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Rapid Communication

Three Classes of Nuclear Import Signals Bind to Plant Nuclei[1]

Glenn R. Hicks, Harley M. S. Smith, Mark Shieh, and Natasha V. Raikhel*

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

Three nuclear localization signals (NLS), including an unusual Mat α2-like NLS from maize (*Zea mays*) R, were found to compete for binding to plant nuclei. In addition, the authentic yeast Mat α2 NLS, which does not function in mammals, was shown to function in plants in vivo. Our results indicate that plants possess a site at the nuclear pore complex that recognizes the three known classes of NLSs.

The specific import of proteins into the nucleus is mediated by NLSs, which are short polypeptide regions enriched in basic amino acids. Most of the identified NLSs can be categorized into one of three classes (Garcia-Bustos et al., 1991; Raikhel, 1992). The SV40 large T-antigen NLS typifies a class of NLSs possessing a single short region enriched in basic amino acids. Another class of NLSs known as bipartite signals is composed of two basic regions separated by a spacer. Finally, the yeast Mat α2 NLS typifies a class of NLSs that are unusual in possessing hydrophobic and basic amino acids. It has been reported that the Mat α2 NLS does not function in mammals, indicating that components of the import apparatuses of yeast and animals are not completely interchangeable (Chelsky et al., 1989; Lanford et al., 1990). It is clear from studies in animals that import occurs through the NPC and involves two steps: an NLS-dependent binding step at the NPC followed by translocation, which requires the hydrolysis of ATP (Forbes, 1992) and in some cases GTP (Moore and Blobel, 1994). A recent report suggests that plants may also require ATP hydrolysis for import (Harter et al., 1994).

A number of studies have characterized NLSs from plant pathogenic viruses (Carrington et al., 1991) and bacteria (Citovsky and Zambryski, 1993). In addition, reports indicate that the SV40 large T-antigen NLS that functions in animals also functions in plants (Raikhel, 1992). We have examined the import of the endogenous plant transcription factors O2 (Varagona et al., 1991) and R (Shieh et al., 1993), both of which contain multiple NLSs of different classes. The O2 protein possesses SV40-like and bipartite NLSs that are functional in vivo (Varagona et al., 1992). The R protein possesses three NLSs that are functional in vivo: two SV40-like signals (NLSs A and M) and a Mat α2-like NLS (NLS C). Like the authentic Mat α2 NLS (KIPIK), NLS C (RKAIGKR) contains basic and hydrophobic amino acids.

Since there are no strict consensus sequences for NLSs, a thorough study of each of the classes of signals is an essential step in identifying components of the import apparatus of plants. The initial event of import, binding at the NPC, has been examined with an in vitro nuclear binding assay using peptides to two classes of NLSs (Hicks and Raikhel, 1993). The bipartite NLS from O2 and the SV40 large T-antigen NLS specifically bind to and compete for a single low-affinity site that is firmly associated with the nuclear envelope and NPC (Hicks and Raikhel, 1993). However, mutant O2 (Varagona and Raikhel, 1994) and mutant SV40 large T-antigen NLSs (Raikhel, 1992) that are impaired in import in vivo do not compete for binding. To determine if the third known class of NLSs, the Mat α2-like NLSs, could compete for binding to the same nuclear site, we examined the association of NLS C from the R protein with purified tobacco (*Nicotiana tabacum*) and maize (*Zea mays*) nuclei. The similarity between NLS C and the Mat α2 NLS also prompted us to test whether the authentic Mat α2 NLS could direct import in plant cells in vivo.

MATERIALS AND METHODS

Materials

The peptide to NLS C (CWT) was synthesized at the Peptide Synthesis Facility (Yale University, New Haven, CT). Synthetic peptides to the functional O2 bipartite NLS (O2WT), the functional SV40 large T-antigen NLS (SV40WT), and the peptide unrelated to NLSs (non-NLS) were previously described (Hicks and Raikhel, 1993). All peptides contained a Cys residue at the amino or carboxy terminus to facilitate radiolabeling with carbon-14. The peptides were as follows: (CWT) CYMISEALRK AIGKR; (O2WT) MPTEERVRKR KESNRESARR SRYRKAAHLK C; (SV40WT) CTPPKKKRKV; (non-NLS) CDGVFAGGG. Oligonucleotides were synthesized at the Macromolecular Facility (Michigan State University, East Lansing, MI). Unless noted, chemicals were obtained from Sigma.

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* Corresponding author; e-mail nraikhel@ibm.cl.msu.edu; fax 1–517–353–9168.

Abbreviations: CWT, peptide to the Mat α2-like import signal from R; NLS, nuclear localization signal; NPC, nuclear pore complex; O2, Opaque2; O2WT, peptide to the bipartite import signal from Opaque2; SV40, simian virus 40; SV40WT, peptide to the import signal from simian virus 40 large T-antigen.

Nuclear Binding Assays

For binding assays, nuclei were prepared from protoplasts of *Nicotiana tabacum* or maize (*Zea mays*) Black Mexican Sweet suspension-cultured cells using the abbreviated protocol previously described (Hicks and Raikhel, 1993), except that DTT was omitted from the nuclei isolation buffer. For carbon-14 labeling of CWT and binding of [^{14}C]CWT to purified nuclei, previously described methods were used (Hicks and Raikhel, 1993), except that 50 mM Tris-HCl, pH 7.3, was replaced by 50 mM Hepes-KOH, pH 7.3, in the binding buffer. Briefly, 1×10^6 tobacco or maize nuclei were diluted to 70 μL with binding buffer, and 200,000 cpm of [^{14}C]CWT (76 μCi/mmol; approximately 5 μM final concentration) in 30 μL of binding buffer was added. Binding was allowed to occur for 5 min on ice, after which time the nuclei were pelleted. The supernatant was removed and the cpm associated with the nuclei were quantitated by scintillation counting. For competitive displacement curves, unlabeled peptides were added from concentrated stocks made in binding buffer. Nonspecific backgrounds were estimated from the addition of 10 mM unlabeled CWT. Assay points were the average of at least duplicate samples, and all experiments were done at least twice.

Constructs and in Vivo Nuclear Import

The GUS and NLS C/GUS fusion constructs were previously described (Shieh et al., 1993). The oligonucleotide used to mutagenize the NLS C/GUS fusion construct to produce the Mat α2 NLS/GUS construct was: 5′-TGC-CGTCGTG CCCTGGATCG ATTCTAGAAT GAACAA-GATC CCGATCAAGG ACCTGCTGAA CCCGCAGAGT GGGTACGGTC AG-3′. Mutagenesis, subcloning, preparation of onion epidermal layers and gold particles, and conditions for biolistic gun transformation were as described (Shieh et al., 1993). The amount of plasmid DNA was 5.0 μg/bombardment, and the sonication of gold particles was for 10 s (cup horn probe, 60% power). Histochemical staining for GUS activity was as described (Shieh et al., 1993), except that the X-glucuronide buffer was altered to improve cell viability (3 mM X-glucuronide, 20 mM $NaPO_4$, pH 7.0, 50 μM potassium ferricyanide, 50 μM potassium ferrocyanide, 0.01% Triton X-100) and incubation with X-glucuronide substrate was at 37°C for 24 h. The intracellular location of GUS activity was observed using an Axiophot microscope (Zeiss, Thornwood, NY) equipped with Nomarski optics. Observations were from at least five separate experiments, and the minimum number of transformed cells examined per experiment was 20.

RESULTS

To determine whether NLS C could specifically bind to purified nuclei, CWT was synthesized that corresponded to the minimum region of R that functionally defined this signal in vivo (Sheih et al., 1993). In addition, the previously described (Hicks and Raikhel, 1993) O2WT and SV40WT peptides, and a peptide unrelated to NLSs (non-NLS), were used. The latter peptide, which corresponds to a defective vacuolar import signal (Dombrowski et al., 1993), served as a "non-NLS" control.

To examine the binding of NLS C, the CWT peptide was carbon-14 labeled and allowed to bind to purified tobacco nuclei. Displacement curves resulting from the addition of the CWT peptide as a competitor indicated that [^{14}C]CWT could specifically bind to plant nuclei at low affinity with an apparent dissociation constant of approximately 100 μM (Fig. 1A). This is similar to the apparent dissociation constant for the binding of [^{14}C]O2WT and [^{14}C]SV40WT to tobacco nuclei (200 μM; Hicks and Raikhel, 1993). Displacement of [^{14}C]CWT by unlabeled SV40WT and O2WT indicated that the three peptides competed for binding to the same nuclear site (Fig. 1, B and C). The non-NLS control peptide was a poor competitor compared to the functional NLSs (Fig. 1C). We also examined the binding of [^{14}C]CWT to nuclei from the distantly related monocot maize. Exper-

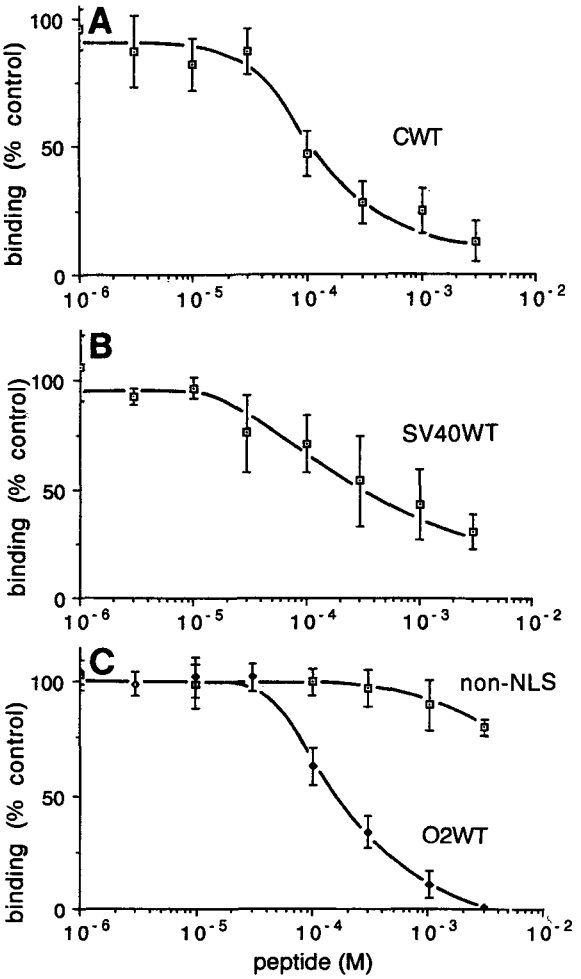

Figure 1. Specific binding of NLSs to purified tobacco nuclei. The [^{14}C]CWT peptide was incubated with nuclei in the presence of CWT (CYMISEALRKAIGKR) (A), SV40WT (CTPPKKKRKV) (B), or O2WT (MPTEERVRKRKESNRESARRSRYRKAAHLKC) or non-NLS (CDGV-FAGGG) (C) competitor peptides. Results are reported as percent binding of control (±SE) versus concentration (M) of added competitor. Average total binding and nonspecific association were, respectively, 16,900 and 11,000 cpm.

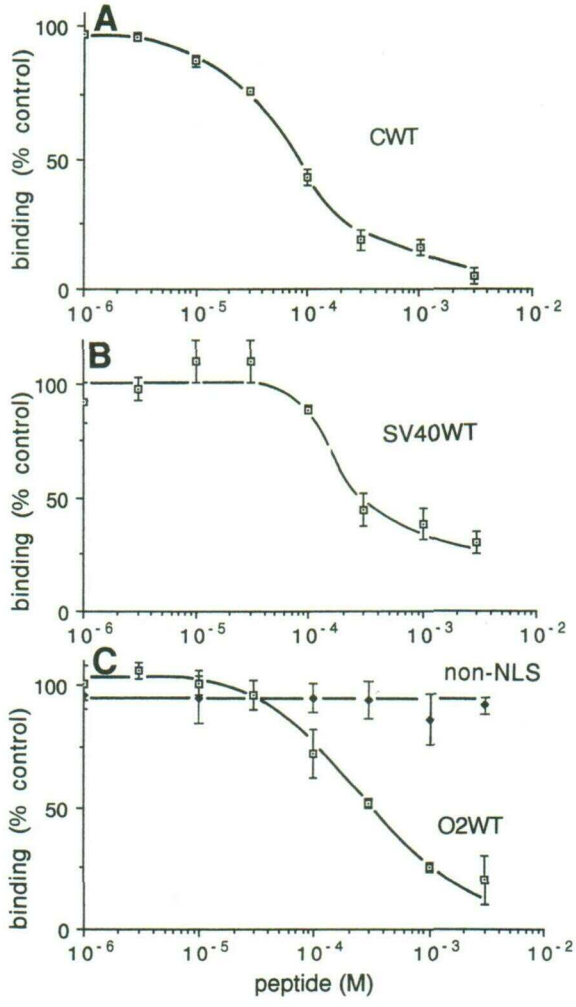

Figure 2. Specific binding of NLSs to purified maize nuclei. The [^{14}C]CWT peptide was incubated with nuclei in the presence of CWT (A), SV40WT (B), or O2WT or non-NLS (C) competitor peptides. Results are reported as percent binding of control (±SE) versus concentration (M) of added competitor. Average total binding and non-specific association were, respectively, 25,400 and 14,000 cpm.

iments using purified nuclei from maize indicated that monocots possess a similar binding site to which [^{14}C]CWT could associate. Addition of CWT as a competitor demonstrated that binding was specific and of an affinity similar to that found with tobacco nuclei (Fig. 2A). The SV40WT and O2WT peptides also competed effectively with [^{14}C]CWT for binding to the nuclear site (Fig. 2, B and C), whereas the non-NLS control peptide displayed no ability to compete (Fig. 2C).

To directly address the functionality of the Mat α2 NLS in plants, we examined whether the authentic Mat α2 NLS could direct the reporter protein GUS to the nucleus in vivo. Gene constructs were made in which the 13-amino acid region encoding either NLS C (MISEALRKAIGKR) or the authentic Mat α2 NLS (MNKIPIKDLLNPQ) were fused to the amino terminus of GUS. The fusion constructs or a construct encoding GUS only were transformed into onion epidermal cells by particle gun bombardment, and the subcellular location of the resulting proteins was determined by histochemical staining for GUS activity. As shown in an earlier study (Shieh et al., 1993), the NLS C/GUS fusion protein was found in the nucleus (Fig. 3A), whereas GUS alone was located in the cytoplasm (Fig. 3B). When the Mat α2 NLS/GUS fusion construct was examined, GUS activity was found in the nucleus as well as in

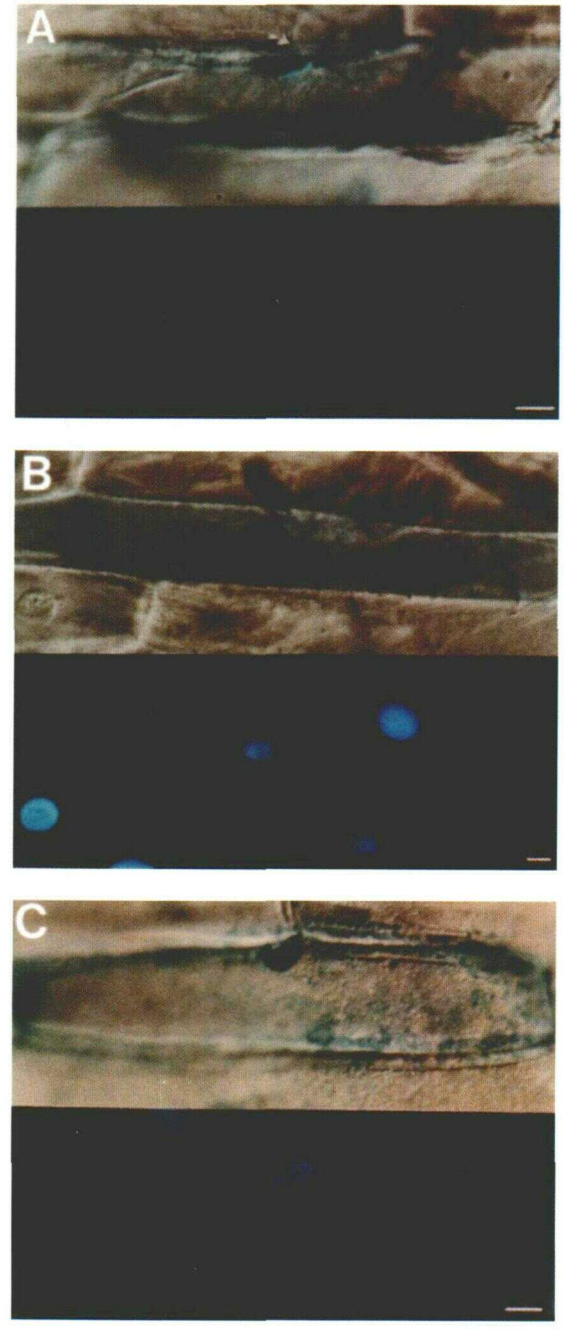

Figure 3. Histochemical localization of the NLS C/GUS fusion (A), GUS (B), or the Mat α2/GUS fusion (C) proteins in transformed onion tissue. Paired micrographs are of the same cells stained for GUS activity (Nomarski images, upper) or with 4′,6-diamidino-2-phenylindole dihydrochloride to indicate the positions of nuclei (fluorescence images, lower). Bar scale = 10 μm.

the cytoplasm (Fig. 3C). Thus, although not as efficient a signal as NLS C when fused to GUS, the Mat α2 NLS did function in plant cells. Overall, our results indicate that the three known classes of NLSs, all of which function in plants, bind to a nuclear site that is present in dicots and monocots.

DISCUSSION

A careful study of the binding of NLSs is an important step prior to identifying the corresponding NLS binding proteins. Although the binding of individual NLSs has been examined in animals and yeast (Forbes, 1992), a systematic approach using the three known classes of NLSs has not been reported. We previously demonstrated that higher plants possess a saturable low-affinity site at the NPC that can specifically bind to the SV40 large T-antigen NLS and the O2 bipartite NLS (Hicks and Raikhel, 1993). Here we found that Mat α2-like NLSs, such as NLS C from the endogenous maize transcription factor R, also bound to nuclei specifically. The SV40WT and O2WT peptides were also found to compete with [^{14}C]CWT for binding. Similar results were obtained using nuclei from maize, indicating that the binding site is probably a component that is common among divergent species.

Several studies have concluded that the Mat α2 NLS does not function in mammals (Chelsky et al., 1989; Lanford et al., 1990). We found that this signal directed a GUS fusion protein to the nucleus of onion cells in vivo. The Mat α2 NLS was not as efficient as NLS C, which may be due to the conformation of the signal, because a mutated NLS C/GUS fusion protein, in which the order of amino acids in the signal are reversed, does not localize to the nucleus in onion cells (M. Shieh and N.V. Raikhel, unpublished data). Although differences probably exist (see below), the import apparatuses of plants and animals also have similarities, since the SV40 large T-antigen NLS functions in both kingdoms (Raikhel, 1992). In this regard, it would be interesting to examine the ability of NLS C to direct import in mammals.

It is generally accepted that animals require soluble factors for NLS recognition (Forbes, 1992; Moore and Blobel, 1994), and this may also be the case in yeast (Schlenstedt et al., 1993). Our results indicate that at least some components of NLS recognition are located at the NPC of plants. We have recently extended our in vitro studies to include the addition of cross-linking reagents during NLS binding. Using this approach, several NLS binding proteins have been specifically radiolabeled with the peptide to the functional O2 bipartite NLS (Hicks and Raikhel, 1995). We are also developing in vitro nuclear import systems so that we can explore the function of the NPC NLS binding proteins and identify other factors necessary for nuclear import in higher plants.

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LITERATURE CITED

Carrington JC, Freed DD, Leinicke AJ (1991) Bipartite signal sequence mediates nuclear translocation of the plant potyviral NIa protein. Plant Cell **3:** 953–962

Chelsky D, Ralph R, Jonak G (1989) Sequence requirements for synthetic peptide-mediated translocation to the nucleus. Mol Cell Biol **9:** 2487–2492

Citovsky V, Zambryski P (1993) Transport of nucleic acids through membrane channels: snaking through small holes. Annu Rev Microbiol **47:** 167–197

Dombrowski JE, Schroeder MR, Bednarek SY, Raikhel NV (1993) A mutational analysis of the carboxyl-terminal propeptide of barley lectin: determining functional elements necessary for proper sorting to vacuoles of tobacco. Plant Cell **5:** 587–596

Forbes DJ (1992) Structure and function of the nuclear pore complex. Annu Rev Cell Biol **8:** 495–527

Garcia-Bustos J, Heitman J, Hall MN (1991) Nuclear protein localization. Biochim Biophys Acta **1071:** 83–101

Harter K, Kircher S, Frohnmeyer H, Krenz M, Nagy F, Schafer E (1994) Light regulated modification and nuclear translocation of cytosolic G-box binding factors in parsley. Plant Cell **6:** 545–559

Hicks GR, Raikhel NV (1993) Specific binding of nuclear localization sequences to plant nuclei. Plant Cell **5:** 983–994

Hicks GR, Raikhel NV (1995) Nuclear localization signal binding proteins in higher plant nuclei. Proc Natl Acad Sci USA **92:** 734–738

Lanford RE, Feldherr CM, White RG, Dunham RG, Kanda P (1990) Comparison of diverse transport signals in synthetic peptide-induced nuclear transport. Exp Cell Res **186:** 32–38

Moore MS, Blobel G (1994) A G-protein involved in nucleocytoplasmic transport: the role of Ran. Trends Cell Biol **19:** 211–216

Raikhel NV (1992) Nuclear targeting in plants. Plant Physiol **100:** 1627–1632

Schlenstedt G, Hurt E, Doye V, Silver PA (1993) Reconstitution of nuclear protein transport with semi-intact yeast cells. J Cell Biol **123:** 785–798

Shieh M, Wessler SR, Raikhel NV (1993) Nuclear targeting of the maize R protein requires two localization sequences. Plant Physiol **101:** 353–361

Varagona MJ, Raikhel NV (1994) The basic domain in the bZIP regulatory protein Opaque2 serves two independent functions: DNA binding and nuclear localization. Plant J **2:** 207–214

Varagona MJ, Schmidt RJ, Raikhel NV (1991) Monocot regulatory protein Opaque-2 is localized in the nucleus of maize endosperm and transformed tobacco plants. Plant Cell **3:** 105–113

Varagona MJ, Schmidt RJ, Raikhel NV (1992) Nuclear localization signal(s) required for nuclear targeting of the maize regulatory protein Opaque-2. Plant Cell **4:** 1213–1227