Monocot Regulatory Protein Opaque-2 Is Localized in the Nucleus of Maize Endosperm and Transformed Tobacco Plants

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Protein targeting to the nucleus has been studied extensively in animal and yeast systems; however, nothing is known about nuclear targeting in plants. The Opaque-2 (O2) gene produces a regulatory protein that is responsible for inducing transcription of the α-zein class of storage proteins in maize kernels. The cloned O2 gene encodes a protein that contains a leucine zipper DNA binding domain that can interact with zein gene promoters. We have used immunolocalization to show that the O2 protein is present in nuclei in the maize endosperm tissues known to produce α-zeins. In addition, neither embryo tissue from wild-type kernels nor endosperm from kernels harboring a null o2 allele contain the O2 protein. Analysis of a transposable, element-induced o2 allele, o2-m20, revealed that sectors of endosperm cells contained the nuclear-localized O2 protein, indicating excision of the transposable element. To study further the nuclear transport of the O2 protein, we have transformed this gene, under the control of a constitutive promoter, into tobacco. Plants were shown to have detectable levels of steady-state O2 mRNA and O2 protein. Immunolocalization of O2 protein in transformed tobacco plants indicated that the O2 protein was transported into tobacco nuclei. Therefore, we have developed a system to study nuclear targeting in plants and have established that the nuclear transport machinery is similar in monocots and dicots.

INTRODUCTION

The cell has many compartments in which different biochemical processes take place. Targeting studies determine how the proteins, or other molecules required for these reactions, are transported to the appropriate cellular compartment. In plants, most targeting studies have focused on the transport of proteins to the chloroplasts (see Keegstra, 1989, for review), mitochondria (see Hartl and Neupert, 1990, for review), endoplasmic reticulum, vacuole, or plasma membrane (see Chrispeels, 1991, for review). Recently, Restrepo et al. (1990) presented data on nuclear targeting of a viral protein to the plant cell nucleus. However, targeting of a plant regulatory protein to the plant cell nucleus has not been addressed.

Xenopus, Drosophila, yeast, and mammalian systems have been used extensively for studies on nuclear targeting (see Dingwall and Laskey, 1986; Silver and Hall, 1988, for reviews). Thus far, two criteria have been identified as necessary for nuclear protein targeting. Proteins smaller than 20 kD to 40 kD are presumed to diffuse freely into the nucleus through the nuclear pore. Larger proteins are actively transported and must contain a nuclear localization signal within the body of the protein. The primary amino acid sequences in these signals are not identical among various proteins; however, they have conserved features such as an abundance of basic amino acids and a proline or glycine.

We have chosen to study plant nuclear transport using the cloned maize regulatory protein Opaque-2 (O2). Plants containing the o2 mutation produce lower levels of the zein seed storage proteins (Mertz et al., 1964; Tsai et al., 1978). The o2 locus has the greatest effect on transcription of the 22-kD class of α-zeins (see Motto et al., 1989, for review); however, o2 also affects the expression of the 19-kD class of α-zeins (Kodrzycki et al., 1989). The α-zeins are localized within the subaleurone and starchy endosperm tissues in maize endosperm (Lending and Larkins, 1989).

The O2 gene was cloned by transposon tagging with the Suppressor-mutator (Spm) element (Schmidt et al., 1987) and also with the Activator element (Motto et al., 1988). Sequence analysis revealed a 47-kD protein that contains a leucine zipper domain common to the fos-jun class of DNA-binding proteins (Hartings et al., 1989; Schmidt et al., 1990). In vitro studies have shown that the

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Figure 1. Localization of O2 Protein in Maize Tissues.

Photomicrographs are shown in pairs with tissues treated with the specific nuclei stain DAPI [(A), (C), (E), (G)] and with immunolocalization, using polyclonal anti-O2 sera and IgG-conjugated rhodamine [(B), (D), (F), and (H)].
O2 protein binds to a 22-kD α-zein gene (Schmidt et al., 1990). Together, these results provide evidence that the O2 protein is a direct regulator of zein transcription and a good candidate for plant nuclear transport studies.

One of the advantages of working with maize is the availability of transposable element alleles. There are several transposable element mutations of the O2 gene (Schmidt et al., 1987; Wienand and Saedler, 1987). These alleles can serve as internal controls for immunocytochemistry because both wild-type and mutant cells are available in the same tissue section. The kernel phenotype of the Spm-induced allele o2-m20 (Schmidt et al., 1987) is opaque with translucent sectors. The opaque tissue is derived from cells that have the Spm element in the O2 gene; the translucent, revertant sectors are derived from a cell in which the element has excised and restored O2 gene activity.

In this report, we have analyzed the nuclear localization of the O2 protein in maize endosperm tissue by immunocytochemistry. In addition, the distribution of the O2 protein in seeds from plants homozygous for the o2-m20 allele was studied. In this allele, we have shown that the O2 protein is only present in some cells and is localized within the nuclei of these cells that we presume represent revertant sectors.

Having confirmed that the O2 protein is localized in the nucleus of maize endosperm, we have used a transgenic approach to develop a system to study nuclear transport of the O2 protein. Our results have established that the O2 protein is expressed and transported to the nuclei of tobacco tissue. Therefore, we have shown that the nuclear targeting machinery is similar in monocots and dicots and have established a system to examine the molecular mechanisms of nuclear targeting.

RESULTS

Localization of the O2 Protein in Maize

Genetic and molecular data suggest that the O2 protein regulates the expression of α-zein storage proteins that are localized in the subaleurone and starchy endosperm tissues of maize kernels. Therefore, the O2 protein should be localized within the nuclei of the endosperm tissues.

We have used polyclonal antibodies raised against an O2-β-galactosidase fusion protein. Characterization of these antibodies will be reported elsewhere (R.L. Parsons, M.J. Aukerman, and R.J. Schmidt, manuscript in preparation). Our first goal was to ascertain that these antibodies would bind to the O2 protein in cells of developing maize kernels harboring the wild-type O2 allele. We could then compare the localization patterns of O2 protein in O2 kernels with those seen in mutant o2 kernels that are known to harbor an o2 null allele devoid of O2 mRNA (Schmidt et al., 1987). Therefore, sections were made from both O2 and o2 endosperm during a time course of 12 days, 15 days, 18 days, and 22 days after pollination (DAP). Embryo tissue does not contain zeins, nor does it produce a detectable steady-state level of O2 mRNA or protein (R.J. Schmidt, unpublished results). Sections from O2 embryos at 22 DAP were used as negative internal controls for O2 immunolocalization studies. Figure 1 shows representative photomicrographs of maize tissue sections. All sections were simultaneously stained with the nuclei-specific dye 4',6'-diamidino-2-phenylindole dihydroxychloride (DAPI) (Figures 1A, 1C, 1E, and 1G) and prepared for immunolocalization of the O2 protein (Figures 1B, 1D, 1F, and 1H). The double-staining procedure allowed for the identification of the nuclei in a tissue, followed by analysis of these nuclei for the presence of the O2 protein.

In Figures 1A and 1B, the O2 protein was localized in nuclei of endosperm cells of 18 DAP O2 kernels. The O2 protein was present in nuclei throughout the starchy endosperm and the subaleurone layer in all kernels harboring the O2 allele during the time course tested (data not shown). No specific staining was seen when O2 endosperm sections were incubated with nonimmune serum (data not shown). Furthermore, the fidelity of these results was reinforced by the absence of staining in sections from O2 embryos (Figures 1C and 1D) and o2 endosperms (Figures 1E and 1F). All sections from o2 endosperm, throughout the time course, showed no immunofluorescence, indicating the absence of O2 protein in these tissues. Control sections treated with nonimmune sera also did not show fluorescence (data not shown). The endosperm nuclei in o2 tissue (Figure 1E) are larger than those in wild-type endosperm tissue (Figure 1A). This change in size is correlated with higher DNA content in the o2 nuclei (Kowles and Phillips, 1985).

Figure 1. (continued).

(A) and (B) 18-DAP O2 endosperm.
(C) and (D) 22-DAP O2 embryo.
(E) and (F) 18-DAP o2 endosperm.
(G) and (H) 26-DAP o2-m20 endosperm. A small revertant sector of cells containing O2 protein extends through the bottom third of the section. Arrows in (G) indicate DAPI-stained nuclei that are not fluorescing in the O2 immunolocalization section in (H). Square white dots are located to the right of two fluorescent nuclei whose size is altered because of a slight change in the plane of focus.
Bar = 10 μm.
The 02-m20 allele contains an Spm element inserted in the 02 gene. Kernels harboring this allele often produce revertant sectors of translucent endosperm against a background of opaque tissue (Schmidt et al., 1987). The translucent sectors presumably contain a wild-type complement of α-zein proteins, indicating excision of the Spm element and restoration of 02 function. Revertant sectors show no consistency in size and can range from whole kernels to several cells.

The 02-m20 phenotype is not visible until late in kernel development. Thus, several 02-m20 kernels at 26 DAP were selected and examined for 02 immunolocalization. Sectors of revertant cells were identified by the presence of 02 protein in the nuclei, as seen in the wild-type tissue. Figures 1G and 1H show a representative sector from an o2-m20 endosperm section. The arrows in Figure 1G indicate nuclei visible with the DAPI stain that do not contain detectable 02 protein. The other nuclei in Figure 1G were also visible in Figure 1H, indicating that the 02 protein was localized within the nuclei of this small sector of revertant cells.

Detection of 02 mRNA and Protein in Transgenic Tobacco

To study nuclear transport of the 02 protein, it was necessary to develop a transgenic system that would mimic the transport of the maize protein. The construct used in transgenic studies was made by modifying the 02 cDNA 1-4 (Schmidt et al., 1990). This cDNA contains three small open reading frames upstream of the 02 coding sequence. Exonuclease digestion was used to remove the three small reading frames, leaving 63 nucleotides upstream of the initiation codon. To facilitate cloning, a HindIII restriction site was added 137 nucleotides downstream of the stop codon. The 1.5-kb HindIII fragment, containing the 02 open reading frame and flanking sequences, was inserted into the plant expression vector pGA643 (An et al., 1988). Figure 2 shows the construct, designated p35SO2, that contains the 02 cDNA downstream of the 35S cauliflower mosaic virus promoter.

We have used Agrobacterium-mediated transformation of tobacco leaf discs (Horsch et al., 1988) as a model system to study nuclear transport of the 02 protein. The p35SO2 plasmid was mobilized into Agrobacterium tumefaciens strain LBA4404 and transformed into tobacco (cv Wisconsin 38) leaf discs. Ten kanamycin-resistant plants were obtained, and the one that expressed the 02 protein at the highest level was used for further studies.

The steady-state levels of 02 mRNA in maize and in transgenic tobacco plants were compared by RNA gel blot analysis. Leaves and roots from kanamycin-resistant tobacco plants were analyzed for expression of the 02 gene. The RNA blot analysis in Figure 3 represents the relative amounts of 02 mRNA isolated from maize and tobacco tissues detected by 32P-labeled HindIII insert from the 02 cDNA. Total RNA from 02 (lane 1) and 02 (lane 2) maize endosperm was compared with leaf and root RNA from one of the plants transformed with the p35SO2 construct (lanes 3 and 6), from a plant transformed with the vector pGA643 (lanes 4 and 7), and an untransformed plant (lanes 5 and 8). The plant transformed with the p35SO2 construct produces a 1.7-kb mRNA identical in size to the wild-type mRNA in maize 02 endosperm. This mRNA is not detected in either vector-transformed plants or in untransformed control plants (Figure 3). The relative levels of p35SO2 mRNA in leaf tissue from tobacco plants transformed with the p35SO2 construct were approximately sixfold lower than corresponding 02 mRNA in maize endosperm, as detected by scanning densitometry. The integrity of all RNA samples was confirmed by hybridizing the blot a second time with a 32P-labeled soybean actin gene probe (Shah et al., 1982, and data not shown).

Crude protein extracts from transformed plants were analyzed by immunoblot analysis using polyclonal 02 sera raised against the 02-β-galactosidase fusion protein. Data from a typical experiment are shown in Figure 4. The 47-
Figure 3. Analysis of the Steady-State Levels of O2 Transcripts in Maize and Tobacco Plants.

The RNA gel blot contains 10 μg of total RNA isolated from maize endosperm harboring the O2 allele (lane 1), maize endosperm harboring the null o2 allele (lane 2), and tobacco leaves and roots from a plant transformed with the O2 construct p35SO2 (lanes 3 and 6), from a plant transformed with the vector pGA643 (lanes 4 and 7), and from an untransformed W38 control plant (lanes 5 and 8). The 1.7-kb O2 transcript is present in O2 maize tissue and in p35SO2-transformed tobacco tissues.

kD O2 protein typically migrates at 65 kD on SDS-PAGE gels (Hartings et al., 1989). Leaves and roots from tobacco plants transformed with the p35SO2 construct (lanes 3 and 6) produced O2 protein identical in size to that found in O2 maize endosperm (lane 1). No O2 protein was detected in the o2 endosperm from maize (lane 2), tissues from plants transformed with the vector (lanes 4 and 7), or from untransformed plants (lanes 5 and 8). Identical blots were incubated with nonimmune sera and showed no cross-reactivity with other proteins (data not shown). There are some cross-reacting bands in all of the tobacco samples analyzed on blots treated with O2-specific antibodies. These faint bands, which differ in size from the O2 protein, suggest that other tobacco proteins have epitopes in common with O2. Possibly other proteins with leucine zipper motifs cross-hybridize with the O2 antibodies. This conserved motif is present in several plant (Gruissem, 1990) and mammalian transcriptional activators (Abel and Maniatis, 1989).

Localization of the O2 Protein in Transgenic Tobacco

We have shown above that the O2 protein is localized in the nuclei in cells from maize tissues expressing the O2 gene. In addition, tobacco plants transformed with the p35SO2 construct have produced O2 mRNA and O2 protein. The subcellular location of O2 protein in transgenic tobacco was ascertained by light immunocytochemistry. Sections were made from leaf, petiole, and stem tissues, and representative nuclei from immunolocalizations of stem tissues are presented in Figures 5B and 5D. As previously described, all sections were simultaneously processed for O2 localization and counterstained with the nuclei-specific stain DAPI (Figures 5A and 5C). Figures 5A and 5B show that the O2 protein was not present in the stem tissue of the untransformed plant. However, O2 fluorescence was present in nuclei from stem tissue isolated from a tobacco plant transformed with the p35SO2 construct (Figures 5C and 5D). Autofluorescence from chloroplasts is visible in both untransformed and transformed tissue; however, this autofluorescence does not interfere with the visualization of specific fluorescence of nuclei in transformed tissue. Although only a few representative nuclei are shown in Figure 5, comparable results were seen for all nuclei examined in stem tissue as well as those in sections of leaves and petioles from transformed and untransformed plants. Nuclei in tissues from both...
transformed and untransformed plants treated with non-immune sera did not fluoresce (data not shown). Therefore, the O2 protein is properly localized within the nucleus of transformed tobacco plants.

DISCUSSION

The o2 locus has been shown both genetically and molecularly to be a transcriptional activator of the 22-kD α-zeins (Kodrzycki et al., 1989; Motto et al., 1989). We have shown that the O2 protein is localized within the nucleus of O2 maize endosperm. Furthermore, the O2 protein was detectable in starchy endosperm and subaleurone tissue and was not present in other kernel tissues, such as embryo and aleurone. This tissue specificity correlates with the localization of α-zein proteins in starchy endosperm and subaleurone tissues (Lending and Larkins, 1989). Immunolocalization studies of O2 protein in the null o2 allele demonstrated that the O2 protein was not present in mutant endosperm. Therefore, the immunolocalization data support results of genetic and molecular studies indicating that the O2 protein is a transcriptional activator of α-zein genes.

The phenotype of the o2-m20 kernels is that of an unstable opaque allele containing sectors of translucent tissue. Presumably, the translucent tissue is derived from cells in which the Spm element has excised from the o2 gene allowing for α-zein production. The Spm element in the o2-m20 allele is located in intron 4 of the o2 gene, suggesting that all excision events will lead to restoration of O2 gene expression (L. Pysh and R.J. Schmidt, unpublished results). Excision events in maize endosperm harboring mutable alleles of anthocyanin pathway genes have an easily recognizable phenotype, a color change. Kernels harboring mutable alleles of the maize waxy gene may be stained with iodine to detect excision events. These two

Figure 5. Localization of O2 Protein in Tobacco Stem Tissues.

Photomicrographs of tobacco stem sections are shown in pairs with DAPI staining to indicate nuclei [(A) and (C)] and immunolocalization using polyclonal anti-O2 sera and IgG-conjugated rhodamine antibody [(B) and (D)].

(A) and (B) Untransformed W38 control plant.
(C) and (D) p35SO2-transformed plant.
Bar = 10 μm.
phenotypic assays allow for the detection of excision events in individual cells. It is impossible to analyze individual cells of o2 mutable alleles using the phenotypic change from opaque to translucent. And, unlike the enzyme encoded by the waxy gene, no biochemical assay is available for detecting products produced as a result of restored O2 function. Thus, we have used immunolocalization of the O2 protein to examine Spm excision from the o2-m20 gene. Results of immunolocalization studies have shown that the O2 protein is present in only some nuclei of cells from o2-m20 endosperms. The simplest explanation is that these nuclei represent cells from a revertant sector where O2 expression has been restored and the O2 protein is properly expressed and compartmentalized.

To develop a system to study nuclear transport in plants, we have transformed the maize O2 cDNA into tobacco. This system allows us not only to study plant nuclear transport but also to analyze nuclear transport of monocot transacting factors in a dicot environment. Some aspects of post-transcriptional modifications, such as splicing, differ between monocots and dicots (Keith and Chua, 1986). These differences may be due to varying AT content in monocot and dicot introns (Hanley and Schuler, 1988; Goodall and Filipowicz, 1989) and/or heterogeneity in splicing machinery (Egeland et al., 1989). Monocot and dicot vacuolar protein targeting machinery was analyzed by using a monocot secretory protein, the barley lectin (Wilkins et al., 1990). It has been shown that barley lectin is properly processed and targeted to vacuoles of tobacco. Therefore, we are confident in using the O2 cDNA clone for our nuclear localization studies in transgenic tobacco.

Results of immunolocalization studies of O2 protein in transformed tobacco plants reported here showed that the maize O2 protein was targeted to nuclei of tobacco. Therefore, the cellular machinery used for nuclear transport in tobacco is competent for correct targeting of this monocot regulatory protein to the nucleus of this dicotyledonous plant. Thus, despite differences in mRNA processing between monocots and dicots, it is possible that protein targeting is similar between these two classes of plants.

Several examples of nuclear transport of proteins in heterologous systems have been reported in animal systems (Silver and Hall, 1988). More recently, Finlay and Forbes (1990) showed that part of the nuclear transport machinery, the nuclear pore-associated proteins, may be interchanged between rat and Xenopus systems, suggesting conservation between classes.

In general, nuclear targeting studies in animal and yeast systems suggest that small proteins (20 kD to 40 kD) diffuse through the nuclear pore where the proteins are selectively retained. Larger proteins are actively transported through the nuclear pore and require a nuclear localization signal (Dingwall and Laskey, 1986; Silver and Hall, 1988). These localization signals are rich in basic amino acids and usually contain a proline or glycine. The O2 protein possesses two regions that contain putative targeting signals, amino acids 119 to 129 and, in the beginning of the basic domain, amino acids 223 to 232 (Schmidt et al., 1990). Experiments are in progress to determine whether the 47-kD O2 protein has the ability to target a reporter protein to nuclei. Further analysis will determine whether these putative targeting regions are actually involved in nuclear targeting.

METHODS

Plant Materials

Maize plants harboring the O2 and o2 null alleles (Schmidt et al., 1987) in a Wisconsin 22 background were maintained under greenhouse conditions. Endosperm and embryo tissues were harvested at 12 DAP, 15 DAP, 18 DAP, and 22 DAP. Maize plants harboring the o2-m20 allele (Schmidt et al., 1987) were field grown, and kernels were harvested at 26 DAP. Tobacco plants (Nicotiana tabacum cv Wisconsin 38) were maintained sterilely as described in Wilkins et al. (1990).

Plant Transformations

The construct used for tobacco transformations p35S O2 (see Figure 1) was made by modifying the O2 cDNA 1-4 described by Schmidt et al. (1990). The O2 cDNA contains a long untranslated leader sequence with three methionine start codons, each of which is followed by an in-frame stop codon. The function of this leader sequence is unknown, but it may impart some sort of translational control on O2 expression. To eliminate possible complications in the translation of the O2 mRNA in transgenic tobacco, we deleted the portion of the untranslated sequence that contains the upstream ATGs. The O2 transcript was cloned into the SK Bluescript vector (Stratagene). This plasmid was linearized using an Spnl/BamH double digest, and part of the 5'-noncoding region was removed using exonuclease III digestion (Henikoff, 1987) according to the Pharmacia nested deletion kit. Clones containing deletions were sequenced using the dideoxy nucleotide method (Sanger et al., 1977). The deletion clone used in this study has 63 nucleotides 5' of the initiating ATG (Figure 1). The construct was further modified by digestion with SmaI and ligation of HindIII linkers (New England Biolabs). This allowed cloning of the entire transcript, as a HindIII fragment, into the Agrobacterium vector PGA643 (An et al., 1988). The O2 gene construct, designated p35SO2, was transformed into tobacco plants as described in Wilkins et al. (1990).

RNA Gel Blot Analysis

For RNA blot analysis of maize kernels, only endosperm tissue was used. Endosperms were dissected from whole seeds and frozen in liquid nitrogen. Tobacco leaves and roots were frozen in liquid nitrogen and stored at -80°C until extraction. Total RNA was extracted from 1 g of tobacco leaves or maize endosperm using the aurin tricarboxylic acid method of Nagy et al. (1988). RNA gel blot analysis was done as described in Ludwig et al. (1989). Ten micrograms of total RNA was subjected to electrophoresis, blotted onto a nylon membrane, and hybridized with a 35S-labeled O2 cDNA probe. The probe was prepared by labeling the O2 cDNA with [35S]dCTP using the random primer method (Feinberg and Vogelstein, 1984). After hybridization, the blot was washed and exposed to autoradiographic film.
phoresis in 1.2% agarose, formaldehyde gels and transferred to nitrocellulose paper. Filters were prehybridized for 4 hr at 65°C in hybridization buffer [0.33 M NaPO₄, pH 7.0, 0.1 M EDTA, 5% SDS, 0.5 mg/L single-stranded salmon sperm DNA (Sigma)]. Hybridization occurred overnight at 65°C with the addition of the O₂ HindIII insert, labeled with ³²P by the random primer method (Feinberg and Vogelstein, 1983). The blots were washed under stringent conditions two times for 45 min each in 0.1 x SSC, 0.5% SDS at 65°C (Ludwig et al., 1989). Autoradiography was done using Kodak XAR film at -80°C for 4 days with an intensifying screen. To test the integrity of the RNA, filters were hybridized a second time with ³²P-labeled soybean actin gene (Shah et al., 1982). Less stringent conditions were used for the actin probe; hybridization and washes were done at 55°C.

**Protein Gel Blot Analysis**

Partially purified maize O₂ protein used for protein gel blot analysis was made by extracting total protein from endosperm tissues at 22 DAP. Frozen, ground endosperm tissue was extracted in a buffer containing 50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 1 mM EDTA, and 20 mM 2-mercaptoethanol. Debris was removed by centrifugation for 10 min at 8000g. Proteins were precipitated from the supernatant using 20% saturated ammonium sulfate and centrifugation. Remaining salts were removed from the pellet by resuspension in the extraction buffer followed by PD-10 column chromatography (Pharmacia).

Crude extracts of proteins isolated from tobacco plants were made by extracting leaf tissues directly in Laemmli buffer (2 µL of buffer/mg of tissue, fresh weight) (Laemmli, 1970). Thirty microliters of extract was used for protein gel blot analysis. SDS-PAGE was done as described in Sambrook et al. (1989), and proteins were transferred to Immobilon P (Millipore), according to Towbin et al. (1979). Filters were blocked overnight in blotto (Sambrook et al., 1989), incubated for 1.5 hr in a 1:2000 dilution of anti-O₂ sera, washed, and incubated for an additional 1.5 hr in a 1:10,000 dilution of goat anti-rabbit alkaline phosphatase-conjugated IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Color development was done as described in Blake et al. (1984).

**Immunocytochemistry**

O₂ protein was detected in maize and tobacco tissues essentially as described in Raikhel and Quatrano (1986). Tissues used for immunocytochemistry were harvested and fixed immediately. Maize kernels were first cut longitudinally, bisecting the embryo. Each half-kernel was cut longitudinally and cross-wise, yielding four quarters. Tobacco tissues for immunocytochemistry were cut into 2-mm to 3-mm cross-sections and fixed immediately after harvest. Fixation was performed in PBS, pH 7.2 (140 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 0.02% sodium azide) containing 4% paraformaldehyde and 0.1 M sucrose. After 24 hr, tissues were washed 3 x 10 min in PBS, pH 7.2, containing 0.5 M sucrose. Frozen sections were prepared as previously described (Raikhel et al., 1989) and placed on slides pretreated with 50 mg/L poly-L-lysine (Sigma). Sections were simultaneously stained for immunocytochemistry and with the nuclei-specific dye DAPI. The specific antibody and nonimmune rabbit serum were used at a concentration of one part per 500. Rhodamine-conjugated goat anti-rabbit secondary antibody (Pierce) was used at a concentration of 1 part per 3000. Sections were mounted with a DAPI-glycerol mixture [20 µg/mL DAPI (Sigma), 90% glycerol, 0.1 x PBS, 10 mM sodium azide], as described by McLean et al. (1990).

The immunocytochemistry was performed by immunofluorescence and, sections were viewed with epifluorescence optics (Axioptot, Zeiss) with a 450-nm to 495-nm filter. The same sections, counterstained with DAPI, were viewed with epifluorescence using a 365-nm filter. Color pictures were made with 1600 ASA Kodak Ektapress film and were processed commercially.

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