundred microliters of 0.25 M Tris–HCl (pH 7.8) were added to the tubes containing the frozen protoplasts. After sonication the samples were centrifuged for 5 min at 4°C to remove cellular debris. Supernatants were transferred to new tubes, incubated at 65°C for 10 min to inactivate endogenous acetylases, and centrifuged for 5 min at 4°C. The new supernatants were transferred to new tubes. The chloramphenicol acetyltransferase (CAT) assay was performed following the method described by Gorman *et al.* (1982). CAT activity was determined by scintillation counting of the acetylated ^{14}C-chloramphenicol forms present in the corresponding spots excised from the TLC plate.

Tobacco stable transformation

Vectors carrying the plasmid constructs were introduced into DH5α *Escherichia coli* K12 strain by transformation and into LBA4404 strain of *Agrobacterium tumefaciens* via triparental mating or transformation of competent *Agrobacterium*. The *Agrobacterium* cells were then used to inoculate sterile leaf discs of *Nicotiana tabacum* L. cv. Petit Havana SR1. Transformed tobacco cells were selected in a shoot-inducing medium containing 100 μg ml^{-1} kanamycin and 500 μg ml^{-1} carbenicillin. Regenerated shootlets were rooted in a root-inducing medium containing 100 μg ml^{-1} kanamycin and 250 μg ml^{-1} carbenicillin. Plants were grown in the greenhouse and F_1 seeds were collected and germinated in a medium with 200 μg ml^{-1} kanamycin. Genomic DNA from transgenic plants was analyzed by Southern blotting to determine the number of incorporated copies of the GUS gene and possible recombinations. This was carried out by digesting genomic DNA by *Hin*dIII and by *Hin*dIII plus *Eco*RI, and hybridizing with the *Hin*dIII–*Sna* BI fragment of the GUS gene as probe.

Histochemical GUS assay

The histochemical localization of GUS in transformed plants was performed essentially as described by Jefferson (1987). Small pieces of several tissues were immersed in a histochemical reaction mixture containing 1 mg ml^{-1} X-Gluc in 50 mM sodium phosphate buffer (pH 7). The histochemical reaction was performed in the dark at 37°C until a blue indigo color appeared. Tissues were rinsed several times in 50 mM of phosphate buffer to stop the reaction, rinsed in 70% v/v ethanol and examined by light microscopy.

Other methods

Root cultures were established in solid and liquid MS medium (Murashighe and Skoog, 1962) supplemented with 3% sucrose and 0.5 mg l^{-1} NAA and cultured in the dark.

In situ hybridization experiments were carried out by the method described by Langdale *et al.* (1988).

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