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The Maize Abscisic Acid-Responsive Protein Rab17 Is Located in the Nucleus and Interacts with Nuclear Localization Signals

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The maize abscisic acid (ABA)-responsive *rab*17 mRNA and Rab17 protein distribution in maize embryo tissues was investigated by in situ hybridization and immunocytochemistry. *rab*17 mRNA and Rab17 protein were found in all cells of embryo tissues. Synthesis of *rab*17 mRNA occurred initially in the embryo axis. As maturation progressed, *rab*17 mRNA was detectable in the scutellum and accumulated in axis cells and provascular tissues. However, the response to exogenous ABA differed in various embryo cell types. The Rab17 protein was located in the nucleus and in the cytoplasm, and qualitative differences in the phosphorylation states of the protein were found between the two subcellular compartments. Based on the similar domain arrangements of Rab17 and a nuclear localization signal (NLS) binding phosphoprotein, Nopp140, interaction of Rab17 with NLS peptides was studied. We found specific binding of Rab17 to the wild-type NLS of the SV40 T antigen but not to an import incompetent mutant peptide. Moreover, binding of the NLS peptide to Rab17 was found to be dependent upon phosphorylation. These results suggest that Rab17 may play a role in nuclear protein transport.

INTRODUCTION

The plant hormone abscisic acid (ABA) mediates plant developmental and physiological processes, such as embryo maturation and the response of vegetative tissues to osmotic stress (reviewed by Zeevaart and Creelman, 1988; Quatrano et al., 1992). The maize ABA-responsive *rab*17 gene is induced during late embryogenesis when ABA levels are high, and it is also ABA and water stress inducible in embryo and vegetative tissues (Vilardell et al., 1990). Several gene products homologous to *rab*17-encoded proteins (also known as dehydrins and late embryogenesis abundant Lea proteins) have been identified in different plant systems (reviewed by Skriver and Mundy, 1990; Close et al., 1993). Based on the highly conserved protein structure and regulation of the *rab* genes, theoretical models implicating protective roles of these proteins in the plant during water stress have been proposed (reviewed by Dure, 1993). Despite considerable research in this field, the function of the ABA-responsive proteins and the mode of action of the hormone remain elusive. Elucidating the functions of these proteins is an important step to understanding the molecular mechanisms of ABA action in the response of the cells to desiccation.

In an earlier study, we showed that the Rab17 protein is the most heavily phosphorylated protein in the mature maize embryo (Goday et al., 1988). The predicted protein sequence of Rab17 contains a cluster of eight serine residues followed by a consensus site for casein kinase II (CKII). Previously, we established that Rab17 is phosphorylated by CKII exclusively in serine residues (Plana et al., 1991). Following this acidic domain, there is a basic amino acid stretch comprising abundant lysines (Vilardell et al., 1990). Interestingly, similar domains have been described in the sequence of a mammalian nucleolar phosphoprotein, Nopp140 (Meier and Blobel, 1992). Nopp140 had been identified independently by two groups (Yamasaki et al., 1989; Meier and Blobel, 1990) as a nuclear localization signal (NLS) binding protein that can function in nuclear cytoplasmic transport (Meier and Blobel, 1992). Furthermore, the NLS binding protein from yeast, NSR1, also contains an extended negatively charged stretch of amino acids with multiple serine residues (Lee et al., 1991) and has been involved in ribosome biogenesis (Lee et al., 1992).

To further understand the basis for ABA induction and the accumulation of the *rab*17 mRNA and Rab17 protein during embryo development, we have followed Rab17 synthesis through the distribution of its mRNA and protein in embryo

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tissues by using in situ hybridization, subcellular fractionation, and immunomicroscopy. Our results indicated that the *rab*17 mRNA and Rab17 protein exist in all embryo cell types, although the relative concentrations differ in the various tissues. The Rab17 protein was located in the nucleus and in the cytoplasm, and qualitative differences in the phosphorylation state of the protein were found in both compartments.

The similar domain arrangements of the Nopp140 and Rab17 proteins prompted us to investigate whether they also had similar functional properties. As a first step, we studied the Rab17 protein interaction with NLSs. The interaction of Rab17 with NLSs indicated specific binding of Rab17 to the wild-type NLS of the SV40 T antigen but not to an import-incompetent mutant peptide. Moreover, as occurs with Nopp140, binding of NLS to Rab17 was found to be dependent on phosphorylation. The relevance of these results is discussed.

RESULTS

Spatial Pattern of *rab*17 mRNA Accumulation during Late Embryogenesis

We reported previously that *rab*17 mRNA is not detectable in young embryos (before 20 days after pollination), progressively accumulates during late embryogenesis, and rapidly disappears upon rehydration of embryos. We also showed that incubation of developing embryos with ABA increases the level of *rab*17 mRNA in RNA gel blots (Goday et al., 1988).

To investigate spatial and temporal patterns of *rab*17 gene expression during embryo maturation, we used in situ hybridization. Fixed embryo sections were hybridized with digoxigenin-labeled *rab*17 antisense or sense strand probes to localize *rab*17 mRNA sequences within maize cell types. Figures 1 and 2 show the distribution of *rab*17 mRNA in embryos of different developmental stages, with and without ABA treatment. Figure 1 shows longitudinal sections of embryos at 22 and 40 days after pollination. Figure 2 shows transverse sections through the radicle and plumule of embryos at 30 and 40 days after pollination.

During normal embryogenesis, *rab*17 mRNA is first detectable in young embryos at 22 days after pollination (Figure 1A). The *rab*17 antisense probe produced weak hybridization staining throughout the axis, which is more intense in leaf primordia and radicle, suggesting that these are the initial sites at which RNA synthesis in young embryos occurs. Hybridization staining was not detected or was very low in the scutellum. At later stages of development, *rab*17 mRNA was detected evenly throughout the embryo axis organs and scutellum (Figures 1C and 2A to 2D), but it showed the most prevalent accumulation in the following tissues: in the embryonic radicle *rab*17 mRNA accumulated in the radicle cortex (Figures 2A and 2C), the protoxylem (Figure 2I), and in initial metaxylem cells enlarging in the central core (Figures 2C and 2D). In the plumule, accumulation of *rab*17 mRNA was observed in leaf primordia,

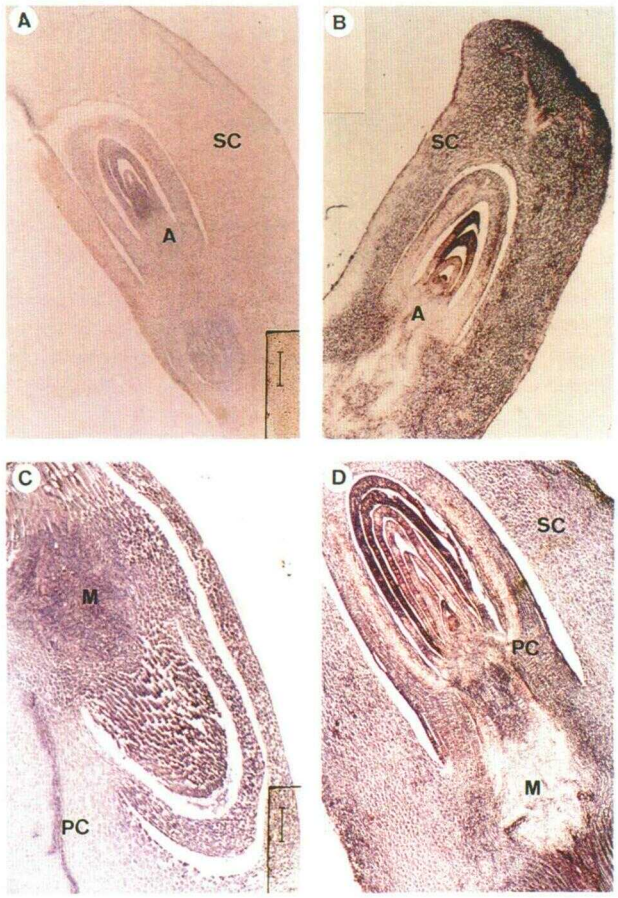

Figure 1. *rab*17 mRNA Localization during Maize Embryo Development and after ABA Treatment.

(A) and **(B)** In situ hybridization of the *rab*17 antisense RNA probe with paraffin-embedded longitudinal sections of embryos at 22 days after pollination as shown in **(A)** and after 24 hr of treatment with ABA as shown in **(B)**.

(C) and **(D)** In situ hybridization of the *rab*17 antisense RNA probe with paraffin-embedded longitudinal sections of embryos at 40 days after pollination as shown in **(C)** and after 24 hr of ABA treatment as shown in **(D)**.

A, axis; SC, scutellum; M, mesocotyl; PC, procambium. Bars in **(A)** and **(C)** = 1 μm.

coleoptile, and provascular elements (Figure 2B). In the scutellum, hybridization staining was more intense in procambium strands (Figures 1C and 2A) and in the surface epidermal cells (Figures 2A and 2C).

By contrast, after ABA treatment, a differential response of the various cell types was observed. Following imbibition in ABA, there was a large increase in the level of detectable mRNA in the scutellum (compare Figures 1A and 1B, Figures 2A and 2E, and Figures 2C and 2G). A similar increase was not detected in most of the axis tissues except the epidermis in which *rab*17 mRNA accumulated (Figures 1D and 2F). Moreover, provascular tissues and mesocotyl cells showed a decrease

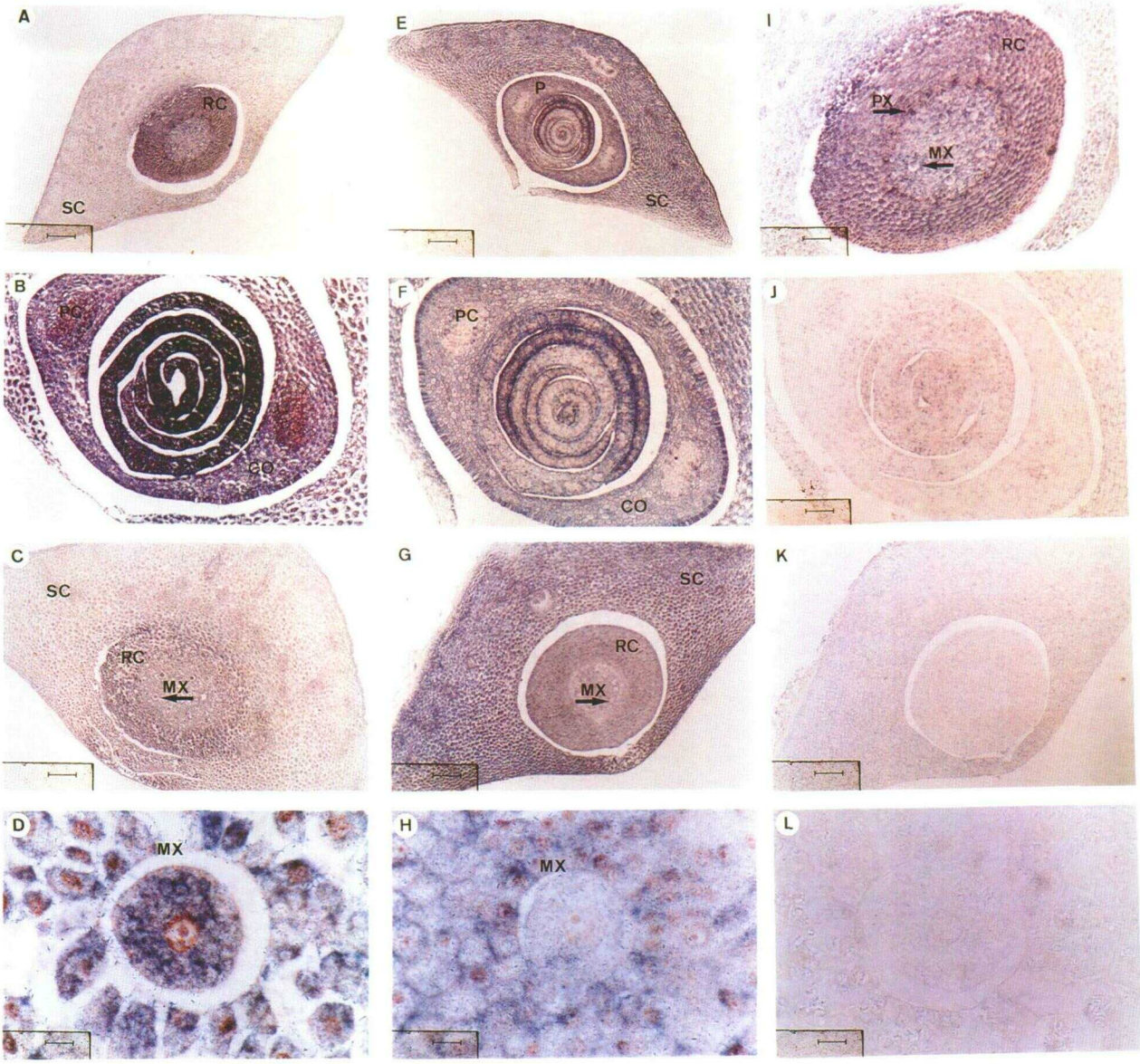

Figure 2. *rab*17 mRNA Localization during Maize Embryo Maturation and after ABA Treatment.

(A) to **(D)** In situ hybridization of the *rab*17 antisense RNA probe with paraffin-embedded sections of normally developing embryos. Transverse sections from the radicle in **(A)** and the plumule in **(B)** of embryos at 30 days after pollination are shown. A transverse section from the radicle in **(C)** and a detailed view of a metaxylem cell in **(D)** of an embryo at 40 days after pollination are shown. Blue staining indicates regions containing RNA–RNA hybrids. A more detailed view of **(A)** is shown in **(I)**.

(E) to **(H)** In situ hybridization of the *rab*17 antisense RNA probe with sections of embryos incubated with ABA for 24 hr. A transverse section from a plumule in **(E)** and a more detailed view in **(F)** of an embryo 30 days after pollination are shown. Shown in **(G)** is a transverse section through a radicle of an embryo at 40 days after pollination, with a more detailed view of a metaxylem cell provided in **(H)**.

(I) A more detailed view of **(A)**.

(J) to **(L)** In situ hybridization of a *rab*17 mRNA control probe. The arrows in **(C)**, **(G)**, and **(I)** point to protoxylem (PX) and metaxylem (MX) cells. Sc, scutellum; PC, procambium; CO, coleoptile; RC, radicle cortex. Bars = 1 μm.

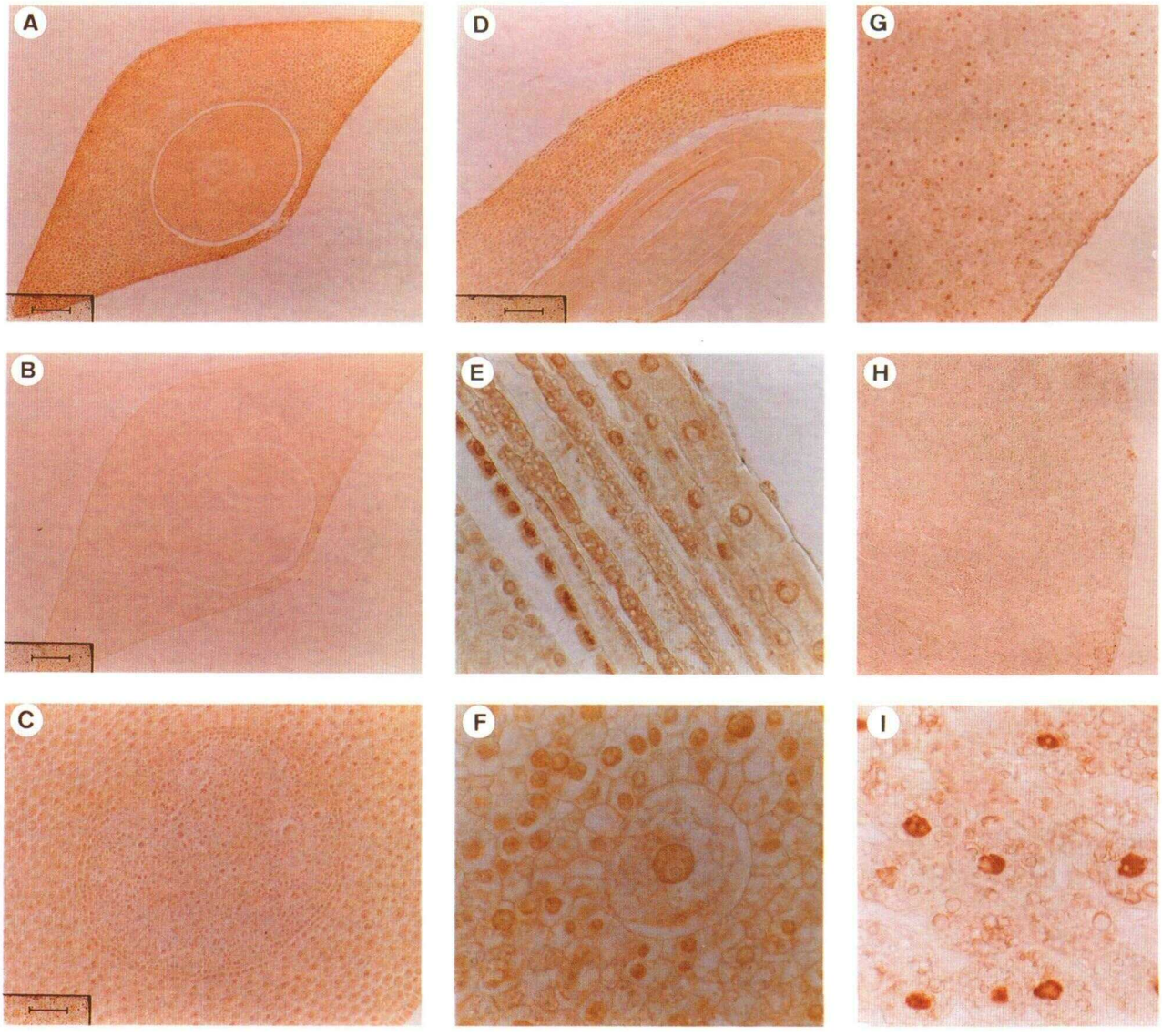

Figure 3. Immunolocalization of Rab17 Protein in Mature Embryos.

Paraffin-embedded sections of embryos at 40 days after pollination were incubated with anti-Rab17 antibodies and an avidin-biotin-peroxidase detection system. Brown staining indicates a Rab17 antibody–specific reaction.

(A) to **(C)** A transverse section through a radicle in **(A)**, control reaction using nonimmune serum in **(B)**, and a detailed view of the radicle central core in **(C)** are shown.

(D) to **(F)** A longitudinal section from a plumule is shown in **(D)**, with a more detailed view of stained nuclei in coleoptile and metaxylem cells provided in **(E)** and **(F)**, respectively.

(G) to **(I)** Longitudinal section from a scutellum in **(G)** and a control reaction using nonimmune serum in **(H)** are shown. In **(I)**, a more detailed view of the scutellar cells is provided.

Bars in **(A)**, **(B)**, **(C)**, and **(D)** = 1 μm.

in hybridization signal after ABA treatment (Figures 1D and 2F to 2H). In situ hybridization with a *rab*17 mRNA control probe was used to monitor background hybridization. The specificity of the reaction was shown by the lack of appreciable reaction of the *rab*17 sense strand probe with the paraffin-embedded sections (Figures 2J to 2L).

The Rab17 Protein Is Located in the Nucleus and the Cytoplasm of Maize Embryo Cells

To determine the localization of Rab17 protein in maize embryos, an immunochemical study was undertaken by using immunocytochemistry. Figure 3 shows bright-field photographs

of paraffin sections from embryos at 40 days after pollination that were incubated with anti-Rab17 antibodies. In agreement with the in situ hybridization data, the Rab17 protein was detected in all types of cells of mature embryos (Figures 3A, 3D, and 3G). Surprisingly, anti-Rab17 antibodies reacted very strongly with the nucleus, whereas nonimmune serum exhibited only background staining. Particularly strong signals were visible in nuclei of the scutellum and epidermal cells (Figures 3E and 3I). Figure 3 also shows high-magnification photographs of coleoptile (Figure 3E), metaxylem (Figure 3F), and scutellum (Figure 3I) cells. Specific labeling of the cytoplasm by the anti-Rab17 antibody, exceeding that seen with nonimmune sera, was also observed. The cytoplasmic signal was evenly distributed in all cell types (Figures 3A and 3D). It was, however, enhanced in embryo leaf (Figure 3D) and root tissues (Figure 3C) and in the scutellum of dry embryos (data not shown). Concomitantly, in these cells, a lower level of nuclear staining was observed.

Distribution and Phosphorylation State of Rab17 in Nuclear and Cytosolic Fractions

To further confirm the intracellular location of Rab17, immunodetection of the protein in subcellular fractions of maize embryos at 40 days after pollination was performed. Embryos were fractionated on a discontinuous Percoll gradient (Luthe and Quatrano, 1980), and the distribution of the Rab17 protein in subcellular fractions was analyzed on protein gel blots with anti-Rab17 antibodies. Figure 4A shows that Rab17 was present in both first pellet and cytosolic fractions (Figure 4A, lanes 2 and 3) and in a unique band in the 80% Percoll fraction (Figure 4A, lane 7) from which nuclei are recovered.

Because Rab17 is a highly phosphorylated protein in the mature maize embryo, to assess whether different phosphorylation states of Rab17 result in protein accumulation in different cellular compartments, proteins from the cytosolic and nuclear fractions were analyzed. Protein gel blot analysis of Rab17 resolved by two-dimensional electrophoresis showed that nuclear (Figure 4B) and cytoplasmic (Figure 4C) Rab17 proteins are a mixture of phosphorylated and unphosphorylated forms. Different levels of phosphorylation were detected in both cellular compartments. In the cytoplasm, the highest phosphorylated forms of Rab17 were more abundant than in the nucleus. Together, these results indicated that phosphorylated Rab17 forms are found partly in the cytosol and partly associated with nuclei structures.

Interaction of Rab17 with NLSs

To determine whether the conserved protein domains and the acidic serine cluster followed by the consensus site for CKII and basic amino acid stretches, which are found in both Nopp140 and Rab17 proteins (see Figure 5), reflected similar functional properties, we studied the ability of Rab17 to interact with synthetic NLS peptides. In plants as well as in animal cells, the wild-type SV40 T antigen NLS is sufficient to promote nuclear localization of cytoplasmic proteins (Kalderon et al., 1984; van der Krol and Chua, 1991). The mutant SV40 NLS, with the single amino acid change of Lys-128 to Thr, fails to direct nuclear import either in mammals or plants. Therefore, we used functional wild-type and mutant synthetic peptides

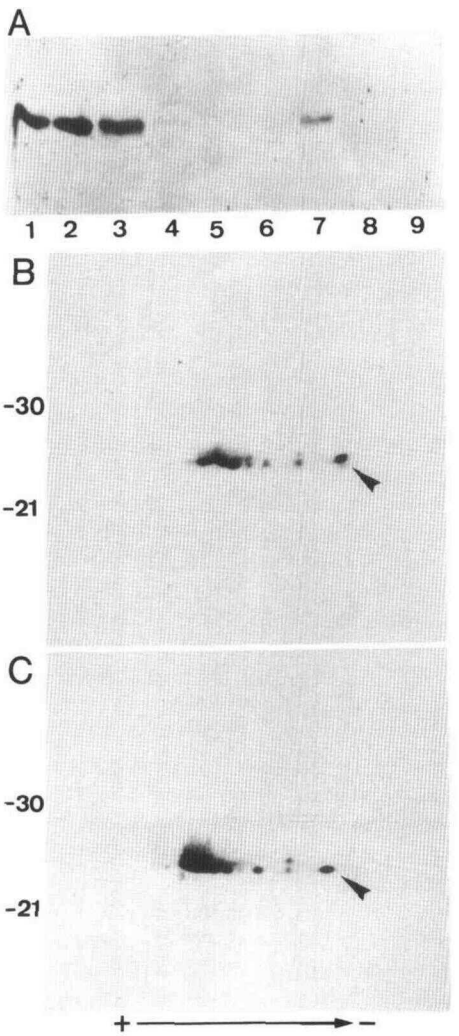

Figure 4. Distribution and Phosphorylation of Rab17 in Nuclear and Cytosolic Fractions.

(A) Immunoblot analysis of Percoll reactions. Lanes contain equal amounts of proteins. Lane 1 contains total embryo proteins; lane 2, the cytosolic fraction; lane 3, the pellet. Lanes 4 to 7 show the distribution of Rab17 in the Percoll gradient; lane 4 contains the supernatant and lanes 5, 6, and 7 contain the 40, 60, and 80% fractions, respectively. Lane 8 contains the sucrose cushion and in lane 9 is the pellet. **(B)** and **(C)** Bidimensional immunoblot showing phosphorylated forms of Rab17 in nuclear **(B)** and cytosolic **(C)** fractions. Isoelectric focusing was run from the acidic (+) to the basic (–) end. Protein markers are shown at left in kilodaltons. Arrows indicate native form of Rab17.

	phosphorylation domain		basic domain	
Rab17				
76	SGSSSSSSSEDD	GMGG	RRKK	GIKEKIKEKLPG
Nopp140				
127	ESSSSEESSEEE	EEKD	KKKK	PPQQKAVKPQAK
170	SESESDSSSEDE	APQT	QKPK	AAATAAKAPTKA
274	SSSSEDSSSEEE	EEQK	KPMK	KKAGPYSSVPPP
479	SSDSESSSSEEE	KKTP	PKPP	AKKKAAGAAVPK
524	SSSSSEDSSEEE	KKKP	KSKA	TPKPQAGKANGV
NSR1				
118	SSSSSSSDSEKE	ESND	KKRK	SEDAEEEEDEES

Figure 5. Conserved Protein Domains between Rab17 and NLS Binding Proteins.

Phosphorylation domain indicates the region (boxed) of Rab17 that was shown to actually contain the phosphate groups. This region contains one acidic serine cluster followed by the consensus site for CKII. Basic domain indicates the four basic amino acids (boxed) that may represent an actual NLS. Numbers at left represent amino acid positions in the protein sequence.

of the SV40 T antigen NLS to assay their binding to Rab17 in different experiments.

First, we used the ligand blotting technique (Lee and Melese, 1989) to detect binding between the Rab17 protein bound on nitrocellulose filters and the wild-type peptide conjugated to human serum albumin (HSA) or the ^{14}C-labeled free wild-type peptide in solution. Binding was detected by antibodies against HSA or, in the case of ^{14}C-labeled free wild-type NLS peptide, by autoradiography. Figure 6A shows the interaction of both the peptide conjugate (lane 3) and the free ^{14}C-labeled peptide (lane 5) with Rab17 (lanes 2 and 4) as well as with other proteins from total protein extracts from maize embryos.

It has been reported that only phosphorylated Nopp140 binds to NLS peptides (Meier and Blobel, 1992). To test whether phosphorylation might be responsible for the interaction with the wild-type NLS peptide, we employed partly purified fractions of phosphorylated or dephosphorylated Rab17. These semipurified fractions contained other proteins that were used as controls for unspecific binding (Figure 6B, lanes 1 and 2). The proteins were probed with the wild-type peptide conjugate. As shown in Figure 6B, phosphorylated but not dephosphorylated Rab17 bound to the wild-type peptide conjugate, demonstrating that binding of NLS to Rab17 is dependent upon phosphorylation (Figure 6B, lanes 3 and 4). To investigate the specificity of this interaction, competition experiments were performed to determine wild-type conjugate binding to Rab17 with free NLS peptides. Competitions were conducted with 10-, 50-, and 100-fold excess of free peptides as compared with the coupled peptide. Figure 6C shows that binding of Rab17 to the peptide conjugate was competed for strongly by the free wild-type peptide and only weakly by the mutant peptide.

Similar results were obtained when the phosphorylated Rab17 protein was incubated with immobilized wild-type or mutant peptides (data not shown). The Rab17 protein was strongly retained in the wild-type peptide column but to a much lesser extent in the mutant peptide column. Together, these results indicated a specific interaction of the wild-type but not the mutant peptide to Rab17.

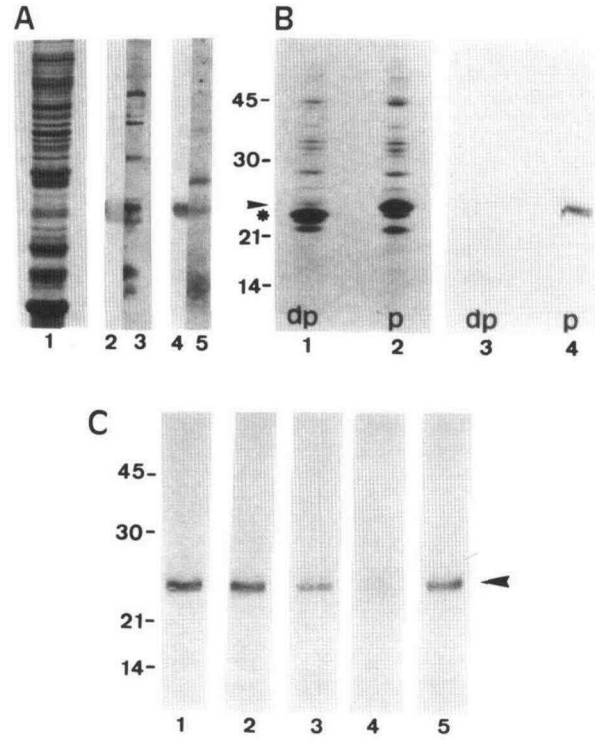

Figure 6. Interaction of Rab17 with NLS Peptides.

Ligand blotting of the Rab17 protein with NLS peptides is shown.
(A) Total protein extracts from mature embryos were stained with Coomassie Brilliant Blue R250 (lane 1). Protein extracts from mature embryos were blotted to nitrocellulose filters and incubated with an anti-Rab17 antibody (lanes 2 and 4), the wild-type peptide–HSA conjugate (lane 3), or the ^{14}C-labeled wild-type peptide (lane 5).
(B) Phosphorylated (p) and dephosphorylated (dp) semipurified Rab17 proteins were stained with Coomassie blue (lanes 1 and 2). Phosphorylated and dephosphorylated semipurified Rab17 proteins were incubated with the wild-type peptide conjugate (lanes 3 and 4). The arrow points to the phosphorylated form, and an asterisk denotes the dephosphorylated Rab17 protein.
(C) Phosphorylated Rab17 was incubated with the wild-type peptide conjugate plus 0- (lane 1), 10- (lane 2), 50- (lane 3), and 100-fold (lane 4) chemical excesses of free wild-type or a 100-fold chemical excess of mutant peptide (lane 5). The arrow points to the Rab17 protein. Protein markers are shown at left in kilodaltons.

DISCUSSION

Distribution of *rab*17 mRNA and Rab17 Protein in Embryo Cells

The function of the Rab17 protein is not yet known. As a first step in the identification of the function of the protein and to assess its importance in the plant responses to ABA and environmental stress, we studied the localization of *rab*17 mRNA and Rab17 protein in developing maize embryos by means of in situ hybridization and immunocytochemistry.

Our data indicated that the *rab*17 mRNA and Rab17 protein are ubiquitously distributed in the various embryo tissues. *rab*17 mRNA displayed distinct patterns of accumulation in developing embryos and after ABA treatment. During normal embryogenesis, synthesis of *rab*17 mRNA occurred initially in the embryo axis. As maturation progressed, *rab*17 mRNA accumulated in the scutellum and axis cells. Interestingly, the most prevalent expression during embryo desiccation was observed in epidermis cells, the radicle cortex, and the coleoptile, suggesting that the outermost tissues are the first to sense environmental stress and respond by increasing *rab*17 mRNA.

In agreement with previous results, *rab*17 mRNA increased after ABA treatment. This effect was stronger in scutellum than in axis cells. Moreover, a surprising decrease in *rab*17 mRNA levels was observed in mesocotyl and provascular cells on ABA treatment. Together, these results indicated that the response to exogenous ABA of the different cell types is not uniform. The actual decrease in *rab*17 mRNA levels that occurred in provascular cells may be explained as a rehydration response of the cells upon imbibition of the embryo in the ABA solution. There is evidence of xylem-transported ABA in many plants (Gowing et al., 1993); therefore, a lower sensitivity to ABA may well exist in the xylem initial cells in embryos. Alternatively, we cannot exclude other possibilities such as differences in the pH or a higher rate of mRNA degradation in these cells.

In summary, the different distribution of the *rab*17 transcripts in specific cells after exogenous ABA treatment reinforced our previous data showing that two distinct molecular mechanisms are responsible for developmental and exogenous ABA induction of *rab* genes (Pla et al., 1991; Vilardell et al., 1994).

Rab17 Is a Nuclear Protein but It Is also Found in the Cytoplasm

Our immunochemical data showed that in maize embryos, Rab17 is associated in part with nuclear structures and in the cytosol. Rab17 protein homologs from different plant species, such as the tomato ABA- and salt-responsive protein TAS14 and dehydrins from barley and maize, have also been found associated with both the cytoplasm and the nucleus (Luna et al., 1991; Close et al., 1993). In contrast, other reports indicate cytosolic localization of Rab21 in ABA-treated rice leaves (Mundy and Chua, 1988) and of desiccation stress protein Dsp16 in desiccated leaf cells of *Craterostigma plantagineum* (Schneider et al., 1993).

The existence of the Rab17 protein in the nucleus raises some questions regarding the nuclear targeting of the protein. The predicted amino acid sequence of Rab17 does not contain any sequence homology with the various NLSs identified so far in different nuclear proteins (Garcia-Bustos et al., 1991; Varagona et al., 1992). However, Rab17 and other homologous proteins sequenced in different plant systems contain the four–amino acid motif RRKK that resembles that SV40-like NLS sequence (K-R/K-X-K/R) (Kalderon et al., 1984). Experiments are currently in progress to assess the functionality of this sequence.

Other transport models for nuclear protein import have been described (reviewed in Silver, 1991; Garcia-Bustos et al., 1991); thus, it is also possible that Rab17 could be cotransported to the nucleus through protein–protein interaction. This hypothesis is especially attractive based on the interaction of phosphorylated Rab17 with synthetic NLS peptides (see below). It has been proposed that cytoplasmic binding proteins may recognize NLSs and deliver proteins to the pore complex or to the nuclear interior before being released and recycled to the cytoplasm (Silver, 1991). In this context, the Rab17 protein has been found in the nucleus and cytosol. Interestingly, specific binding of synthetic NLS peptides to the nuclear envelope has recently been shown in plants (Hicks and Raikhel, 1993). Experiments are in progress to enable the localization of Rab17 protein–NLS complexes within the cell.

Bidimensional gel patterns of Rab17 from nuclear and cytosolic fractions indicated that the degree of accumulation of phosphorylated forms differed in the two cellular compartments. The relative amounts of the most highly phosphorylated forms of the protein were higher in the cytoplasm than in the nucleus. To date, it is not known whether the distribution of distinct phosphorylated forms of Rab17 in both cellular compartments may reflect functional differences. It is not yet known if Rab17 is phosphorylated in the nucleus or in the cytoplasm. It is known that CKII activity exists in both cellular compartments (Klimczak et al., 1992; Krek et al., 1992). Furthermore, it has been suggested that CKII may introduce functional determinants for nuclear localization (Rihs et al., 1991). Further work is needed to establish the phosphorylation mechanism of Rab17 and how the different phosphorylation states of the protein affect its function.

Rab17 Interacts with Synthetic NLS Peptides

Several NLS binding proteins have been identified and characterized on the basis of their ability to interact with synthetic NLS peptides by using various methods that include chemical and photoaffinity cross-linking, ligand blot assays, and purification through antibody affinity columns (reviewed by Forbes, 1992). NLS binding proteins, such as the 60- and 70-kD proteins in rat liver cells (Adam et al., 1989; Yamasaki et

al., 1989) and the 66-kD protein in HeLa cells (Li and Thomas, 1989), have been found in the cytoplasm, the nuclear envelope, and the nucleoplasm. For example, in yeast, NBP70 was found in the cytoplasm and in the nucleus (Stochaj and Silver, 1992), and NSR1 was found in the nucleolus where it is involved in ribosome biogenesis (Lee et al., 1992). Recently, the rat liver Nopp140 has been shown to shuttle in tracks between nucleolus and cytoplasm (Meier and Blobel, 1992).

Our results showed that the Rab17 protein recognized free or HSA-conjugated synthetic peptides containing the SV40 T antigen NLS in ligand blotting assays. The preferential interaction between Rab17 and the wild-type NLS peptide was dependent on Rab17 phosphorylation. Only the phosphorylated form of Rab17 bound to the NLS peptide and not the dephosphorylated form. One possibility is that the interaction of Rab17 with the wild-type peptide may be due to the association of the positively charged NLS with the phosphorylated serine domain. However, the combined specificity and binding data in competition experiments make this unlikely. An excess of the free wild-type peptide, but not the free mutant peptide, abolished binding of the wild-type peptide conjugate to Rab17. Moreover, similar results have been found in Nopp140 in which NLS binding was dependent on phosphorylation (Meier and Blobel, 1992), as it was for NBP70 (Stochaj and Silver, 1992) and NSR1 (Forbes, 1992). The conserved protein domains found in the Nopp140, NSR1, and Rab17 proteins suggest that similar NLS binding mechanisms may be operative. In this context, the highly phosphorylated domain could facilitate rapid binding to the NLS of specific proteins in various physiological conditions and/or in different cellular compartments. Unambiguous localization of the Rab17 protein NLS binding sites as well as the physiological relevance of its NLS binding property remain to be determined.

Possible Function of Rab17 Protein: An Hypothesis

ABA-induced transcription is a highly specific process that allows expression of only certain genes in response to the hormone. Elaborated regulatory mechanisms are thought to modulate gene induction and repression by ABA and during water stress. Strong evidence indicates that the activity of transcriptional activators and repressors in animal systems is regulated by their transport from the cytoplasm to the nucleus in response to developmental cues or environmental factors (reviewed in Hunter and Karin, 1992). In this way, factors are retained in the cytoplasm in an inactive complex with inhibitory proteins. In response to various stimuli, the complex dissociates and the binding activity of the factor is detected in the nuclei. Although the precise function of Rab17 remains to be elucidated, based on its pattern of expression, NLS-binding ability, and nuclear cytosolic localization, the following hypothesis for Rab17 protein function is proposed. We suggest that Rab17 may function by interacting with specific proteins through association with their NLSs during stress conditions, either sequestering them in the cytoplasm and rendering them inactive through lack of access to their target sequences or functioning as a nuclear import/export carrier. In this context, different levels of phosphorylation of Rab17 may represent a means of regulating association/dissociation of the complex(es).

In this study, we identified Rab17 in both the nucleus and cytoplasm and showed its specific interaction with wild-type NLS peptides. These results should provide a starting point to address questions about the biological role of the Rab17 and to investigate the mechanisms of ABA action on gene expression.

METHODS

Plant Material

Embryos of maize (*Zea mays*) pure inbred line W64A were dissected manually and used immediately after collection or incubated with 50 μM abscisic acid (ABA) for 24 hr.

Microscopy

For immunolocalization, IgG fractions were purified from the immune serum obtained against the ABA-responsive protein Rab17 (Goday et al., 1988) by precipitation with 40% $(NH_4)_2SO_4$ followed by DEAE–cellulose chromatography. Sections from paraffin-embedded material were blocked with 3% goat serum in phosphate-buffered saline (PBS; 10 mM phosphate, 150 mM NaCl, pH 7.4) for 30 min at 22°C and incubated with anti-Rab17 immune serum (diluted 1:800), the purified IgG fraction (50 μg/mL), and nonimmune serum (diluted 1:800). Immunoreactivity was visualized by the avidin–biotin complex (Vectastain Elite ABC Kit; Vector, Burlingame, CA) using diaminobenzidine as substrate.

For in situ hybridization, digoxigenin-labeled RNA probes were prepared according to the manufacturer's instructions (Boehringer Mannheim). In situ hybridization was performed as described by Jackson et al. (1991). Antibody binding and substrate reaction steps were performed as described by Coen et al. (1990). Sense and antisense probes were transcribed from the *rab*17 cDNA clone in the pBluescript SK+ vector (Stratagene). To make the in situ hybridization data quantitatively comparable, sections from normal and ABA-treated embryos of the same developmental stage were hybridized on the same slides. In all cases, no signal over background was observed using control sense strand probes.

Subcellular Fractionation

Isolation of maize embryo nuclei on discontinuous Percoll gradients was performed according to Luthe and Quatrano (1980). Maize embryos (40 days after pollination) were homogenized in a chilled mortar and pestle in a buffer containing 0.44 M sucrose, 2.5% (w/v) Ficoll 400 (Pharmacia), 5% (w/v) dextran 40, 25 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.5% Triton X-100, and 2 mM spermine. After filtration through Miracloth and centrifugation at 5.850*g* for 5 min, the supernatant, defined as cytoplasmic fraction in the text, was removed, and the resulting pellet was further purified on 40, 60,

and 80% (v/v) discontinuous Percoll layers on a 2 M sucrose cushion. Microscopic observation after staining with Hoechst N33342 (Sigma), toluidine blue O (Merck, Germany), and I_2-KI solution was used to confirm the presence of nuclei devoid of major contaminants in the 80% Percoll fraction. Protein extracts were analyzed by two-dimensional electrophoresis and protein gel blotting using anti-Rab17 antibodies as described previously by Goday et al. (1988). Histone-specific antibodies were used as markers for subcellular fractionation.

Peptide Conjugates

Peptides containing the nuclear localization signal (NLS) of the SV40 T antigen, wild-type peptide (AAPKKKRKVEC), and mutant peptide (AAPKTKRKVEC) were synthesized and radiolabeled with carbon-14 by acetylation of the N-terminal alanine at the Universitat de Barcelona Biopolymer Facility Service (Barcelona, Spain). Peptides were chemically bound to human serum albumin (HSA) by specific coupling through cysteine residues in the peptide using the procedure described by Albericio et al. (1989). Approximately six to eight peptides were coupled per HSA molecule, as determined by amino acid analysis of conjugate hydrolysates.

Ligand Blotting Assays

Ligand blotting was performed essentially as described by Lee and Melese (1989). Partial purification and dephosphorylation of the Rab17 protein was done as described previously (Plana et al., 1991). Protein extracts, without boiling, were resolved by SDS-PAGE and transferred to nitrocellulose filters as described previously by Goday et al. (1988). Nitrocellulose filters were blocked with 0.5% Tween-20/0.5% gelatin in PBS; the blots were cut in half and incubated in parallel with the anti-Rab17 antibody and the peptide–HSA conjugate (10 μg/mL) for 18 hr. Binding of the wild-type peptide–HSA conjugate was detected by antibodies against HSA (Sigma), and either enzyme-labeled or radiolabeled secondary reagents were used. The ligand blot of embryo proteins with free ^{14}C-labeled wild-type peptides was detected by autoradiography. In competition experiments, 10- to 100-fold molar excess (8 to 80 μM) of free peptides were included during the incubation.

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