Plant Molecular Biology **15**: 905–912, 1990.

Analysis of an ABA-responsive rice gene promoter in transgenic tobacco

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Received 23 May 1990; accepted in revised form 20 August 1990

Key words: rice, developmental regulation, embryo-specific

Abstract

We have analyzed in transgenic tobacco the expression of a chimeric gene containing 5′ sequences of the rice *rab-16B* gene fused to the β-glucuronidase (GUS) reporter gene. This construct, a translational fusion (−482 to +184) including 14 amino acids of the RAB-16B protein, is expressed only in zygotic and pollen-derived embryos. In zygotic embryos, GUS activity begins to accumulate 10 days after flowering (daf), and increases until seed maturation at 25 daf. Immunological measurements of endogenous abscisic acid (ABA) accumulation in these seeds showed a close parallel between hormone levels and GUS activity. However, GUS activity could not be reproducibly induced by treatment of immature embryos with ABA (10 μM). Neither GUS activity nor GUS mRNA could be detected in leaves of transgenic tobacco even after ABA treatment. In contrast, GUS activity could be induced to high levels in pollen-derived embryos by treatment with ABA. Our results show that 482 bp of 5′ sequences of the rice *rab-16B* promoter can confer in transgenic tobacco developmentally regulated expression in embryos but not ABA-responsive expression in vegetative tissues.

Introduction

The phytohormone abscisic acid (ABA) mediates the response of plants to osmotic stress and acts as a developmental cue during embryogenesis and seed maturation [6, 17 for review]. ABA has been shown to induce in vegetative tissues the expression of genes which are normally expressed during late embryogenesis [2, 8, 10, 11]. Some of these genes can also be induced in vegetative tissues by water-stress and it has been suggested that their gene products play a role in osmoprotection or regulation during desiccation [7].

As part of our studies on the regulation of gene expression by ABA, we are analyzing the regulatory regions of membranes of the rice *rab-16* (*r*esponsive to *AB*A) gene family. The four members of this family, *rab-16A–D*, encode conserved proteins of 15–17 kDa, and are tandemly arrayed in a locus of 30 kb [19]. Like other ABA-responsive genes, the *rab-16* rice genes are induced in various rice plant organs by the hormone and by osmotic stress. Previous work using rice protoplasts as a transient expression system allowed for the identification of an upstream region of the *rab-16A* gene that is responsive to

ABA [13]. This region contains sequence motifs conserved in regions of *rab-16B–D* and in some ABA-responsive genes from other species [2, 19]. Furthermore, these motifs are part of sequences that interact with nuclear protein factors, suggesting they may be ABA-responsive regulatory elements [12].

The *rab-16A* and *rab-16B* genes also show stage-specific expression in embryos during seed development [11, 19]. Ideally, the upstream sequences responsible for this organ specificity could be assayed in transgenic rice. Although this transformation technology has been established [15], it is not yet practical for large-scale expression studies. We therefore used transgenic tobacco as an expression system. To this end, we prepared chimeric constructs of the 5′ upstream sequences of the *rab-16A* and *rab-16B* genes fused to the β-glucuronidase (GUS) reporter gene. We report here on the activity of these constructs in tobacco tissues.

Materials and methods

DNA constructions

Sequences from the 5′ regions of the rice *rab-16A* gene (− 442 to + 132, including amino acids 1–15 of the RAB-16A protein) and of the *rab-16B* gene (− 482 to + 184, including amino acids 1–14 of the RAB-16B protein) were fused to the GUS coding sequence at the unique *Sal* I site of pBI101 (Clontech). The resulting translational fusions encode the N-terminal amino acids of the RAB 16A & B proteins noted above followed by 10 amino acids encoded by the pBI101.1 polylinker and the GUS 5′ leader in frame with the GUS open reading frame. The *Eco* RI and *Hin*d III fragments of these vectors (*rab* upstream/GUS/NOS 3′) were isolated, made blunt-ended, and cloned into the *Hpa* I site of the vector pMON505 [3]. The vector also contains at its polylinker site a chimeric gene comprising the 35S promoter (− 941 to + 81) fused to the chloramphenicol acetyl transferase (CAT) coding sequence. Transformants were selected by assaying for CAT activity.

Transgenic plants

Transformation of *Nicotiana tabacum* cv. SR1 was performed as previously described [4]. R1 progeny of selfed, primary transformants were selected on MS medium containing 100 μg/ml kanamycin and 50 μg/ml carbenicillin. Seedlings were transferred to plastic containers in MS medium, then transferred to soil and grown at 26 °C, 16 h light and 22 °C, 8 h dark.

Organ treatments and GUS enzyme assays

GUS activity was assayed in whole 7-day old seedlings by histochemical staining [4]. Seedlings were removed from containers and incubated at room temperature for 24 h in the dark in distilled water with or without 10^{-5} M ABA. They were then blotted dry and transferred to 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) solution. GUS activities in organs (seeds, flowers, leaves, stems and roots) of 6-week old plants, flowering plants and plants bearing capsules, were measured fluorometrically [4]. Fully expanded 4th and 5th leaves were excised, cut into pieces (ca. 5 × 5 mm) and incubated with ABA. Flowers, stems, and roots were treated similarly. Mature seeds were not treated with ABA. Histochemical staining of encapsulated, developing seeds was performed on 200 μm sections of capsule prepared with a cryotome.

Measurements of ABA and GUS in developing seeds

The developmental stages of R2 seeds used for the assays were determined by tagging R1 flowers when petals had fully expanded (0 daf). At various intervals hereafter, seeds were removed from the capsules, divided into two portions, frozen in liquid N_2 and stored at − 80 °C. Samples for ABA determination were lyophilized before use.

Extraction and immunoassay of ABA were performed under dim light by standard procedures [18]. Samples were extracted in 80% acetone, and insoluble material removed by cen-

trifugation. Acetone was removed under vacuum and the residue suspended in 1% $NaHCO_3$. This solution was partitioned against ethyl acetate and the pH of the aqueous phase adjusted to 2.5 with HCl. ABA was extracted from the aqueous phase with methylene chloride, which was then dried under vacuum. The final residue was resuspended in 50 mM Tris-HCl, pH 7.5, and ABA was measured by immunological assay according to the manufacturer's instructions (Phyto detek-ABA, Idetic, Inc.). Control experiments showed that the recovery of ABA by this extraction procedure is better than 95%.

RNA extraction and northern blot analysis

Total RNA was extracted from frozen seed samples as described by Nagy *et al.* [13], electrophoresed in formaldehyde gels, and blotted according to standard protocols. rRNA concentrations and integrity were monitored after electrophoresis by staining. GUS mRNA was detected with a probe derived from the 480 bp *Xho* I/*Sna* BI fragment of X-GUS-90 [3] which contains 380 bp of the GUS coding sequence. Osmotin mRNA, used as a control, was detected with a 45-base antisense oligonucleotide derived from the coding region (bases 76–121) of the osmotin gene [16].

Pollen embryo culture

Anthers containing uninucleate stage pollen were collected from homozygous plants and placed in tobacco anther culture medium with activated charcoal [14]. The same medium supplemented with 0.2 mg/l 2,4-D was used to produce pollen calli. Pollen calli, or embryos, were collected and stained with X-gluc after treatment with 10^{-5} M ABA for 24 h.

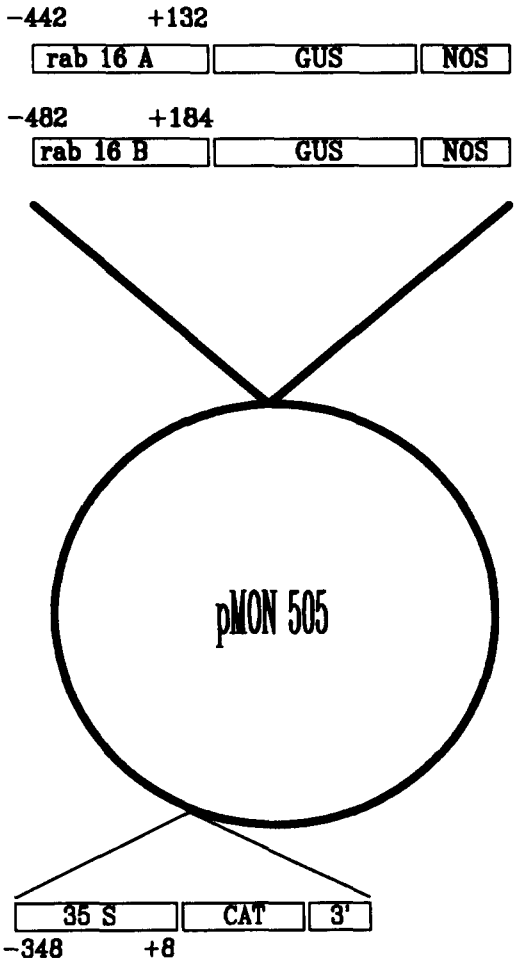

Fig. 1. Structure of the *rab-16A* and *rab-16B*/GUS fusion genes. See Materials and methods for details.

Results

Expression of rab-16A and rab-16B/GUS fusion genes in transgenic tobacco

Figure 1 shows the structure of the translation fusions between the upstream regions of the *rab-16A* and *rab-16B* genes and GUS. No GUS activity was detected in any tissue of R1 plants containing the *rab-16A* upstream sequences. Transcriptional *rab-16A*/GUS fusion genes were also not expressed in any organs of transgenic plants (J. Mundy, unpublished). Similarly, in transgenic plants obtained with *rab-16B* fusion, no enzyme activity could be measured in ABA-

Table 1. GUS enzyme activity in mature R2 seeds of transgenic tobacco.

		Independent transgenic plants		
Wild-type	A	B	C	D
26	25063	21458	16014	14347

Means of GUS enzyme activity (pmol 4MU/min/mg protein) are indicated. The values are the average of three different plants derived from the same primary transformant.

treated or untreated vegetative tissues, at any time during the plants' life cycle. Control experiments showed that osmotin mRNA in leaf tissue accumulated within 24 h after ABA treatment (data not shown). We therefore conclude that there is no transcription of the *rab-16B*/GUS fusion gene in vegetative tissue. However, the assays showed that there was considerable GUS activity in seeds of these R1 transgenic plants whereas there is essentially none in seeds of wild-type plants (Table 1). Similar values were obtained for seeds harvested from the primary transformants (Ro; data not shown).

In order to precisely localize the GUS activity in seeds, the enzyme was assayed histochemically in sectioned, mature capsules. These experiments showed that GUS was expressed in the entire embryonic tissue (Fig. 2A). Closer examination showed no activity in placental tissues or in the seed coat or endosperm. Interestingly, no activity was detected in 1-week old seedlings, suggesting that the expression of the reporter gene is arrested and that the accumulated GUS mRNA and protein are degraded early during germination.

Developmental expression of the rab-16B/GUS fusion gene

To further analyze the time-course of expression of the *rab-16B*/GUS gene, we measured the GUS activity histochemically and fluorometrically at 5-day intervals during seed development. No significant levels of GUS activity were detected until 10 daf (Fig. 3). However, between 10 and 25 daf, the activity increased dramatically about 180-fold. Histochemical staining clearly shows this increase in Fig. 2B–D. Northern analysis revealed that GUS mRNA accumulates at 15 daf, reaching a maximum level at around 20–25 daf (Fig. 4A). The tobacco osmotin mRNA [16] shows a similar pattern of accumulation (Fig. 4B).

The induction and time course of accumulation of GUS protein and mRNA parallel the increase in the endogenous ABA level (Figs. 3 & 4). This suggests that the *rab-16B* upstream sequences may be responsive to the levels of endogenous ABA in seeds. This is further supported by the fact that osmotin mRNA, from the ABA-responsive tobacco gene [16], shows the same

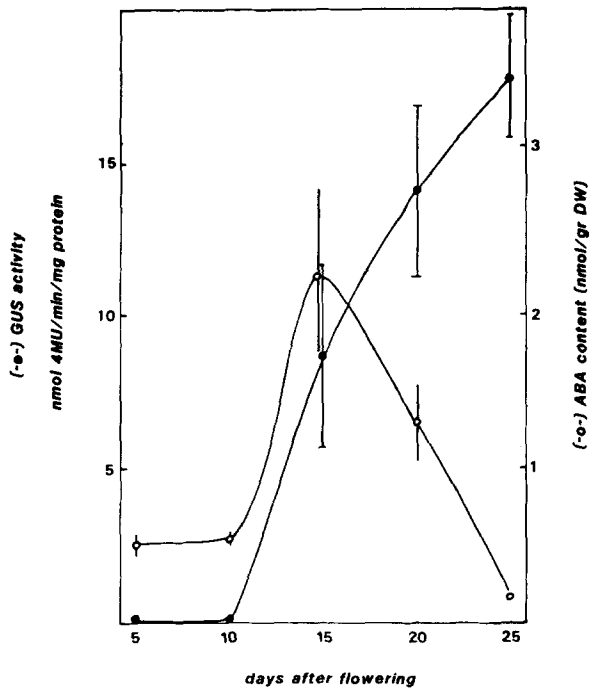

Fig. 3. GUS enzyme activities and endogenous ABA levels in seeds of transgenic R1 tobacco plants. Vertical bars indicate standard error of mean. Seeds were used from at least 5 capsules pooled at each time point from 5 R1 plants, derived from 4 independent primary transformants.

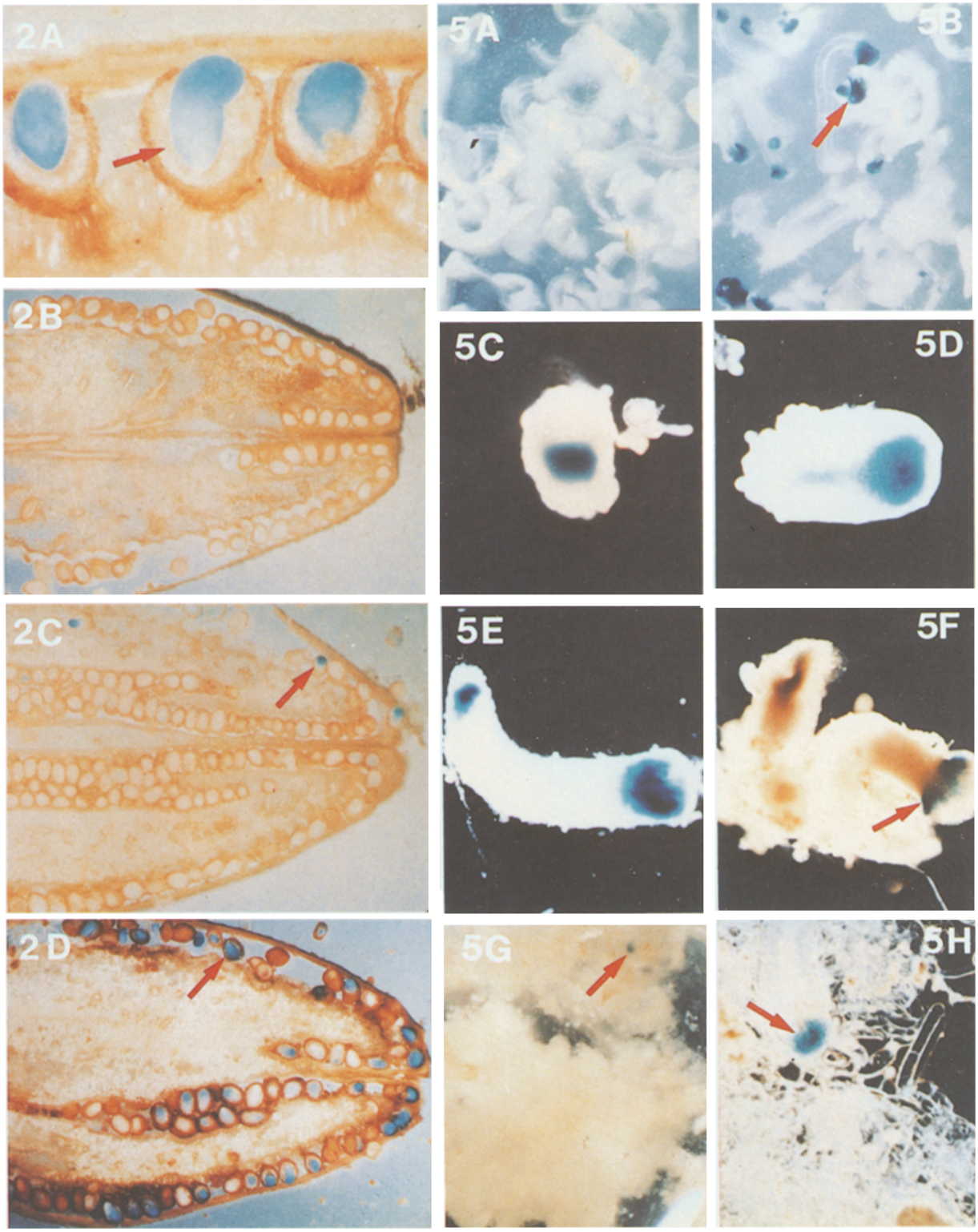

Fig. 2. Histochemical staining of GUS enzyme activity in developing seeds of R1 transgenic plants containing the *rab-16B*/GUS gene. Longitudinal sections of capsules: A, at close view 20 daf; B, 10 daf; C, 12 daf; D, 20 daf. Arrows show the staining.

Fig. 5. Histochemical staining of GUS activity in pollen-derived embryos. A, Controlled water treatment; B, 10^{-5} M ABA treatment; C–F, pollen embryos at various stages of development; G–H, pollen calli. Arrows indicate GUS enzyme activity detected only in embryonic tissue.

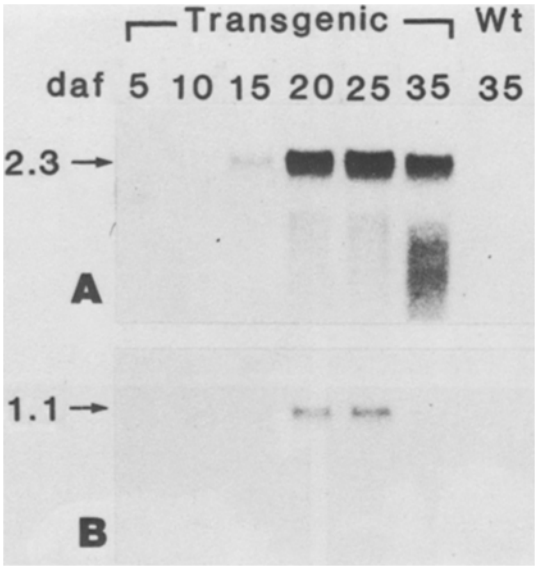

Fig. 4. Northern blot analysis showing accumulation of GUS mRNA and osmotin mRNA in developing seeds. R2 seeds were pooled for each time point (5, 10, 15, 20, 25, and 35 daf) from 4 independent R1 transformants (A, B, C, and D for Table 1). The sizes of the transcripts are shown at left in kb. A, GUS mRNA; B, osmotin mRNA.

pattern of accumulation as the mRNA derived from the *rab-16B*/GUS fusion gene (cf. Fig. 4A–B).

To directly test whether the fusion gene is responsive to ABA, immature seeds were treated with 10^{-5} M ABA. Surprisingly, these experiments did not show a reproducible increase in GUS activity in response to the hormone (Table 2). In ABA-treated, 10 daf seeds, GUS activity remained very low even though relatively high levels (70 × control) of ABA appear to have been taken up or adsorbed onto the seeds. To test whether this was due to the impermeability of the seed coat to ABA, seeds were nicked with a razor prior to hormone treatment. Again, this treatment did not result in increased GUS levels, perhaps because the *rab-16B* sequences are responsive to ABA only after a specific stage of embryo development has been reached (see below). Thus, in contrast to our results from transient expression assays in rice protoplasts [12], ABA-responsive expression from the *rab-16B* promoter in transgenic tobacco appears to be under tissue-specific control.

ABA-responsive expression of the rab-16B/GUS gene in pollen-derived embryos

To circumvent the possibility that seed structure or the developmental programming of gene expression in zygotic embryos affects their response to exogenously applied ABA, embryos were derived from pollen cultures. Such embryos respond well to ABA treatment, as witnessed by

Table 2. Influence of exogenous ABA treatment on GUS activities and ABA contents in developing transgenic tobacco seeds

Transgenic plants	daf	GUS activity (pmol 4MU/min/mg protein)		ABA content (pmol/g dry weight)	
		− ABA	+ ABA	− ABA	+ ABA
B	9	101	49	94	4264
	10	65	44	192	7353
	12	688	595	346	1556
	15	13548	13936	3021	3640
C	9	83	21	124	8750
	12	4792	5059	2583	3804
	15	6913	7857	1228	1467

Seeds at different times after flowering (daf) were removed from single capsules, divided into four parts. Two parts were used as reference for the determination of endogenous ABA content and GUS enzyme activity. The other two were incubated in 10^{-5}M ABA for 24 h, and the ABA content and GUS enzyme activity were determined. Samples for ABA determination were rinsed three times with 80% acetone followed by excessive washing with Tris-HCl buffer, pH 7.5, in order to remove ABA which remained on the surface of the seeds.

the high levels of GUS enzyme induced in them by the hormone (Fig. 5A–B). These experiments provide evidence that the *rab-16B* 5′ sequences are indeed responsive to ABA. This response was seen in specific cell types of pollen embryos stained at various stages. Activity was detected in early, globular embryos, <0.2 mm diameter (Fig. 5C), late heart-stage embryos (Fig. 5D), late torpedo-stage embryo (Fig. 5E), and in the cotyledons of germinating embryos (Fig. 5F). No enzyme activity could be detected at the plantlet stage (data not shown).

ABA treatment did not induce GUS activity in pollen-derived calli (Fig. 5G–H). However, occasional embryos found among the calli expressed GUS activity in response to the hormone (see arrows in Fig. 5G–H). We conclude that the *rab-16B* 5′ sequences do not respond to ABA treatment in either undifferentiated cells, or in vegetative plants tissues. Taken together, our results indicate that both embryo-specific and ABA-responsive elements interact to control the expression of the *rab-16B*/GUS fusion gene in transgenic tobacco.

Discussion

To initiate studies on the regulation of expression of the *rab-16A* and *rab-16B* genes, translational fusions between the 5′ sequences of these two genes and the GUS reporter gene were introduced into tobacco plants via Ti-mediated transformation (Fig. 1). Recent studies of the *rab-16A* promoter function in transfected rice cells have identified several conserved motifs as putative ABA-responsive regulatory elements [12]. These motifs are contained within the 5′ sequences used in the present work. From our work on the expression of the *rab-16A* and *rab-16B* genes in rice, we expected that the fusion genes might be expressed in seeds and in vegetative organs under conditions of osmotic stress, or following application of ABA. Surprisingly, neither construct produced detectable GUS activity in vegetative organs at different stages of growth or following water stress or hormone treatments. These results suggest that the transcriptional machinery of tobacco does not recognize all of the control elements that direct expression of the *rab-16A* and *rab-16B* genes in rice. We note that the wheat *rbcS* gene is also not transcriptionally active in transgenic tobacco, a dicotyledonous plant [9].

The *rab-16B*/GUS gene was expressed, however, in developing, transgenic embryos between 15 daf and seed maturity (Figs. 2, 3, & 4; Table 1). During this time, accumulation of GUS mRNA and enzyme correlated closely with the levels of endogenous seed ABA (Fig. 3). Similar expression patterns in transgenic tobacco seeds have been described for GUS gene fusion containing the 5′ sequences of *Em*, an ABA-responsive wheat gene [10]. Expression of the *Em*/GUS gene could also be induced earlier in embryogenesis by the application of ABA. By contrast, we were unable to induce expression of the *rab-16B*/GUS gene in embryos isolated before 15 daf, or to increase GUS activity significantly in older seeds by ABA treatment (Table 2). Although we do know that the hormone treatments used increased immuno-detectable ABA in the treated seeds, it is possible that the levels of endogenous ABA in older seeds were already saturated. In this case, increases in GUS activity would be difficult to detect. These results suggest that at least two factors regulate the expression of the *rab-16B*/GUS gene in transgenic, zygotic embryos: (a) the developmental stage of the embryo, and (b) the endogenous ABA levels in seeds.

To examine these possibilities more closely, pollen-derived embryos were produced from anther culture. Earlier work has shown that exogenous ABA can repress precocious germination in cultured embryos [1] and that the phytohormone also inhibits germination of tobacco pollen embryos [14]. These observations suggest that pollen-derived embryos contain low endogenous levels of ABA and are responsive to exogenous application of the hormone. In keeping with these observations, our transgenic pollen embryos did not show detectable GUS activity in control incubations, while high levels of enzyme activity could be induced in them by treatment with 10^{-5} M ABA (Fig. 5). These results indicate that 482 bp of the 5′ sequences of the *rab-16B* gene

can confer ABA-responsive, organ-specific expression in transgenic pollen embryos. This suggests that the ABA-responsive DNA elements of this gene are not recognized in other tobacco tissues due to differences in the transcriptional machinery between rice and tobacco.

The *rab-16A* and *rab-16B* genes are normally expressed later during embryo development in rice [11]. Why then is the *rab-16A*/GUS gene not expressed in transgenic tobacco embryos? Comparison of the *rab-16A* and *rab-16B* sequences used in this study show that the former contains a sequence, missing in the latter, which has been implicated in embryo-specific gene expression in dicots [5, 19]. This sequence ($A^{ACG}CCCA$) is present in several copies in a short promoter fragment of the soybean α-subunit of β-conglycinin that is sufficient to direct the expression of a reporter gene in embryos of petunia and tobacco. It is thus probable that our *rab-16B* construction is active in tobacco zygotic embryos because it fortuitously contains sequences recognized by the transcriptional apparatus of dicotyledonous seeds.

In conclusion, we show here that 482 bp of 5′ sequences of *rab-16B* can confer developmentally regulated expression in embryos of transgenic tobacco. Regulatory signals controlling *rab-16B* gene expression in vegetative rice tissues are apparently not recognized in tobacco. However, the tobacco system should enable us to dissect the embryo-specific DNA elements in future experiments.

Acknowledgements

We thank Cathy Ward and Ellen Leheny for expert assistance with the transformation and propagation of tobacco plants, and Wendy Roine for typing. This work was supported by a grant from NIH (BRSG S07 RR07065) and a grant from The Rockefeller Foundation.

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