A rat pancreatic ribonuclease fused to a late cotton pollen promoter severely reduces pollen viability in tobacco plants

R.B. Bernd-Souza[1], M.F. Grossi de Sa[1], D.D. Ellis[2] and B.H. McCown[2]

Abstract

The effects of an animal RNase fused to the late cotton pollen-specific promoter *G9* in a plant system were investigated. Expression of the chimeric genes *G9-uidA* and *G9-RNase* in tobacco plants showed that the 1.2-kb promoter fragment of the *G9* gene was sufficient to maintain tissue and temporal specificity in a heterologous system. GUS (beta-glucuronidase) expression was detected only in pollen from anther stage 6 through anthesis, with maximal GUS activity in pollen from stage 10 anthers. Investigating the effects of the rat RNase on pollen viability at stage 10, we found that pollen viability was reduced from 79 to 8% and from 89 to 40%, in pollen germination and fluoresceine diacetate assays, respectively, in one *G9-RNase* transgenic line, suggesting a lethal effect of the RNase gene. This indicates that the rat RNase produces deleterious effects in this plant system and may be useful for engineering male sterility.

INTRODUCTION

The use of genetic cell ablation through the expression of RNases, toxic genes or antisense mRNA along with promoters directing the expression to different cell types has aided in the molecular dissection of plant developmental processes. The cell ablation techniques have been used to study male gametogenesis, by visualization of the developmental events in the absence of specific cells, and can be used as a tool to produce male sterility (Mariani *et al.,* 1990; McCormick, 1991; Nasrallah *et al.,* 1991; Denis *et al.,* 1993; Goldberg *et al*., 1993; Roberts *et al.,* 1995; Twell, 1995; Zhan *et al*., 1996; Beals and Goldberg, 1997).

Male gametogenesis occurs in the sporophytic tissue of the anther and begins with the meiotic division of a diploid sporogenic cell (pollen mother cell) into a tetrad of haploid microspores, which later undergo an asymmetric mitotic division, resulting in pollen grains. These pollen grains contain a small generative cell within a larger vegetative cell. The pollen grains mature and undergo another mitotic division, before or after germination, giving rise to two sperm cells that complete the process with the double fertilization of the egg and the central cell (Bedinger, 1992; McCormick, 1993).

The genes expressed during male gametogenesis have been classified as "early" genes, which become active soon after meiosis is complete, or as "late" genes, when become active after microspore mitosis (Mascarenhas, 1990). About 75% of the mRNAs present in mature pollen of *Tradescantia* occur in two or more abundant classes, compared to only 35% of mRNAs in shoots (Mascarenhas, 1988). This, and the fact that the late pollen-specific cotton gene *G9* was isolated by differential screening (John and Petersen,

1994), make it probable that *G9* is a high-abundance transcript (Scott *et al.,* 1991). Thus, the 5' flanking region of the *G9* gene can promote pollen-specific expression, certainly at levels high enough for cell ablation. The native *G9* gene shows maximal expression on the day of anthesis (John and Petersen, 1994), when there is an existing stored pool of presynthesized RNAs (mRNA, rRNA and tRNA) that will be used for protein translation during pollen germination (Mascarenhas, 1988; Clarke and Newbigin, 1993). It indicates that *G9* is an adequate promoter to monitor the efficacy of an RNase gene. If the RNase gene works properly in the plant system, it may cause a reduction in pollen germination by depletion of protein synthesis.

The RNases used to investigate the role of specific anther genes/tissues and to induce male sterility have fungal or bacterial origin (Mariani *et al.,* 1990; Denis *et al.,* 1993; Roberts *et al.,* 1995; Zhan *et al*., 1996; Beals and Goldberg, 1997), and were reported to present thermo-instability (Denis *et al.,* 1993). We investigated the effects on male gametogenesis and pollen viability, of an animal RNase fused to the *G9* cotton pollen promoter, and the possibility of using this system to generate male sterility.

MATERIAL AND METHODS

Agrobacterium tumefaciens vectors

Two *A. tumefaciens* LBA4404 binary vectors were obtained from Agracetus, Inc. (Middleton, WI, USA). Both vectors contained the neomycin phosphotransferase (*NPT*II) selection gene and 1.2 kb of the *G9* cotton gene promoter (accession number U09805; John and Petersen,

[1]EMBRAPA - Uva e Vinho, R. Livramento, 515, 95700-000 Bento Gonçalves, RS, Brasil.
Send correspondence to R.B.B.-S. Fax +55-54-451-2792, E-mail: bernd@cnpuv.embrapa.br
[2]Department of Horticulture, University of Wisconsin - Madison, 1575 Linden Drive, Madison, WI, 53706-1590 USA.

1994) within the T-DNA regions. The *G9* promoter fragments were linked to different coding regions in the two vectors, one to a rat pancreatic RNase gene (accession number J00771) in plasmid TV4*G9*pRNaseA (*G9-RNase*), and the other to a *uidA* gene (Jefferson, 1987) in plasmid TV4p2119*G9*P (*G9*-GUS).

Plant transformation

Aseptically grown immature leaves of *Nicotiana tabacum* 'Havana 425' were cut into squares (2 cm x 2 cm), and placed in MSH medium (Murashige and Skoog, 1962, 0.1 mg NAA/l, 1.0 mg benzyladenine/l, 7 g agar/l) for 24 h. Leaf discs were transferred to 15-ml centrifuge tubes, inoculated with an overnight culture of *A. tumefaciens* diluted 1/5 (OD_{550} ~0.6), and vortex mixed on high for 15-20 s. The liquid was removed and the discs blotted dry and placed on Whatman filter paper on Petri plates containing MSH broth over a feeder tobacco cell culture (maintained in MS medium containing 2 mg 2,4-D/l) for three days. The discs were then transferred to MSHA selective medium (MSH supplemented with 500 mg carbenicillin/l, 250 mg cefotaxime/l and 100 mg kanamycin/l) and subcultured onto the same medium every seven days until callus and/or bud formation (~2-3 weeks). Calli and shoot buds were excised from the discs and individually subcultured in MSHA without cefotaxime for 3-4 weeks until the buds were elongated enough to root on hormone-free MS0 selective medium (MSH without hormones and supplemented with 500 mg carbenicillin/l and 100 mg kanamycin/l). Rooting occurred after 1-3 weeks, at which time the plants were acclimated to a lower humidity and transferred to soil. The studies were performed with primary transformants (T_0).

Genomic DNA isolation

Total DNA was prepared from young leaves as follows: two leaf discs (2 cm in diameter) were placed in a 1.5-ml Eppendorf tube with 50 µl of sand (white quartz, 50-70 mesh, Sigma S-9887) and freeze-dried in liquid nitrogen for at least 30 s. Five hundred microliters of prewarmed (60ºC) isolation buffer (2% (w/v) hexadecyl-trimethylammonium bromide, 1.4 M NaCl, 0.2% (v/v) β-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0) was then added to the tube, and ground (American model LR - 41A stirrer). After 30-min incubation at 60ºC with occasional swirling, 500 µl of chloroform-isoamyl alcohol (24:1 v/v) was added to each tube, the contents of which were then mixed by inverting the tube 10-15 times. The tubes were centrifuged for 20 s and the supernatant (aqueous phase) was then transferred to a clean tube prior to the addition of 2/3 total volume cold isopropanol. The nucleic acids precipitated as a white cloud, which was removed with a glass hook and transferred to 1 ml of wash buffer (76% v/v ethanol and 10 mM ammonium acetate). The washing was repeated and the samples then centrifuged

before air-drying. The pellet was resuspended in 100 µl of TE buffer (10 mM Tris-HCl, pH 7.4, and 1 mM EDTA) containing RNase A at a final concentration of 100 µg/ml. The amount of DNA was estimated by fluorometry.

PCR and Southern blots

PCR reactions were performed in 0.6-ml siliconized reaction tubes (Slickseal) in a Perkin/Elmer PCR cycler. The PCR mix tube contained 50-500 ng genomic DNA (or 1-100 pg of plasmid DNA), 2 mM dNTPs, 0.5 M KCl, 100 mM Tris, pH 8, 16 mM $MgCl_2$, 10 mM of each primer (3' and 5'), and 4.5 units Taq polymerase/µl (Promega) overlain with 1-2 drops of mineral oil. The PCR reaction consisted of 30 cycles of 60 s at 96ºC, 45 s at 55ºC, 60 s at 72ºC, followed by a 3-min hold at 72ºC and a continuous hold at 4ºC. The primers for *uidA* gene (*DR 37/48*) and RNase gene (*MEJ100/101*) amplifications were furnished by Agracetus, Inc.

Ten micrograms of genomic DNA was digested with appropriate restriction enzymes (*Bam*HI and *Xba*I), and samples were electrophoresed on a 1% agarose gel. DNA was transferred to nylon membranes (Hybond N, Amersham) prior to hybridization using [^{32}P] probes amplified by PCR and labeled by random priming (Ready-to-Go kit, Pharmacia Biotech). The hybridization conditions were 5X SSPE, 5X Denhart's solution, 0.5% SDS and 50 µg salmon sperm DNA/µl, at 65ºC, overnight. The membranes were washed once (30 min) at room temperature with 2X SSPE containing 0.1% SDS, once with 1X SSPE containing 0.1% SDS as above and once at 65ºC for 1 h with 0.5X SSPE containing 0.1% SDS. The membranes were then auto-radiographed at -80ºC with intensifying screens.

GUS and MUG assays

The histochemical staining for beta-glucuronidase (GUS) and the fluorogenic enzymatic assay for 4-methyl umbelliferyl beta-D-glucuronide (MUG) were adapted from Jefferson (1987). For histochemical examination, manually cut transverse sections from anthers and ovaries, pieces of leaves, petals, sepals, shoots, roots, and pollen grains were examined for GUS expression. The plant material was incubated in sodium phosphate buffer (100 mM $NaH_2PO_4.H_2O$, pH 7.0, 10 mM Na_2EDTA, 0.1% Triton X-100), containing freshly prepared 5-bromo-4-chloro-3-indoyl glucuronide (X-gluc; 0.5 mg/ml) dissolved in DMSO. The stained sections and pollen grains were examined using a stereo microscope.

For the MUG assays, one flower of each anther developmental stage was used. The flowers were placed on ice immediately after collection and then transferred to Eppendorf tubes with 125 µl of lysis buffer (50 mM Na_2HPO_4, 10 mM NaEDTA, 10 mM β-mercaptoethanol, 0.1% Triton X-100, 0.1% sodium laryl sarcosine, 25µg phenylmethyl-sulfonyl fluoride/ml). The tissue was fully

ground with a glass pestle (American model LR - 41A stirrer), followed by centrifugation for 3 min. Total protein was quantified using the BCA protein assay reagent (Pierce). To quantify methyl-umbelliferone (MU), 30 µl of the supernatant was added to 1 ml pre-warmed (37ºC) assay buffer (1 mM MUG in lysis buffer). Aliquots (200 µl) were withdrawn after 15 min, added to 800 µl stop buffer (0.2 M Na_2CO_3 in H_2O), and vortexed. The resulting fluorescence was read in a fluorometer (model TKO100, Hoefer Scientific Instruments), with excitation at 365 nm and emission at 455 nm. The assays were done at least three times.

Pollen viability assays

The anther developmental stages were classified as described by Koltunow *et al*. (1990). Pollen germinability was evaluated by excising the anthers from a flower of each developmental stage and dividing the anthers into two samples to evaluate germination viability. Anthers were cut with a scalpel and pollen grains gently squeezed into an Eppendorf tube containing 500 µl of liquid Micro Art's germination medium. The suspension was mixed and immediately poured onto a Petri dish with solid germination medium. The pollen grains were then spread equally over the solid medium. Following incubation in the dark at room temperature for 4 h, the pollen grains were observed with a Nikon Diaphot-TMD inverted microscope and scored as germinated (based on the presence of a pollen tube) or nongerminated. The germination medium consisted of sucrose (150 g), H_3BO_3 (100 mg), $CaNO_3$ (300 mg), $MnSO_4$ (100 mg) and $MgSO_4$ (500 mg) per liter of distilled H_2O. For the solid medium, 5% purified agar was added, heated in a microwave oven and poured on Petri dishes. The medium was used on the day it was prepared.

For fluoresceine diacetate (FDA) staining, the pollen grains were prepared essentially as described above. Five microliters of a stock solution of FDA (5 mg/ml acetone) was added to Eppendorf tubes containing 500 µl of liquid Micro Art's germination liquid medium with pollen grains. The mixture was incubated for 60 min in the dark at room temperature. After incubation, a drop of the pollen suspension was placed on a slide with a cover slip and the number of fluorescing pollen grains was determined. The observations were made with a Nikon Diaphot-TMD inverted microscope fitted with a Nikon UV-1A epi-fluorescence filter combination (400 dichroic mirror, 356/10 excitation filter, 400 barrier filter). A minimum of 100 pollen grains were counted in each of two samples on three or more occasions.

Histology

Whole flowers were fixed in CRAF V (1% chromic acid, 10% acetic acid, 40% formaldehyde) for 48 h. The anthers were removed, washed in running distilled H_2O for 24 h and dehydrated in an alcohol (ethanol and tertiary bu-

tyl alcohol) series. After embedding in paraffin, 15-µm sections were cut, mounted, deparaffinated and stained with safranine and fast green according to Johansen (1940) and Saas (1940), respectively. The sections were examined and photographed by light microscopy.

RESULTS

G9-GUS tissue expression

Tobacco plants were transformed with a 1.2-kb promoter fragment from the *G9* cotton gene containing 370 bp of the original coding region fused to the GUS reporter gene. The T-DNA used to transform the tobacco plants with the *G9*-GUS chimeric gene is shown in Figure 1A. The transformed plants were analyzed by PCR to confirm GUS integration (Figure 2), and five transgenic plants were chosen for further analysis. Histochemical GUS assays were performed on stage 2 pollen grains when microspores separated from the tetrad, through anthesis, as well as on sections of the anther, ovary and nonreproductive cells
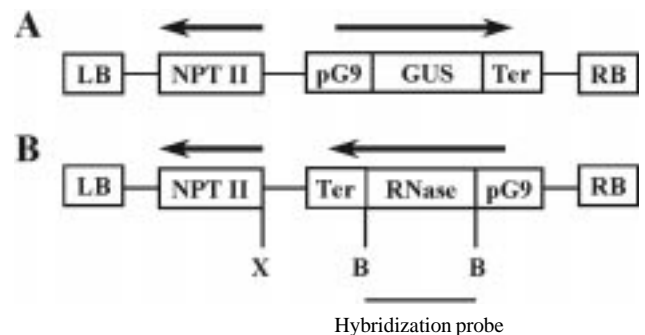

Figure 1 - Diagram of the T-DNA region of the *Agrobacterium tumefaciens* vectors used for tobacco transformation. The vectors contained the chimeric genes *G9*-GUS (*G9* promoter fused to GUS reporter gene) (**A**) or *G9*-RNase (*G9* promoter fused to the rat RNase coding region) (**B**). The sites for the restriction endonucleases *Bam*HI (B) and *Xba*I (X) and the DNA fragment used as the hybridization probe in Southern blots are shown. Neomycin phosphotransferase (*NPT*II) was used as a marker. LB, RB, T-DNA left and right borders, respectively.
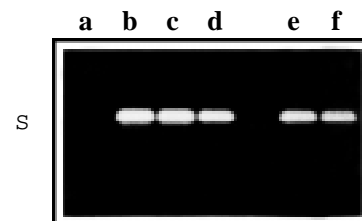

Figure 2 - Polymerase chain reaction gel of tobacco clones transformed with *G9*-GUS. Lanes **a**, Non-transformed plant, negative control; **b-f**, GUS-positive transgenic clones. The arrowhead shows a 710-bp fragment amplified with the GUS primers (*DR 37/48*) furnished by Agracetus Inc.

from flowers, leaves, shoots and roots. Blue staining in-
dicative of GUS activity was seen only in pollen and not in
the cells of other tissues (Figure 3). GUS activity was de-
tected as early as stage 6, when there are still remnants of
a degenerated tapetum, and in subsequent stages through
anthesis. These results demonstrate that the 1.2-kb pro-
moter fragment is sufficient to direct pollen specific ex-
pression. In addition, enzymatic assays (MUG) were done
with a transformed clone and a nontransformed tobacco
plant (Figure 4). The peak expression of the reporter gene
in tobacco occurred at stage 10, when the flower begins
to open, and contrasted with the maximal mRNA concen-
tration of the original *G9* gene in cotton found at the day
of anthesis (John and Petersen, 1994).

Generation of *G9-RNase* transgenic plants and T-DNA insert numbers

The T-DNA used to transform the tobacco plants
with the *G9-RNase* chimeric gene is shown in Figure 1B.
Total DNA from five of the Kan[r] transformed tobacco
clones was digested with *Bam*HI, generating an RNase
internal fragment. A Southern blot probed with a 700-bp
PCR-amplified internal fragment of the RNase coding
region (Figure 5A) confirmed integration of the RNase
gene into the plant genome. Nuclear DNA from the five
G9-RNase transgenic tobacco clones was digested with
*Xba*I, which has a single cleavage site in the T-DNA re-
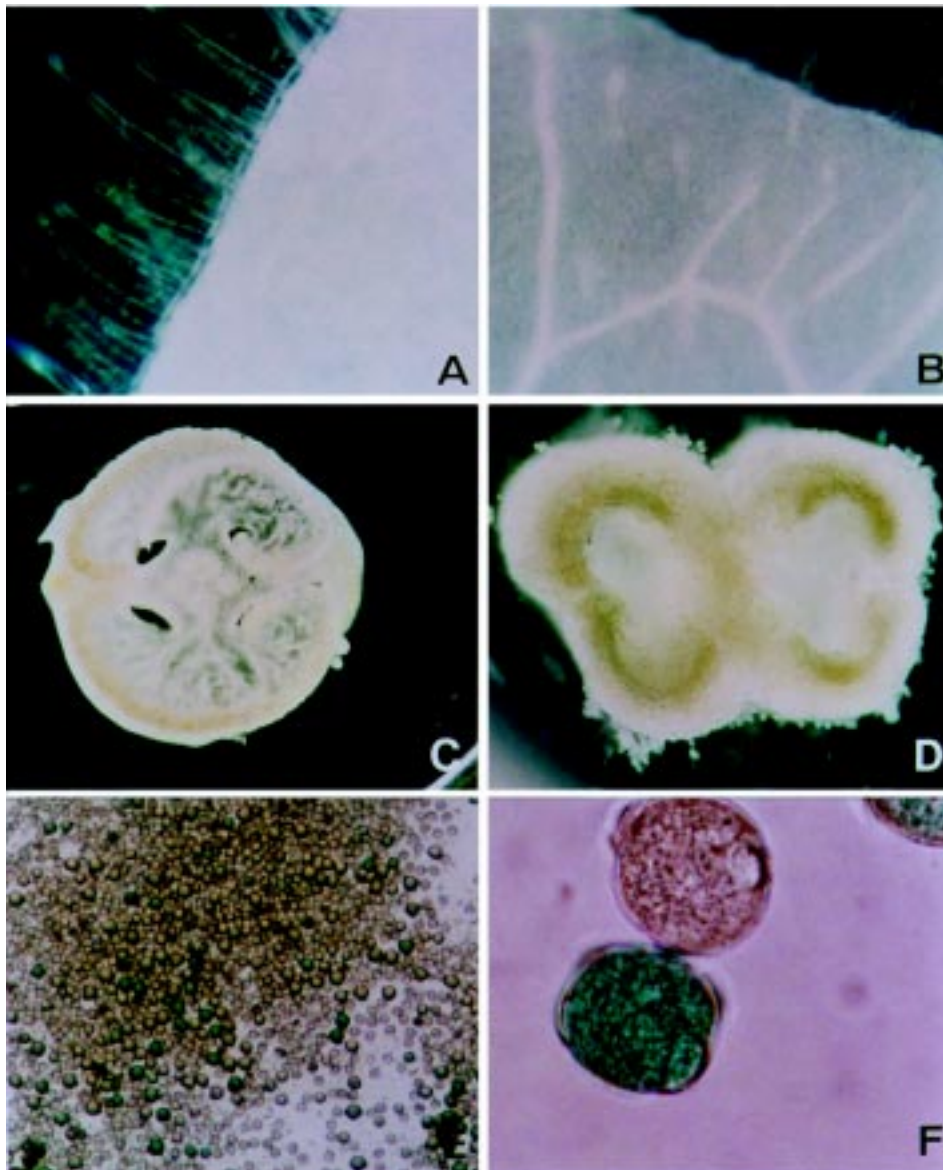gion, providing T-DNA/plant genome fragments of dif-

Figure 3 - Histochemical GUS assays of different plant organs in *G9*-GUS transgenic tobacco at anther stage 10. **A**, Leaf; **B**, petal; **C**, ovary; **D**, anther with blue-colored microspores; **E,F**, mature pollen grains.
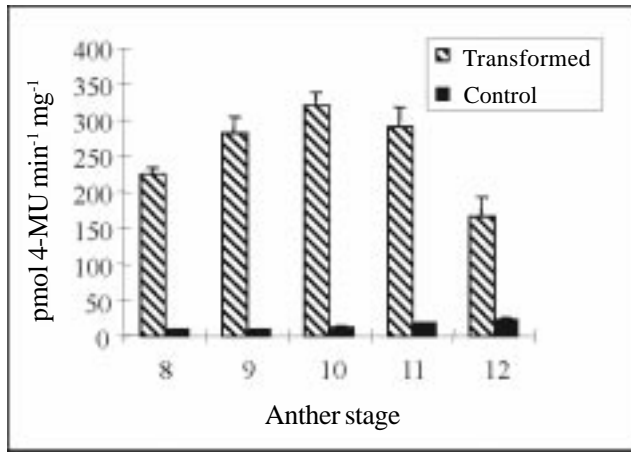
Figure 4 - Temporal pattern of *G9*-GUS expression, measured by the MUG fluorogenic assay in tobacco anthers at stages 8-12. The control is a nontransformed *Nicotiana tabacum* 'Havana 425' plant. The product of GUS enzyme activity (MU) was quantified with a fluorimeter with excitation at 365 nm and emission at 455 nm. The results are the average of at least three independent measurements.
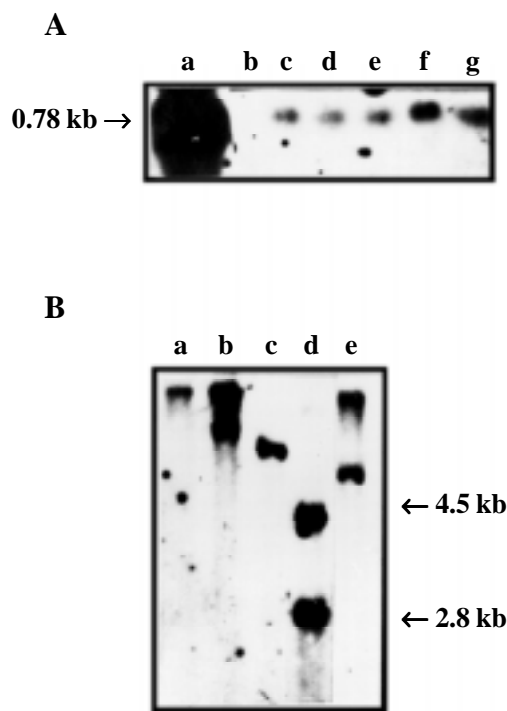

Figure 5 - Autoradiographs of Southern blots of genomic DNA extracted from *G9-RNase*-transformed tobacco plants and digested with restriction endonucleases. **A**, *Bam*HI, providing an RNase internal fragment; lanes **a**, plasmid positive control; **b**, nontransformed plant, negative control, and **c-g**, transgenic clones *RN1-5*, respectively; **B**, *Xba*I, providing T-DNA/plant DNA border fragments which reflect the number of T-DNA loci in each plant; lanes **a-e**, transgenic clones *RN1-RN5*, respectively. The DNA fragment used as hybridization probe in the Southern blots is indicated as a black bar beneath the restriction enzyme map of the T-DNA region in Figure 1B.

ferent sizes. A Southern blot probed with the RNase internal fragment (Figure 5B) allowed the detection of the number of copies of the RNase gene. Three of the clones, *RN2*, *RN4* and *RN5*, had two insertions of the RNase gene, and two clones, *RN1* and *RN3*, had only one insertion.

Pollen grain germination

To study the effect of the rat RNase gene on pollen viability, the germinability and fluoresceine diacetate staining tests were performed on anther stage 10 pollen, when the *G9* promoter confers maximal expression (as shown with *G9*-GUS transgenic tobacco). Three transformed clones (*RN2-RN4*) were compared morphologically to wild type tobacco. Whenever germination occurred, the pollen tube growth was similar (equivalent length and rate) to the nontransformed pollen grains (Figure 6A). On average, 79% of the nontransformed tobacco pollen grains germinated on the artificial medium (Figure 7A). All transformed clones had an average pollen germination which was lower than the wild type control. Clone *RN3*, which had only one copy of RNase, had a pollen viability (72%) close to the control value, whereas the clones *RN2* and *RN4*, with two copies of the RNase gene, had significantly less pollen germination (55 and 8%, respectively).

FDA test

The degradation of FDA by intracellular esterases yields fluoresceine, which can be visualized under ultra-violet light (Figure 6B). FDA degradation was examined using four transformed clones, two of them, *RN4* and *RN5*, containing two copies of the RNase gene, and two, *RN1* and *RN3*, containing one copy of the RNase gene. Independent of the number of insert copies, three transgenic clones, *RN1*, *RN3* and *RN5*, showed no significant difference from the non-transformed control (Figure 7B). The clone with the least pollen germination, *RN4*, also showed a much lower percentage of pollen viability (40%) in this test than did the non-transformed control (89%). This clone was chosen for morphological studies.

Phenotype of a *G9-RNase*-transformed clone

Macroscopically (naked eye observations), the transformed clone *RN4* had a vegetative and floral appearance that was indistinguishable from nontransformed plants. The flowers showed normal development, with pistil, stamens and pollen shedding similar to the control plants (Figure 8A).

Microscopically (light microscopy), the pattern of anther development and the aspect of its tissues in the transformed clone also resembled the wild type, with the tapetum degeneration being analogous (Figure 8B-D). The pollen grains were morphologically similar in size and shape, with comparable patterns of staining in the nucleus, cytoplasm, and exine.
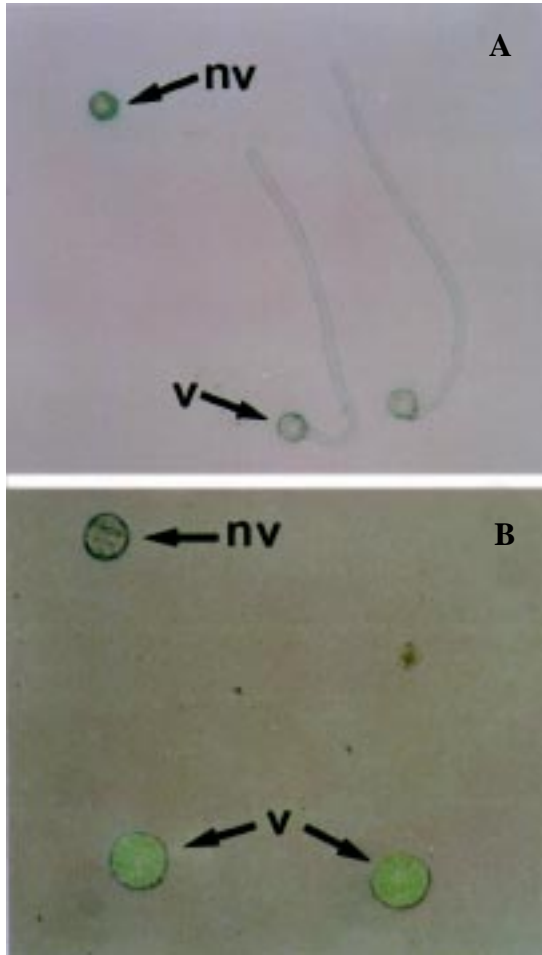
Figure 6 - Phenotype of mature pollen grains of the transgenic tobacco clone *RN4* under light microscopy. **A**, Germination test. Arrows show a viable (v) germinated pollen grain (emitting pollen tube on Micro Art's media), and a nonviable pollen grain (nv). **B**, FDA test. Arrows show fluorescent viable pollen grains (v) and a nonviable pollen grain (nv).

Figure 7 - Viability of *G9-RNase* transgenic tobacco clones *RN1-RN5* and nontransformed *Nicotiana tabacum* 'Havana 425' (*WT*) at anther developmental stage 10. **A**, Percentage of viable pollen grains germinating on Micro Art's media (see Figure 6A). **B**, Percentage of viable fluorescent pollen grains (see Figure 6B). A minimum of 100 pollen grains were counted per sample, in at least three independent replications.

DISCUSSION

We investigated the gene expression patterns in tobacco anthers during the developmental stages defined by Koltunow *et al.* (1990), beginning at stage 1, when all major tissues (epidermis, endothecium, connective, vascular bundle, stomium and tapetum) have differentiated, meiosis is completed, and microspores are bound together as tetrads. From stage 1 to stage 5, the anthers increase in weight and length, and then they maintain their volume relatively constant up to stage 11, after which they decrease to the next stage (stage 12) when the flower opens fully and the anthers dehisce. The pattern of mRNA synthesis in developing anthers shows tapetal dominance during sporogenesis and early microspore development, in contrast to microspore dominance in later stages of development (Scott *et al.*, 1991).

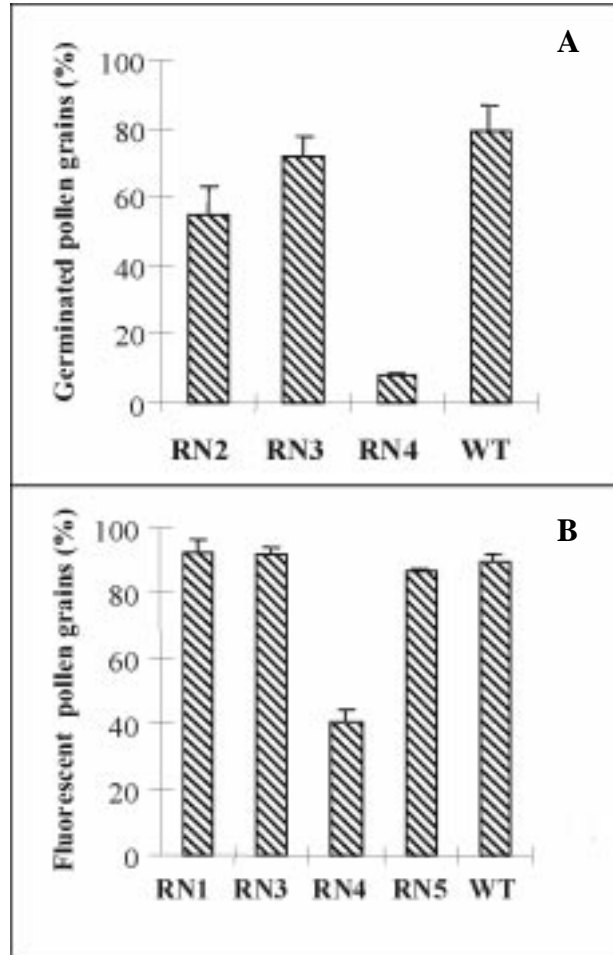Differential hybridization, a method widely used for screening anther-specific genes, favors the isolation of high-abundance transcripts with a bias for tapetum or microspore specificity, depending on the anther developmental stage of the c-DNA library (Scott *et al.*, 1991). The cotton *G9* gene is a late microspore-specific gene which shows increasing mRNA accumulation along with pollen maturation, with maximal transcriptional activity on the day of anthesis (John and Petersen, 1994). In tobacco, Schrauwen *et al.* (1990) showed that immediately after mitosis of the haploid microspore there is a rise in cell activity, which marks a very dynamic period of the pollen developmental process, with an increase of 50% in the cell dry weight and 300% in the mRNA content.

In our experiments, the *G9* promoter fused to the reporter gene *uidA* showed maximal expression at stage 10 in transgenic tobacco (Figure 4), coinciding with the transcriptional dynamic period observed by Schrauwen *et al.* (1990), but occurring slightly before the peak of ex-
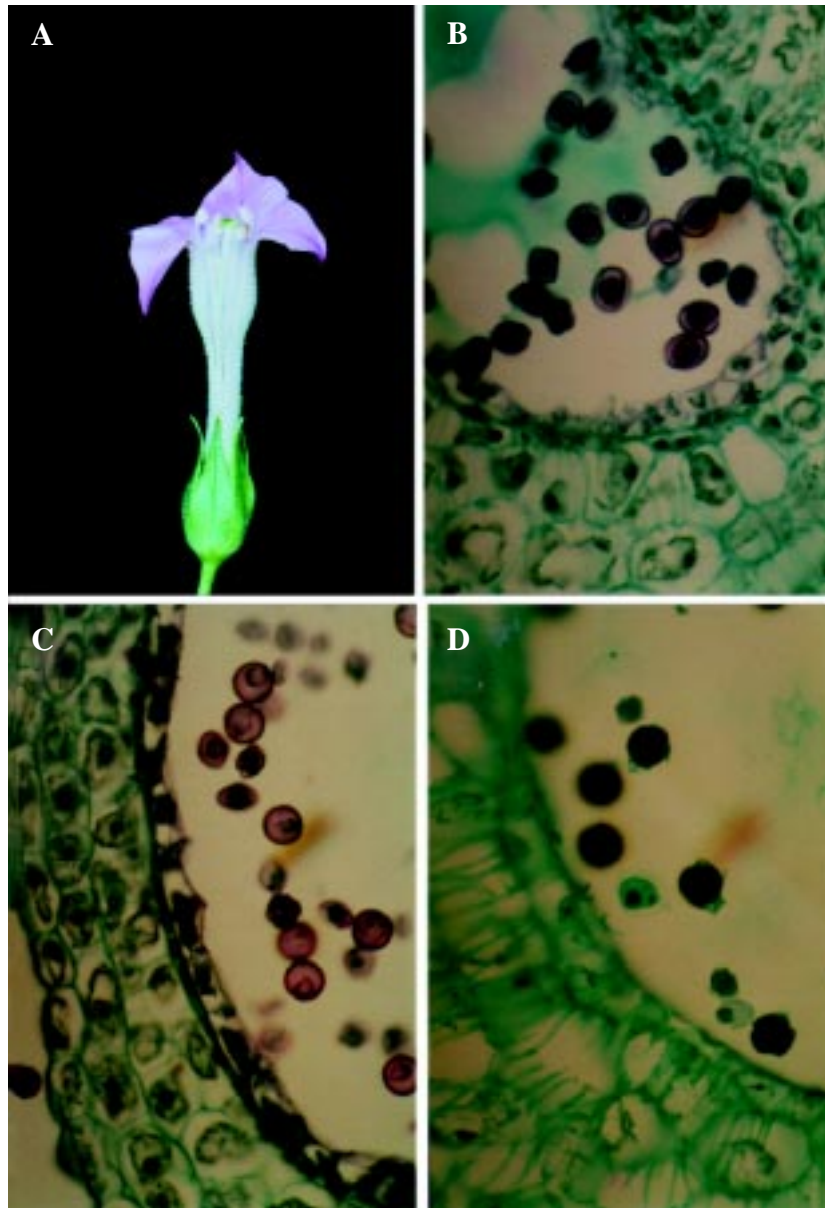
Figure 8 - Floral morphology of the transgenic tobacco clone *RN4*. **A**, Flower at anthesis, phenotypically similar to a non-transformed flower. **B-D**, Anther locula at different developmental stages. Early uninucleate microspores (stage 2), meiosis complete and microspores have separated from tetrad (**B**); late uninucleate microspores (stage 5) (**C**); binucleate pollen grains (stage 10) (**D**). Magnification for B-D, 320X.

pression found for the endogenous cotton *G9* gene (John and Petersen, 1994). A similar temporal difference in the expression of a chimeric-GUS construct was also reported by Koltunow *et al.* (1990) for *TA29* in tobacco, where endogenous transcripts are present from anther stages 1 to 6, with maximal levels at stage 3. In contrast, when a 279-bp 5' sequence of *TA29* was fused to *uidA*, intense enzyme activity was seen at anther stage 4, and no blue color was observed in cells of stage 1 and stage 6 anthers of the same transformants. Furthermore, in the last two stages (11 and 12) of development prior to fertilization, the anthers decreased in volume, which might have affected

the results of the MUG assays. In spite of the small temporal variance in *G9*-GUS expression, the 1.2-kb promoter was sufficient to maintain pollen specificity in tobacco, as shown by histochemical staining of the floral and vegetative tissues (Figure 3).

Our studies of the deleterious effect of rat RNase on pollen viability were performed at anther stage 10, since this stage showed a high level of chimeric *G9* expression. In addition, the anther volume is stable at this stage and the unopened flower/anther minimizes undesired side effects. Of the various methods for assaying pollen viability (see a list in Trognitz, 1991), we chose pollen germina-

tion and fluoresceine diacetate staining, as well as morphological observations of whole plants and their anther development. We analyzed five transgenic clones, *RN1-RN5*, in which RNase integration was confirmed by Southern blots (Figure 5A). Macroscopically, the transformants were indistinguishable from nontransformed plants in that they had a normal vegetative and floral development (Figure 8A), and the amount of pollen shed was similar to the controls. Pollen from transformed (Figure 6) and nontransformed plants was also almost identical in appearance. Histological sections from anther stages 1-12 (Figure 8B-D) showed no morphological differences from the control plants, including the pattern of coloration following safranine-fast green staining. However, there were significant differences, in the viability tests, in the quantity of pollen that hydrated but could not germinate or lost metabolic activity.

Three clones (*RN2*, *RN4* and *RN5*) had two copies of RNase gene, but only one transgenic clone, *RN4*, showed a major reduction in pollen viability. It may be that the two copies of the RNase in this clone may have been integrated in a place of the plant genome that promoted high expression. Such position effects have been previously suggested by other authors (Morota and Uchimiya, 1988; Peach and Velten, 1991).

The pollen grains of clone *RN4* showed an 8% germination capacity. A similar case of severely reduced male fertility in tobacco caused by an RNase was found by Zhan *et al.* (1996) using a promoter of a rice pollen-specific gene (*PS1*) fused to Barnase. Another case of male sterility caused by an RNase was reported by Denis *et al.* (1993). In neither of these cases were the effects correlated with insert copy number.

The maximal concentration of *G9* RNA in clone *RN4* pollen may have occurred on the day of anthesis, suggesting a post-anthesis function (John and Petersen, 1994). This is compatible with the function of polygalacturonase in degrading complex carbohydrates found between the cells of the stylar transmitting tract (Scott *et al.*, 1991). On the day of anthesis, however, there is a stored pool of presynthesized mRNAs that are translated during germination (Mascarenhas, 1988). Therefore, the rat RNase gene may not have shown its cytotoxicity on pollen metabolism until the emission of the pollen tube, thereby leading to a severe reduction in pollen viability.

In conclusion, the rat RNase was properly expressed in this plant system and may therefore be useful for engineering male sterility.

ACKNOWLEDGMENTS

RESUMO

Foram investigados os efeitos da expressão de uma ribonuclease de origem animal em um sistema vegetal, ligando-se esta ao promotor do gene pólen-específico *G9* de algodão. Examinouse a expressão dos genes quiméricos *G9-uidA* e *G9-RNase* em plantas de tabaco e determinou-se que o fragmento de 1.2 kb do promotor do gene *G9* foi suficiente para manter a especificidade temporal e espacial da expressão, em sistema heterólogo. A expressão do gene GUS foi detectada somente em pólen, do estágio 6 do desenvolvimento da antera até a antese, com atividade máxima em pólen de anteras no estágio 10. Estudos neste estágio com linhagens transgênicas contendo *G9-RNase* mostraram que um clone transgênico apresentava reduções na viabilidade do pólen de 79 para 8% e de 89 para 40% nos testes de germinação e coloração com diacetato de fluorosceína, respectivamente, sugerindo letalidade na expressão do gene de RNase. Estes resultados indicam que a RNase animal apresenta um efeito deletério em planta e oferece possibilidade de uso na obtenção da esterilidade masculina.

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