Nuclear Import in Permeabilized Protoplasts from Higher Plants Has Unique Features

Glenn R. Hicks, Harley M. S. Smith, Stephane Lobreaux, and Natasha V. Raikhel

Michigan State University–Department of Energy Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824-1312

The import of proteins into the nucleus is a poorly understood process that is thought to require soluble cytosolic factors in vertebrates and yeast. To test this model in plants and to identify components of the import apparatus, we developed a direct in vitro nuclear import assay by using tobacco protoplasts that were permeabilized without detergents such as digitonin or Triton X-100. Substrates were imported specifically by a mechanism that required only guanine nucleotides. Moreover, in vitro import did not require exogenous cytosol. To investigate this novel finding, we isolated a full-length cDNA encoding an Arabidopsis homolog of vertebrate and yeast nuclear localization signal receptors and produced an affinity-purified antibody. The plant receptor was tightly associated with cellular components in permeabilized protoplasts, even in the presence of 0.1% Triton X-100, indicating that this factor and probably others were retained to an extent sufficient to support import. The lectin wheat germ agglutinin bound to the nucleus; however, it did not block translocation in our system, indicating that direct interaction with polysaccharide modifications at the nuclear pore complex was probably not essential for import in plants. Other features of in vitro import included reduced but significant import at low temperature.

INTRODUCTION

In eukaryotes, nuclear activities are isolated from other cellular functions by the nuclear envelope (NE). This separation of functions requires the transmembrane import and export of vital macromolecules, such as proteins, mRNAs, tRNAs, ribosomes, and small nuclear ribonucleoprotein particles. These diverse molecules enter and exit the nucleus through a regulated macromolecular channel known as the nuclear pore complex (NPC) (reviewed by Davis, 1995; Hicks and Raikhel, 1995b). The nuclear import of proteins is mediated by nuclear localization signals (NLSs), which have been classified as SV40-like, bipartite, or Mat a2-like (reviewed by Boulikas, 1993, 1994; Hicks and Raikhel, 1995b). Several other NLSs are yet to be classified (Dingwall and Laskey, 1991; Boehm et al., 1995; Siomi and Dreyfuss, 1995).

In animals and yeast, nuclear import has been described experimentally as a two-step process involving energy-independent NLS binding at the NPC followed by temperature-sensitive translocation, which requires ATP and GTP hydrolysis. Moreover, import in vertebrates is blocked by the lectin wheat germ agglutinin (WGA) (reviewed by Hicks and Raikhel, 1995b; Melchoir and Gerace, 1995). The receptor responsible for NLS binding in mammals has been identified in cytosolic and NE fractions (Adam et al., 1989; Adam and Gerace, 1991). It has been subsequently purified from Xenopus (Gorlich et al., 1994), mammals (Adam, 1995; Imamoto et al., 1995b), and yeast (Enenkel et al., 1995) and shown to be necessary for nuclear import in mammals (Gorlich et al., 1994; Imamoto et al., 1995b; Moroianu et al., 1995; Radu et al., 1995a; Weis et al., 1995) and yeast (Azuma et al., 1995; Loeb et al., 1995). The receptor, also termed importin α (Gorlich et al., 1995a), karyopherin α (Radu et al., 1995a), or PTAC 58 (Imamoto et al., 1995b), has close identity to the yeast protein SRP1 (Yano et al., 1992). Moreover, two human homologs (Cortes et al., 1994; Cuomo et al., 1994) and one Drosophila homolog (Kussel and Frasch, 1995; Torok et al., 1995) have been reported. The receptor is thought to form a cytosolic heterodimer with a second factor of 97 kDa, termed p97 (Adam and Adam, 1994; Chi et al., 1995), importin β (Gorlich et al., 1995a, 1995b), karyopherin β (Moroianu et al., 1995; Radu et al., 1995a), PTAC 97 (Imamoto et al., 1995a), or Kap95p (lovine et al., 1995). The heterodimeric complex is thought to be responsible for the NPC binding step of nuclear import (Moroianu et al., 1995; Radu et al., 1995a, 1995b).

In addition to the NLS receptor complex, other components of the import apparatus have been identified (reviewed by Melchoir and Gerace, 1995): the GTPase Ran/TC4 (Melchoir et al., 1993, 1995; Moore and Blobel, 1993), a factor known...
as p10 (Moore and Blobel, 1994) or NTF2 (Paschal and Gerace, 1995), and Hsp70 (Imamoto et al., 1992; Shi and Thomas, 1992). Ran/TC4 and p10 associate directly with the NPC during import (Dingwall et al., 1995; Melchoir et al., 1995; Paschal and Gerace, 1995; Wu et al., 1995; Yokoyama et al., 1995), and recent evidence indicates that p10 also interacts with GDP-bound Ran/TC4 and importin β (Nehrbass and Blobel, 1996). Additional interactions between the importin αβ heterodimer, import substrate, Ran/TC4, and NPC proteins have been described (reviewed by Gorlich and Mattaj, 1996; Rexach and Blobel, 1996).

In plants, unlike vertebrates, the three known classes of NLSs have been demonstrated to bind to purified nuclei at a site that is tightly associated with the NE and NPCs (Hicks and Raikhel, 1993; Hicks et al., 1995). Molecular components of the NLS binding site have been identified (Hicks and Raikhel, 1995a). However, functional characterization of these and other potential components has been hampered by the lack of direct in vitro methods to detect nuclear import. We have developed an import assay using cells in which the plasma membrane, but not the NE, is permeabilized. This assay avoids the use of the cholesterol-binding detergent digitonin, because plants do not possess significant amounts of this sterol. In contrast to other organisms studied, the nuclear import receptor in plants, a homolog of importin α, was found to be resistant to depletion from permeabilized cells. This was particularly evident at the nucleus, even after treatment with Triton X-100. These and several other findings indicate that in vitro import in higher plants exhibits features that may yield insight into this essential process.

RESULTS

Nuclear Import Can Be Directly Observed in Permeabilized Cells from Higher Plants

Description of the Methodology

Suspension-cultured tobacco cells were treated enzymatically to remove cell walls, and the resulting protoplasts were treated to remove vacuoles. These vacuoles typically account for 80% of the total cell volume and contain hydrolytic activities. For a schematic diagram of the steps used in preparing protoplasts and monitoring import, see Figure 1A. Evacuolization was achieved by centrifugation of protoplasts through a Percoll density gradient in the presence of 100 mM CaCl₂. The reduced size of evacuolated protoplasts (15 to 45 μm; see Figure 2) compared with untreated protoplasts (50 to 60 μm) probably increased the resistance of the evacuolated cells to mechanical lysis during and after permeabilization of the plasma membrane. The evacuolated protoplasts were separated from vacuoles by centrifugation through sucrose, and several wash steps were applied to reduce the concentration of CaCl₂, whose presence prevents permeabilization. Selective permeabilization of the plasma membrane was achieved by incubating evacuolated protoplasts on ice in buffer having a reduced concentration (from 500 to 225 mM) of mannitol. The resulting permeabilized cells had a rougher surface morphology than did unpermeabilized cells (Figure 3A). The DNA stain 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) selectively entered and stained the nucleus of permeabilized cells (Figure 3). Import substrates that were rhodamine labeled were then added, and nuclear import was allowed to proceed at 23°C and observed by fluorescence microscopy. Cells were directly examined to eliminate the possibility of artifacts due to fixation.

Nuclear Accumulation Is Specific

An artificial, and presumably constitutively translocated, import substrate was constructed by chemically coupling a synthetic peptide to the bipartite NLS from the O2 protein of maize (Figure 1B, O2WT) to human serum albumin (HSA). As

Figure 1. Nuclear Import in Vitro and the NLS Peptides Used to Produce Import Substrates.

(A) Diagram depicting cell permeabilization and nuclear import assay. 
(B) Amino acid sequences of peptides corresponding to the wild-type (O2WT) and mutant (O2mut) NLSs of O2 are shown in single-letter code. Amino acids that were substituted in the wild-type NLS to produce the mutant signal are shaded. A cysteine was added to the C terminus for chemical coupling.
a negative control, a mutated version of this NLS (Figure 1B, O2mut) was coupled to HSA. Consistent with the import activity of these NLSs in vivo (Varagona and Raikhel, 1994), the substrate containing the functional NLS (O2WT-HSA) showed significant accumulation in the nucleus (Figures 2A to 2C), whereas the mutant NLS substrate (O2mut-HSA) showed little accumulation (Figures 2D to 2F). Functional and mutant SV40 large T-antigen NLSs coupled to HSA also displayed similar amounts of accumulation (data not shown) consistent with their activities in vivo (Varagona and Raikhel, 1994).

**NE Is a Barrier to Diffusion in Permeabilized Protoplasts**

Addition of HSA without an NLS resulted in no detectable accumulation in the nucleus (Figures 2G to 2I), indicating that the NE was intact and served as an effective diffusion barrier to a protein of this mass (65 kD). The integrity of the NE to diffusion was also confirmed by the exclusion of a monoclonal DNA antibody from the nucleus. When added to permeabilized tobacco protoplasts at 23°C, the DNA antibody was effectively excluded for at least 45 min (Figures 3A to 3C).

![Figure 2. Import in Permeabilized Tobacco Protoplasts Is Specific.](image)

Protoplasts were incubated for 10 min at 23°C with import substrates and then visualized directly by Nomarski optics ([A], [D], and [G]) or epifluorescence ([B], [E], and [H]) to detect rhodamine-labeled import substrates. Protoplasts were stained with DAPI ([C], [F], and [I]) to indicate the position of the nucleus.

(A) to (C) Permeabilized tobacco protoplasts were incubated with O2WT-HSA.
(D) to (F) Permeabilized protoplasts were incubated with O2mut-HSA.
(G) to (I) Permeabilized protoplasts with HSA only.
Bar in (A) = 32 μm for (A) to (I).
The Plant Cell

Figure 3. The NE in Permeabilized Protoplasts Is Intact.
Protoplasts were visualized directly by Nomarski optics ([A] and [D]) or for rhodamine ([B] and [E]) or DAPI ([C] and [F]) fluorescence. (A) to (C) Permeabilized tobacco protoplasts were incubated with DNA antibodies for 45 min. (D) to (F) Protoplasts from the same preparation that were incubated with 0.1% Triton X-100 and then DNA antibodies. Bar in (D) = 32 μm for (A) to (F).

However, when permeabilized protoplasts were treated with 0.1% Triton X-100 to disrupt the NE before the addition of the antibody, an intense immunofluorescence signal was observed at the nucleus (Figures 3D to 3F). Similar results were obtained on ice, and immunofluorescence signals of lesser intensity also were observed in the nuclei of some permeabilized cells at Triton X-100 concentrations as low as 0.01% (data not shown).

Substrates Are Imported into the Nucleus
To demonstrate that import substrates accumulated within the nucleus rather than at the nuclear periphery, permeabilized protoplasts were sectioned optically by using a laser-scanning confocal microscope. When examined after the addition of O2WT-HSA (Figure 4A, left panel), the substrate was clearly concentrated within the nucleus (Figure 4A, right panel). In comparison to import (Figure 4B, right panel), the binding of O2WT-HSA to the NE and NPCs of purified tobacco nuclei, via a previously published in vitro binding assay (Hicks and Raikhel, 1993), results in a punctate pattern of NPC labeling (Figure 4B, left panel). These data demonstrate that permeabilized tobacco protoplasts specifically accumulate substrates within the nucleus. Furthermore, import does not occur by simple diffusion; rather, it occurs by a facilitated process.

Import Requires Energy
Import Requires Energy
Import Requires Energy

Import Is Inhibited by Guanine Nucleotide Analogs
Import was examined in the presence of nucleotides and nucleotide analogs to determine if there was a specific energy requirement for import. When compared with control protoplasts with no added nucleotide (Figures 5A to 5C), the addition of the nonhydrolyzable GTP analog guanosine-5'-O-(3-thiotriphosphate) (GTPγS) to a final concentration of 1.5 mM resulted in nearly complete inhibition of import (Figures 5G to 5L). Similarly, the presence of 1.5 mM of the GDP analog guanosine-5'-O-(2-thiodiphosphate) (GDPβS) resulted in a similar inhibition of import (Figures 5J to 5L). Partial inhibition of import was observed by using these analogs at final concentrations as low as 100 μM (data not shown). Interestingly, the addition of GTP did not stimulate import (Figures 5D to 5F), indicating that sufficient endogenous GTP was present within the permeabilized protoplasts to maintain detectable import. More importantly, this result demonstrated that inhibition was specific to the nonhydrolyzable guanine nucleotide analogs. Neither the addition of the nonhydrolyzable ATP analogs adenosine-5'-O-(3-thiotriphosphate) (ATPγS) or adenylylimidodiphosphate (AMP-PNP) to concentrations as high as 2
mM nor attempts to deplete endogenous ATP by using hexokinase and glucose resulted in inhibition (data not shown). These results indicate that guanine nucleotides are essential for nuclear import in vitro and suggest that ATP is not necessary.

**Import in Vitro Is Independent of Exogenous Cytosol**

**Added Cytosol Is Not Essential for Import**

Import in permeabilized protoplasts was detected in the absence of exogenous cytosol (Figures 2 to 5). This result is unique because permeabilization of vertebrate cells and fungal spheroplasts results in the depletion of essential import factors, necessitating the addition of cytosol to restore import (reviewed by Hicks and Raikhel, 1995b; Melchoir and Gerace, 1995). As a working hypothesis, we proposed that one or more essential import factors were retained within our permeabilized protoplasts. This hypothesis was tested by determining the intracellular location of an endogenous plant-specific marker of nuclear import, an Arabidopsis homolog of known NLS import receptors.

**A cDNA and Antibody to the Import Receptor**

A partial cDNA with deduced amino acid identity to yeast SRP1 and vertebrate importin α homologs was obtained as an expressed sequence tag (Newman et al., 1994) and used as a probe to identify a full-length cDNA from Arabidopsis of 2.2 kb, termed Arabidopsis importin α (alMPα). The open reading frame of alMPα encodes a polypeptide of 532 amino acids (Figure 6). The protein contains eight tandem repeats that are found in other importin α homologs as well as in several otherwise unrelated proteins, including armadillo (Peifer et al., 1994; Smith and Lefevre, 1996). The repeats are 42 amino acids in length, highly hydrophobic, and may be involved in protein–protein interactions. Interestingly, most of the basic residues at the N terminus of alMPα and other importin α homologs are highly conserved and contain a region that could function as an NLS (Figure 6; Gorlich et al., 1996; Weis et al., 1996). At the amino acid level, alMPα is 45 to 56% identical with homologs found in Xenopus, Drosophila, mice, humans, and yeast (Table 1). Importin α homologs are conserved in different kingdoms and have been shown to be essential for import in vertebrates and yeast. Thus, alMPα probably has a similar receptor function in the nuclear import of proteins in plants.

It was necessary to produce antibodies to alMPα because antibodies to the mammalian and yeast import receptor homologs were not specific against plant proteins (see Discussion). A glutathione-S-transferase (GST)–alMPα fusion protein was purified for antibody production, and the resulting alMPα antibody was purified by affinity chromatography using immobilized His-tagged alMPα. Immunoblot analysis of protein from Arabidopsis roots, leaves, stems, and flowers resulted in the detection of a protein at ~56 kD (Figure 7A, left panel, lanes 1 to 4). A control blot incubated with preimmune serum did not detect any protein (Figure 7A, right panel, lanes 1 to 4). The alMPα antibodies also detected specific proteins in nuclear and cytosolic fractions from tobacco protoplasts (Figure 7B, left panel, lanes 1 and 2). The tobacco proteins were similar in mass to the Arabidopsis protein and recombinant His-tagged alMPα, which displays a small increase in mass due to the His tag (Figure 7B, right panel, lanes 1 and 2). A tight protein doublet of ~56 kD was detected in both fractions. Interestingly, however, the faster migrating components of the respective doublets had slightly different masses (compare Figure 7B, right panel, lanes 1 and 2). The preimmune serum did not detect any protein (Figure 7B, right panel). In addition, the alMPα antibodies specifically detected recombinant mammalian importin α (Weis et al., 1995), suggesting that alMPα is a close homolog of this essential import factor (data not shown). Thus, we conclude that the purified alMPα antibodies are probably specific for the NLS import receptor in Arabidopsis and tobacco.
aIMPa Is Not Depleted from Permeabilized Tobacco Protoplasts

To determine whether essential import factors were retained in permeabilized protoplasts, we incubated purified aIMPa antibodies or preimmune serum with protoplasts followed by rhodamine-labeled second antibodies. Compared with the preimmune control, which gave no detectable fluorescence (Figures 8E and 8F), the specific antibodies detected endogenous aIMPa in the cytoplasm (Figures 8A and 8B). Because the NE is a barrier to diffusion in unfixed cells (Figure 3), permeabilized protoplasts were treated with Triton X-100 to disrupt the NE and to allow the aIMPa antibodies to gain access to the nuclear interior. Under this condition, endogenous aIMPa was reduced but still detectable in the cytoplasm (Figures 8C and 8D). Furthermore, aIMPa was strongly detected at the nucleus. The preimmune control displayed no immunostaining (Figures 8G and 8H). The nuclear and cytoplasmic locations are consistent with the results of biochemical localization (Figure 7B) and indicate that one import factor and probably others are sufficiently retained in permeabilized tobacco cells such that import can occur without exogenous cytosol.

Figure 5. Import in Permeabilized Protoplasts Requires Guanine Nucleotides.

Import was allowed to occur for 10 min, at which time the cells were visualized directly by Nomarski optics ([A], [D], [G], and [J]) or for rhodamine ([B], [E], [H], and [K]) or DAPI ([C], [F], [I], and [L]) fluorescence.

(A) to (C) Import of O2WT-HSA in control cells with no additions.

(D) to (F) Import in the presence of 1.5 mM GTP.

(G) to (I) Import in the presence of 1.5 mM GTPγS.

(J) to (L) Import in the presence of 1.5 mM GDPβS.

Bar in (A) = 32 μm for (A) to (L).
The sequence of alMPα was aligned with the sequences of Xenopus imp2 (GenBank accession number ACC L36340), Drosophila pendulin (ACC U12269), mouse imp58 (ACC D55720), human rchl (ACC U09559), Xenopus impl (ACC L36339), yeast y-srpl (ACC S75295), and human h-imp (ACC Q00281). Regions of identity are indicated by dashes, whereas gaps introduced for alignment are indicated by dots. The positions of the putative NLS (shaded regions) and the eight hydrophobic repeats are also indicated.

**Table 1. Comparison of Amino Acid Identities for alMPα and Other Importin α Homologs**

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*For accession numbers, see Figure 6.*

**Figure 6. Amino Acid Sequence of alMPα and Other Protein Homologs.** The sequence of alMPα was aligned with the sequences of Xenopus imp2 (GenBank accession number ACC L36340), Drosophila pendulin (ACC U12269), mouse imp58 (ACC D55720), human rchl (ACC U09559), Xenopus imp1 (ACC L36339), yeast SRP1 (y-srpl; ACC Q00281), and human importin (h-imp; ACC S75295). Regions of identity are indicated by dashes, whereas gaps introduced for alignment are indicated by dots. The positions of the putative NLS (shaded regions) and the eight hydrophobic repeats are also indicated.

**Other Unique Properties of Import**

**Adaptation to Lower Growth Temperatures**

Time-course experiments were performed at 23°C and on ice, using the same preparation of permeabilized tobacco proplasts. In a typical experiment at 23°C, significant accumulation of O2WT-HSA was observed within 5 min after addition of the substrate (Figures 9A to 9C); the intensity of the intranuclear signal was similar at 20 min (Figures 9D to 9F) and lasted as long as 1 hr (data not shown). On ice, no import was observed for 5 min or more (Figures 9G to 9I); however, significant intranuclear accumulation occurred by 20 min (Figures 9J to 9L) and remained at a similar intensity for up to 2 hr (data not shown). These data demonstrate that import is rapid and efficient at 23°C. However, it is also sensitive to...
low temperature, confirming that the process is facilitated. Interestingly, inhibition of import by cold or nucleotide analogs (Figure 5) did not result in the detectable accumulation of import substrates at the NE, as reported for animals and yeast.

**Lectins Do Not Block Import in Permeabilized Cells**

The lectin WGA binds to N-acetylglucosamine, and a subset of NPC proteins in vertebrates are known to be modified by this saccharide (reviewed by Davis, 1995). Upon binding to the NPC, WGA blocks import in vertebrates but not in yeast. This blockage is probably the result of steric inhibition of NPC function (reviewed by Forbes, 1992; Miller and Hanover, 1994). Because WGA has also been shown to bind to plant NPCs (Heese-Peck et al., 1995), its effect was examined in permeabilized tobacco protoplasts. When compared with control protoplasts (Figures 10A to 10C), the addition of up to 6.7 mg/mL WGA did not result in any inhibition of import (Figures 10D to 10F). The WGA was capable of entering the permeabilized protoplasts, because rhodamine-labeled WGA (at a 100-fold lower concentration) was directly observed to bind to the nucleated. Purified alMPα antibodies detected a protein of ~56 kD in all tissues (left panel). The preimmune control blot is also shown (right panel). (B) Protein from tobacco cytosol (lanes 1) and nuclei (lanes 2) as well as purified His-tagged alMPα (lanes 3) was immunoblotted. Purified alMPα antibodies detected protein doublets of ~56 kD in cytosol and nuclear extracts (left panel). The preimmune control blot is also shown (right panel). Molecular mass standards are indicated at left in kilodaltons.

**Figure 7.** Purified Antibodies to alMPα Are Specific in Both Arabidopsis and Tobacco.

(A) Total protein (50 μg/gel lane) from Arabidopsis flowers (lanes 1), stems (lanes 2), leaves (lanes 3), or roots (lanes 4) was immunoblot-

**Figure 8.** Endogenous alMPα Is Not Depleted from Permeabilized Tobacco Protoplasts.

Protoplasts were visualized directly by Nomarski optics ([A], [C], [E], and [G]) or epifluorescence ([B], [D], [F], and [H]). (A) and (B) Permeabilized protoplasts incubated with purified alMPα antibodies for 45 min displayed cytoplasmic staining. (C) and (D) Protoplasts from the same preparation but treated with 0.1% Triton X-100 displayed cytoplasmic and nuclear staining after incubation with alMPα antibodies for 45 min. (E) and (F) Control permeabilized protoplasts incubated with preimmune serum for 45 min. (G) and (H) Control protoplasts treated with Triton X-100 and then incubated with preimmune serum. Bar in (A) = 32 μm for (A) to (H).
Figure 9. Import in Tobacco Protoplasts Is Temperature Dependent.

Protoplasts were visualized directly by Nomarski optics ([A], [D], [G], and [J]) or for rhodamine ([B], [E], [H], and [K]) or DAPI ([C], [F], [I], and [L]) fluorescence.

(A) to (F) Import at 23°C was examined for 5 min ([A] to [C]) and 20 min ([D] to [F]) after the addition of O2WT-HSA.

(G) to (L) Import on ice in protoplasts from the same preparation was also examined 5 min ([G] to [I]) and 20 min ([J] to [L]) after substrate addition. Bar in (A) = 32 μm for (A) to (L).

cleus (Figures 10G to 10I). Other lectins, many of which bind to N-acetylglucosamine in combination with other sugars, were also examined. These lectins included *Ricinus communis* agglutinin I, concanavalin A, peanut agglutinin, soybean agglutinin, *Ulex europaeus* agglutinin I, and jacalin; none resulted in inhibition of nuclear import (data not shown). This lack of inhibition suggests that direct interaction between imported proteins and polysaccharide modifications at the NPCs is probably not essential for nuclear transport in plants.

DISCUSSION

Important advances in our understanding of nuclear import have been possible through the study of nuclear transport in vitro by using permeabilized mammalian cells (Adam et al., 1990). We have adopted this basic approach in plants and have obtained novel results. An efficient method was developed to permeabilize the plasma membrane selectively in the absence of detergents, which could potentially disrupt the NE or other cellular components. The only other report of in vitro import in plants is that of Harter et al. (1994), who developed an indirect assay in which an antibody to an endogenous protein is added to Triton X-100–permeabilized evacuolated parsley protoplasts. The antigen–antibody complex is imported into the nucleus, and protease-resistant intranuclear antibody is detected by immunoblotting using protein A.

We used evacuolated protoplasts from tobacco suspension-cultured cells because they can be obtained easily and in high yield. Several reports indicate that evacuolated protoplasts regenerate vacuoles and are fully viable (Griesbach and Sink, 1983; Hortensteiner et al., 1994). Thus, essential processes such as nuclear trafficking remain functional in evacuolated protoplasts. The mechanism by which lowered osmoticum causes permeabilization is not yet understood. Some vesiculation has been observed at the plasma membranes of
permeabilized cells, which may cause disruptions in the membrane and allow entry of substrates and antibodies. Attempts to permeabilize protoplasts by using digitonin or streptolysin, which are used to permeabilize animal cells, have been unsuccessful (S. Lobreaux and N.V. Raikhel, unpublished data), presumably due to a lack of cholesterol in the plasma membranes of plants. Accounting for variability between preparations, the proportion of the total number of tobacco protoplasts that are permeabilized by using the osmotic approach is estimated to be between 30 and 70% (G. R. Hicks, S. Lobreaux, and N. V. Raikhel, unpublished data). Permeabilization has also been achieved using maize BMS and Arabidopsis protoplasts (G. R. Hicks, S. Lobreaux, and N. V. Raikhel, unpublished data). Thus, evolutionary comparisons should be possible not only between the plant and other kingdoms but also between monocot and dicot plant species.

In mammals, both GTP and ATP have roles in import, with ATP hydrolysis occurring at the same or possibly a step subsequent to GTP hydrolysis (Melchoir et al., 1995). Only the hydrolysis of GTP was observed to be necessary in our system, because nonhydrolyzable guanine nucleotide analogs were strongly inhibitory. Zupan et al. (1996) agreed, finding that the microinjection of a nonhydrolyzable GTP analog inhibited import in *Tradescantia* stamen hairs. We found that nonhydrolyzable ATP analogs and attempts to deplete ATP failed to block import. Both GDP[S] and GTPyS could prevent the proper function of Ran/TC4. This small GTPase has been shown to be essential for import in vertebrates and yeast (Melchoir et al., 1993; Moore and Blobel, 1993; Schlenstedt et al., 1995), and its association with nonhydrolyzable GTP analogs is known to be inhibitory (Melchoir et al., 1993; Moore and Blobel, 1993). In plants, Ran/TC4 homologs have been cloned from tomato (Ach and Gruissem, 1994) and tobacco (Merkle et al., 1994) and are located at the nucleus in vivo (Ach and Gruissem, 1994). However, there is no direct evidence for a role in nuclear import.

A potential complicating factor in determining the nucleotide requirement is the possibility of metabolic interconversion of ATP and GTP by a nucleoside diphosphokinase activity that is found in plants (Perata et al., 1992). This activity may ex-

Figure 10. Lectin Binds to the Nucleus but Does Not Block Import.

Protoplasts were visualized directly by Nomarski optics ([A], [D], and [G]) or for rhodamine ([B], [E], and [H]) or DAPI ([C], [F], and [I]) fluorescence. (A) to (F) Import of O2WT-HSA in tobacco protoplasts was examined in the absence ([A] to [C]) or presence ([D] to [F]) of 6.7 mg/mL WGA. (G) to (I) The same preparation of protoplasts was also incubated with rhodamine-labeled WGA to detect lectin binding at the nucleus. Bar in (A) = 32 μm for (A) to (I).
plain the results of Harter et al. (1994), who reported import in vitro in the presence of ATP and inhibition upon treatment with apyrase. These contrasting results could also be due to differences in permeabilization and import methodologies.

Nuclear import in vitro requires exogenous cytosol in vertebrates (Newmeyer et al., 1986; Adam et al., 1990) and yeast (Schlenstedt et al., 1993). In contrast, import in permeabilized protoplasts did not require exogenous cytosol. This was explored by using the presumed NLS import receptor, alMPα, as an intracellular marker and localizing the endogenous protein in permeabilized protoplasts. Although we have not demonstrated in this report that alMPα is a functional NLS receptor in plants, this is likely because the yeast and animal receptors share a similar degree of identity with each other and alMPα, and the yeast receptor functions in mammals (Enenkel et al., 1995). In addition, alMPα antibodies cross-react with mammalian importin α (Welis et al., 1995), indicating that the proteins have similar epitopes and are probably functional homologs. The alMPα antibodies do not inhibit import (G.R. Hicks, H.M.S. Smith, and N.V. Raikhel, unpublished data); however, this result is consistent with those reported in mammalian cells in which available antibodies also do not inhibit import. The antibodies block the binding of NLSs to the NPC in an assay using purified nuclei (Hicks and Raikhel, 1993), indicating activity in vitro (H.M.S. Smith, G.R. Hicks, and N.V. Raikhel, unpublished data).

Endogenous alMPα was detected in the cytoplasm and at the nucleus in permeabilized protoplasts, and these locations were confirmed by immunoblot analysis. Nuclear/cytosolic localization has been observed for other importin α homologs (Imamoto et al., 1995b; Kussel and Frasch, 1995; Torok et al., 1995) and is due to the shuttling of the import receptors into and out of the nucleus. This behavior is probably essential for their function in translocation (reviewed by Gorlich and Mattaj, 1996). Studies using permeabilized mammalian cells demonstrate that importin α homologs are depleted (Morianu et al., 1995). Because alMPα is retained in permeabilized protoplasts, even in the presence of 0.1% Triton X-100, other import factors may also be retained. This finding suggests that import factors in plants are more tightly associated within the cell compared with analogous components in vertebrates and yeast.

Import in permeabilized protoplasts without exogenous cytosol does not preclude a role for soluble cytoplasmic factors. Our data only support the conclusion that sufficient quantities of factors are retained to support import in vitro. At a minimum, the regulated transport of specific proteins probably requires soluble factors, such as kinases (reviewed by Hicks and Raikhel, 1995b; Jans, 1995). Interestingly, addition of cytosol from several plant sources to permeabilized cells results in a reversible inhibition of import via a proteinaceous factor that prevents NLS binding at the NPC (G.R. Hicks and N.V. Raikhel, unpublished data). Thus, the lack of cytosol dependency in permeabilized protoplasts may provide opportunities to identify novel regulatory components. Furthermore, we speculate that cytoplasmic or nucleoplasmic alMPα may be associated with cellular components, such as the cytoskeleton (Agutter and Prochnow, 1994), by interaction with the hydrophobic repeats. More detailed localization studies using our antibodies will address this question.

Protein doublets were detected in tobacco by the alMPα antibodies. Two proteins having masses similar to that of alMPα have also been identified in tobacco as nuclear NBPs that bind to import substrates in a specific manner (Hicks and Raikhel, 1995a). The relationship of the NBPs to alMPα can be studied using the alMPα antibodies. It was necessary to produce antibodies to alMPα because antibodies to mammalian importins α and β, Ran/TC4, p10 (Adam et al., 1990; Chi et al., 1995; M.S. Moore, unpublished data), and yeast SRP1 (Yano et al., 1992) were nonspecific on immunoblots of Arabidopsis and tobacco proteins (H.M.S. Smith, G.R. Hicks, and N.V. Raikhel, unpublished data).

As expected for a facilitated process, import is temperature dependent. More unusual, however, is the finding that import occurred on ice, although at a slower rate than it did at 23°C. We hypothesize that plants have adapted appropriately because they must survive direct exposure to lower temperatures than most animals and maintain cellular processes such as macromolecular trafficking. Protein import into plant mitochondria has been demonstrated to possess a temperature optimum that is ~10°C lower than in Neurospora or rat. For example, at 5°C, mitochondria from spinach roots can import substrate at ~30% of the optimum rate observed at 20°C (Knorr et al., 1994). Even more dramatic, protein import into chloroplasts at 4°C has been shown to be nearly as efficient as import at 25°C (Leheny and Thdg, 1994).

Another unusual feature of import in permeabilized protoplasts is the finding that WGA and other lectins had no detectable effect on import. Concentrations of WGA that have been reported to inhibit import in animals are typically in the range of 50 to 100 μg/mL (Finlay et al., 1987; Adam et al., 1990). Higher plant NPCs appear to be modified by novel O-linked oligosaccharides that are more complex than those of vertebrates (Heese-Peck et al., 1995). In addition to evidence by electron microscopy (Heese-Peck et al., 1995), we have shown that rhodamine-labeled WGA can directly associate with the nucleus of permeabilized tobacco protoplasts. However, due to the complex modification at plant NPCs, WGA may bind to a site(s) that is inappropriate to block import. The other lectins examined may fail to block import for similar reasons. The lack of lectin inhibition suggests that the carbohydrate, although probably important for NPC function, is not directly involved in import; this result is consistent with findings in vertebrates (Miller and Hanover, 1994). It is possible, however, that import substrates of greater mass could be blocked by WGA in plants, which would be consistent with the finding that WGA inhibits the in vitro cotranslocation of antigen–antibody complexes (Harter et al., 1994) as well as VirE2 single-strand DNA complexes introduced into plant cells by microinjection (Zupan et al., 1996). In fact, WGA can inhibit the import of different proteins with variable efficiencies in vertebrates (Pruschy et al., 1994).
A common observation in vertebrates is that cold or depletion of ATP results in the inhibition of translocation and the binding of import substrate at the NE. The addition of GTP analogs has been observed to have this effect also (Melchior et al., 1993; Moore and Blobel, 1993). In addition, cold and depletion of ATP result in NE binding in yeast (Schlenstedt et al., 1993). Upon inhibition of import by cold or guanine nucleotide analogs, NE binding was not observed in plants. Specific association of import substrates with plant NPCs does occur on ice in an in vitro binding assay using translocation-incompetent nuclei (Hicks and Raikhel, 1993).

Thus, NPC binding of import substrates does occur, and it is temperature independent. However, in permeabilized protoplasts, the translocation component of import may be particularly efficient compared with NPC binding, which is supported by the observation of slowed yet significant import on ice. Also, the binding component of import in plants may be particularly inefficient relative to translocation, as suggested by the low affinity of binding of three different NLSs to NPCs (Hicks and Raikhel, 1993; Hicks et al., 1995). A combination of these two factors may result in a tight coupling of NPC binding and translocation as well as a lack of separable import steps in permeabilized protoplasts. The use of permeabilized protoplasts is an important step in understanding molecular trafficking at the nucleus, and the approach has illuminated some interesting features of nuclear protein import in plants. Future studies will reveal important molecular details of this essential process in higher plants.

**METHODS**

**Reagents**

Chemicals were obtained from Sigma (St. Louis, MO), unless otherwise noted. Peptide synthesis and chemical conjugation to human serum albumin (HSA; Calbiochem, San Diego, CA), using the reagent maleimido-benzoyl N-hydroxysuccinimide ester (Pierce, Rockford, IL), was described by Hicks and Raikhel (1993). Peptides had the following sequences: functional O2 nuclear localization signal (NLS) (O2WT) and mutant O2 NLS (O2mut) (see Figure 1B); functional SV40 large T-antigen NLS, CTPPKKKRKV; and mutant SV40 large T-antigen NLS, CTPPKTKRKK. The NLS--HSA or HSA substrates were fluorescently labeled with tetramethylrhodamine-5-(and-6-)iodoacetamide (Pierce). Substrates at 3 mg/mL (22 nmol) in 500 mL of PBS were incubated with a 25-fold molar excess of the reagent for 3 hr at 23°C in the dark. The reaction was quenched by the addition of DTT to 10 mM and incubation for 10 min in the dark. Unconjugated reagent was removed by gel filtration through a 2-ml, G-25 column in PBS. This was followed by extensive washing (at least four 25-fold dilutions in PBS) and concentration of substrates in a Centricon-10 microfiltration device (Amicon, Beverly, MA).

Substrates were aliquoted and stored at -80°C. Nucleotides and nucleotide analogs were from Boehringer Mannheim (Indianapolis, IN), and all lectins were purchased from Vector Laboratories (Burlingame, CA). Monoclonal DNA antibodies (1D12; Kotzin et al., 1984), recombinant karyopherin α (M.S. Moore, unpublished data), and karyopherin α antibodies (M.S. Moore, unpublished data) were generously supplied as gifts.

**Cell Culture and Evacuolation**

*Nicotiana tabacum* suspension-cultured cells were maintained and protoplasts were prepared as described by Hicks and Raikhel (1993). Protoplasts were evacuated by a modification of the method of Griesbach and Sink (1983). All steps were performed at room temperature (23°C) unless otherwise noted. Protoplasts from a 3- to 4-day-old 30-mL tobacco culture were pelleted at 50g and suspended in 6 mL of gradient suspension buffer (5 mM Mes-KOH, pH 5.7, 100 mM CaCl₂, 9% mannitol). The protoplasts (typically 2 × 10⁷ total) were equally distributed and layered onto 11.5 mL of gradient buffer (0.12 g Hepes, 1.47 g CaCl₂/2H₂O, 9 g mannitol in Percoll to a volume of 100 mL) in each of six 14 × 95 mm ultracentrifuge tubes (Ultraclear; Beckman, Palo Alto, CA). The samples were centrifuged at 32,000 rpm (140,000g) for 45 min in an SW40 rotor (Beckman instruments), and the resulting single flocculent bands containing evacuolated cells were removed with a Pasteur pipet and pooled. The cells were then filtered through a thin wad of loosely packed glass wool in a 1.5 mL microcentrifuge tube whose bottom was cut off; this step removed cell aggregates and debris. Four volumes of gradient suspension buffer were added, and the cells were pelleted at 50g for 10 min and then suspended in 2 mL of 21% sucrose buffer (5 mM Hepes-KOH, pH 7.3, 100 mM CaCl₂, 21% sucrose). The sample was divided into 1-mL aliquots, and each aliquot was underpipetted with 2 mL of 21% sucrose buffer. After centrifugation at 50g for 8 min, the upper phase containing vacuoles was discarded, and the pellet of evacuolated cells was suspended in 40 mL of wash buffer 0.5 (WB 0.5) (20 mM Hepes-KOH, pH 7.3, 2 mM magnesium acetate, 50 mM potassium acetate, 0.5 M mannitol) and immediately pelleted at 50g for 4 min. After suspension in 40 mL of WB 0.5, the cells were incubated for 1 hr, repelleted, and then incubated in WB 0.5 for an additional 15 min. The cells were quantitated with a hemocytometer after centrifugation and suspension in 10 mL of WB 0.5. Typical yields of evacuolated protoplasts exceeded 10⁶.

**Permeabilization and Import**

Following quantitation, the evacuolated cells were pelleted and suspended gently in ice-cold permeabilization and import buffer (PIB) (20 mM Hepes-KOH, pH 7.3, 2 mM magnesium acetate, 50 mM potassium acetate, 5 mM sodium acetate, 0.225 M mannitol, 1 mM DTT, 0.125 mM spermine, 0.125 mM spermidine, 2 mM phenylmethanesulfonyl fluoride (PMSF), 5 µg/mL each of aprotinin, pepstatin A, aminocaproic acid, and leupeptin) at a density of 7000 cells/µL and incubated on ice for 30 min. For each import assay, 50 µL of cells was diluted into 200 µL of ice-cold PIB in a microcentrifuge tube and incubated on ice for 10 min. Transfer was via a pipette tip that was cut to widen the orifice. The samples were then pelleted at 50g at 4°C (Sorval RT6000B, rotor H1000B) for 10 sec. The supernatants were carefully removed, and the permeabilized cells were gently suspended in a final volume of 14 µL of ice-cold PIB containing 1 µL of 10 mg/mL HSA (to reduce nonspecific association of import substrates). The samples were incubated at 23°C for 15 min, at which time 1 µL of fluorescent NLS-HSA or HSA substrate stock was added to yield a final concentration of 0.3 µM (O2 NLS substrates) or 0.1 µM (SV40 NLS substrates). The final assay volume was 15 µL, and import was allowed to proceed for the times indicated. Samples were then chilled on ice and diluted by the
addition of 100 μL of ice-cold PIB and 2 μL of 20 μg/mL 4:6-diamidino-2-phenylindole dihydrochloride (DAPI) stock in PBS, 10 mM NaN₃ in 90% glycerol. An aliquot of diluted cells was transferred to a hemocytometer and viewed with an Axiophot microscope (Carl Zeiss, Thornwood, NY), using Nomarski or the appropriate fluorescence optics. Micrographs were produced with T-MAX 400 film (Kodak). Where indicated, samples were optically sectioned (2 μm/section) using a laser-scanning confocal microscope (model 10; Carl Zeiss) equipped with phase contrast, fluorescence optics, and a 514-nm argon laser. Micrographs were produced with Kodacolor Gold 100 film (Kodak). The import assay described above will be referred to as the standard assay.

To determine nucleotide requirements for import, the standard assay was used; however, before addition of Q2WT-HSA substrate, the permeabilized cells were incubated for 15 min in PIB or PIB plus GTP, guanosine-5'-O-(3-thiotriphosphate) (GTPS), guanosine-5'-O-(2-thiodiphosphate) (GDP3S), adenylyl-imidodiphosphate (AMP-PNP), or adenosine-5'-O-(3-thiotriphosphate) (ATPS). Stocks at the appropriate concentrations were prepared in 0.5 M Hepes-KOH, pH 7.3, such that the addition of 1 μL resulted in the final concentrations indicated.

For ATP depletion, cells were incubated for 30 min at 23°C in the presence of PIB only; 10 mM glucose in PIB; or 10 mM glucose, 300 units per mL hexokinase (Boehringer Mannheim) in PIB before the addition of Q2WT-HSA substrate. For time-course experiments at 23°C, import in the standard assay was allowed to proceed for the times indicated before examination. For matched experiments on ice using the same preparations of cells, the standard assay was followed except that the permeabilized cells were incubated on ice before and after the addition of Q2WT-HSA substrate for the times indicated. These samples were viewed using a hemocytometer that was precooled on ice. For lectin studies, the standard assay was used, except that cells were incubated before the addition of Q2WT-HSA substrate with wheat germ agglutinin (WGA), Ricinus communis agglutinin I, concanavalin A, peanut agglutinin, soybean agglutinin, Ulex europaeus agglutinin I, or jacalin at the final concentrations indicated. Lectins were added from 10 mg/mL stocks in PIB without DTT, spermine, spermidine, and protease inhibitors. For WGA localization, the standard assay was followed; however, instead of import substrate, 1 μL of 1 mg/mL tetramethylrhodamine-S-(and -6)-sulfoxycyanate (TRITC)--WGA (EY Laboratories, San Mateo, CA) was added. After incubation at 23°C for 15 min, cells were diluted and visualized as in the standard assay.

Immunolocalization

For immunolocalization using antibodies against DNA or Arabidopsis thaliana importin α (alMPa), the standard assay was used except that the permeabilized cells (at the step before substrate addition) were suspended in PIB or PIB plus Triton X-100 at the concentrations indicated at 23°C or on ice. Primary antibodies and HSA (to 2.7 mg/mL to reduce nonspecific association of antibodies) were then added, bringing the volume to 50 μL. Both the DNA antibodies and the alMPa antibodies were added to final dilutions of 1:50. The final concentration of the alMPa antibodies was 9 μg/mL. After incubation at 23°C (DNA antibodies) or on ice (DNA antibodies or alMPa antibodies) for 45 min, the cells were diluted with 150 μL of ice-cold PIB, pelleted for 10 sec at 50g, and suspended in PIB containing HSA and the appropriate second antibodies in a final volume of 50 μL. A 1:500 final dilution of TRITC-labeled rabbit anti–mouse IgG (Pierce) was added to detect DNA in the nucleus, and a 1:50 final dilution of TRITC-labeled goat anti–rabbit IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to detect endogenous alMPa. After incubation for 45 min on ice, the cells were diluted with 150 μL of PIB, pelleted for 10 sec, and suspended gently in 50 μL of PIB plus 1 μL of DAPI stock. Localization was examined by immunofluorescence as in the standard assay.

Cloning and Sequencing of alMPa

A 900-bp partial cDNA exhibiting significant homology with yeast SRP1 and animal importin α homologs was obtained from the Michigan State University–Department of Energy Plant Research Laboratory Arabidopsis Sequencing Project (Newman et al., 1994). The cDNA was used as a probe to screen the PRL2 Arabidopsis cDNA library (Newman et al., 1994) in λZipLox (Gibco BRL, Gaithersburg, MD). The library was made from mRNA of etiolated seedlings, roots, leaves, and flowers. The probe was synthesized with α-32P-dATP (3000 Ci/mmol; NEN Research Products, Boston, MA) by using Klenow (Boehringer Mannheim) and random hexanucleotide primers. Approximately 2 x 108 plaques were screened, and 12 additional cDNAs were purified by standard methods (Sambrook et al., 1989). The cDNAs were excised by standard methods from λZipLox for sequencing, as described by Newman et al. (1994). A full-length cDNA of 2.2 kb was obtained that hybridized to an mRNA of similar length (H.M.S. Smith and N.V. Raikhel, unpublished data), and 3' and 5' deletions were made (Henikoff, 1987) and sequenced using Sequenase 2.0 (United States Biochemical Corp., Cleveland, OH). Deduced amino acid sequences were aligned by the Genetics Computer Group (Madison, WI) sequence analysis software package (version 7.0).

Expression and Purification of GST-alMPa

The alMPa cDNA was cloned into pGEX 5-2 (Pharmacia, Piscataway, NJ) by using EcoRI and NotI sites to produce an N-terminal glutathione-S-transferase (GST) fusion protein. The plasmid was transformed into DH5α, and the production of fusion protein was induced overnight at 37°C, according to the manufacturer's procedures. For protein purification, cells were chilled on ice for 20 min before centrifugation at 7500g and suspended in 20 mL of ice-cold PBS containing 1 mM PMSF. Cells were lysed by two treatments at 1100 p.s.i. in a French press. Triton X-100 was added to 1%, and the extract was centrifuged at 1200g. Then, 40 mL of the supernatant was mixed with 2 mL of glutathione-agarose (Pharmacia) for 3 hr at 4°C. The beads were washed 10 times with 40 mL of ice-cold PBS containing 1 mM PMSF. The fusion protein was eluted with 5 mL of elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM reduced glutathione) and concentrated using a Centricon-10 device (Amicon). The purified protein had a concentration of 25 mg/mL.

For antibody production, the fusion protein was purified further by 10% SDS-PAGE and prepared as follows: gel strips containing a total of ~6 mg of fusion protein were equilibrated in extraction buffer (50 mM ammonium bicarbonate, pH 7.8, 0.05% SDS) for 5 min at 23°C, ground with a mortar and pestle, and mixed overnight as a suspension in extraction buffer at 37°C. The suspension was centrifuged twice at 1300g, filtered through a 45-μm cellulose acetate filter, and washed by repeated dilution with 50 mM ammonium bicarbonate, pH 7.8, in a Centricon-30 device (Amicon). The sample was lyophilized twice and dissolved in 30 mL of PBS at a concentration of 0.9 mg/mL.

For expression as a His-tagged fusion protein, alMPa was cloned into PET14b (Novagen, Madison, WI) by using Ndel and BamHI sites. The plasmid was transformed into BL21 (DE3) and then induced and
The column was then washed sequentially with 10 mM Tris-HCl, pH 7.2 (Bio-Rad Laboratories). Purified protein (3.5 mg) was mixed with volumes of 50 mM Tris-HCl, pH 7.2, three volumes of 100 mM glycine, of Affigel-10 in 50 mM Hepes-KOH, pH 7.2, in a 3.5" column and sodium acetate, and 0.225 M mannitol.

A 45-μm cellulose acetate filter; specific antibodies were bound to the rock for 4 hr at 4°C. The column was washed with three column volumes of 10 mM Tris-HCl, pH 7.2, plus 0.5 M NaCl, and finally equilibrated in 50 mM Hepes-KOH, pH 7.2. Immune serum was diluted 1:10 in Hepes-KOH, pH 7.2, and filtered through a 45-μm cellulose acetate filter; specific antibodies were bound to the alMPa affinity column by passage through the column three times. The column was then washed sequentially with 10 mM Tris-HCl, pH 7.2, 10 mM Tris-HCl, pH 7.2, plus 0.5 M NaCl, and 50 mM Hepes-KOH, pH 7.2. Specific alMPa antibodies were eluted with 100 mM glycine, pH 2.5, and 1-mL fractions were collected and neutralized with 0.6 mL of 1 M Hepes-KOH, pH 7.5, 2 mM magnesium acetate, 50 mM potassium acetate, and 0.225 M mannitol.

Antibody Production and Purification

Preimmune serum was collected; then one rabbit was immunized with 0.5 mg of purified GST-alMPa fusion protein in the presence of an adjuvant (TiterMax; Vaxel, Norcross, GA) for each of three injections over a 6-week period and a final injection of 1.0 mg. For affinity purification of alMPa antibodies, His-tagged alMPa was coupled to Affigel-10 (Bio-Rad Laboratories). Purified protein (3.5 mg) was mixed with volumes of 50 mM Tris-HCl, pH 7.2, three volumes of 100 mM glycine, of Affigel-10 in 50 mM Hepes-KOH, pH 7.2, in a 3.5" column and rocked for 4 hr at 4°C. The column was washed with three column volumes of 50 mM Tris-HCl, pH 7.2, three volumes of 100 mM glycine, pH 2.5, and finally equilibrated in 50 mM Hepes-KOH, pH 7.2. Immune serum was diluted 1:10 in Hepes-KOH, pH 7.2, and filtered through a 45-μm cellulose acetate filter; specific antibodies were bound to the alMPa affinity column by passage through the column three times. The column was then washed sequentially with 10 mM Tris-HCl, pH 7.2, 10 mM Tris-HCl, pH 7.2, plus 0.5 M NaCl, and 50 mM Hepes-KOH, pH 7.2. Specific alMPa antibodies were eluted with 100 mM glycine, pH 2.5, and 1-mL fractions were collected and neutralized with 0.6 mL of 1 M Hepes-KOH, pH 7.5, 2 mM magnesium acetate, 50 mM potassium acetate, and 0.225 M mannitol.

Immunoblots

Ten percent of SDS-PAGE and blotting to nitrocellulose were performed by standard methods (Sambrook et al., 1989). Blots were blocked with nonfat dry milk and incubated overnight at 23°C with purified alMPa antibodies or preimmune serum (both stocks at 90 pg/mL) at a 1:3000 dilution. Blots were developed by using a 1:5000 dilution of goat anti-rabbit alkaline phosphatase-conjugated IgG (Kirkegaard and Perry Laboratories). Proteins were extracted from Arabidopsis roots, leaves, stems, and flowers by grinding in liquid nitrogen and were suspended in extraction buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS) for SDS-PAGE (50 kg per gel lane). Tobacco nuclei were purified by characterization of cytoplasmic nuclear protein import factor p97. J. Cell Biol. 111, 807-816.


Nuclear import in permeabilized protoplasts from higher plants has unique features.
G R Hicks, H M Smith, S Lobreaux and N V Raikhel

*Plant Cell* 1996;8;1337-1352
DOI 10.1105/tpc.8.8.1337

This information is current as of August 4, 2017

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