THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 12, Issue of March 23, pp. 9322–9329, 2001

Printed in U.S.A.

Molecular Cloning of a Novel Importin α Homologue from Rice, by Which Constitutive Photomorphogenic 1 (COP1) Nuclear Localization Signal (NLS)-Protein Is Preferentially Nuclear Imported*

Received for publication, July 19, 2000, and in revised form, December 19, 2000
Published, JBC Papers in Press, December 20, 2000, DOI 10.1074/jbc.M006430200

Chang-Jie Jiang‡§, Kazuhiro Shoji¶, Rikyu Matsuki¶, Akiko Baba‡, Noritoshi Inagaki‡, Hiroshi Ban‖, Toshisuke Iwasaki‖, Naoko Imamoto, Yoshihiro Yoneda‡‡, Xing-Wang Deng§§, and Naoki Yamamoto¶¶**

*From the ‡National Institute of Agrobiological Resources, Tsukuba, Ibaraki 305-8602, Japan, the ¶Central Research Institute of Electric Power Industry, Chiba 270-1194, Japan, the ‖Department of Biology, Faculty of Science, Niigata University, Niigata 950-2181, Japan, the **National Institute of Genetics, Structural Biology Center, Mishima, Shizuoka 411-8540, Japan, the ‡‡Department of Cell Biology and Neuroscience, Graduate School of Medicine, Osaka University, Suita, Osaka 565-0871, Japan, the §§Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, Connecticut 06520-8104, and the ¶¶Department of Biology, Faculty of Science, Ochanomizu University, Bunkyo-ku, Tokyo 112-8610, Japan*

Nuclear import of proteins that contain classical nuclear localization signals (NLS) is initiated by importin α, a protein that recognizes and binds to the NLS in the cytoplasm. In this paper, we have cloned a cDNA for a novel importin α homologue from rice which is in addition to our previously isolated rice importin α1a and α2, and we have named it rice importin α1b. *In vitro* binding and nuclear import assays using recombinant importin α1b protein demonstrate that rice importin α1b functions as a component of the NLS-receptor in plant cells. Analysis of the transcript levels for all three rice importin α genes revealed that the genes were not only differentially expressed but that they also responded to dark-adaptation in green leaves. Furthermore, we also show that the COP1 protein bears a bipartite-type NLS and its nuclear import is mediated preferentially by the rice importin α1b. These data suggest that each of the different rice importin α proteins carry distinct groups of nuclear proteins, such that multiple isoforms of importin α contribute to the regulation of plant nuclear protein transport.

The most characteristic feature of an eukaryotic cell is the presence of a nuclear envelope, which separates the cell into two major compartments, the nucleus and the cytoplasm. Communication between these two compartments takes place through the nuclear pore complex (NPC)[1] (for review, see Refs. 1–3). The NPC allows molecules smaller than 40–60 kDa to diffuse across, while larger proteins and RNA-protein complexes must be actively transported through the NPC in a signal-mediated and energy-dependent manner. Nuclear proteins involved in nuclear activities, such as DNA replication, transcriptional RNA synthesis, and RNA splicing, must enter into the nucleus. Conversely, RNA, such as mRNA synthesized in the nucleus, must be transported into the cytoplasm where it is translated to protein. In plants, nucleocytoplasmic transport has been implicated in functional regulation of a number of plant photomorphogenesis related protein factors (4). For example, constitutive photomorphogenic 1 (COP1), a repressor of photomorphogenesis, has been shown to shuttle between the nucleus and cytoplasm in response to a change of light environment. COP1 exists predominantly in the cytoplasm in the light while it accumulates in the nucleus in the dark, suggesting that nuclear protein transport is an underlying mechanism for the regulation of COP1 activity (5).

Multiple pathways of nucleocytoplasmic transport have been identified, each likely to be involved in carrying a distinct group of proteins (for review, see Refs. 3 and 6). Among them, the best characterized is the import of proteins containing a classical nuclear localization signal (NLS) that consists of either a short stretch of 3–5 basic amino acids or two basic domains separated by a spacer, referred to as monopartite and bipartite NLS, respectively (7). Yeast mating factor (Matα-2) contains a NLS consisting of basic and hydrophobic amino acid residues and has also been shown to be functional in plants (8). The NLS-containing proteins are initially recognized and bound in the cytoplasm by NLS-receptor, a heterodimer consisting of importin α and importin β subunits. Importin α binds the NLS specifically, forming a stable pore targeting complex (PTAC) (9–11), whereas importin β mediates the docking of the PTAC to the cytoplasmic face of the NPC (12–14). Translocation of the docked PTAC into the nucleus is mediated by the small GTPase, Ran (15, 16).

Both importin α and β have been identified in a wide range of species, including vertebrates, yeast, and plants. However,

* This work was supported by the Special Coordination Fund for Promoting Science and Technology, Enhancement of Center-of-Excellence, to the National Institute of Agrobiological Resources from the Science and Technology Agency of Japan, the Ministry of Agriculture, Forestry and Fisheries of Japan (BDP-00-I-1), and Scientific Research C Grant-in-Aid 0868076 from the Japanese Ministry of Education, Science, Sports and Culture (to N. I. and Y. Y.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s)AB03431l.

§ To whom correspondence should be addressed: Dept. of Plant Physiology, National Institute of Agrobiological Resources, Tsukuba, Ibaraki 305-8602, Japan. Tel.: 0298-38-8383; Fax: 0298-38-8383; E-mail: cjjiang@abr.affrc.go.jp.

[1] The abbreviations used are: NPC, nuclear pore complex; COP1, constitutive photomorphogenic 1; NLS, nuclear localization signal; PTAR, pore targeting complex; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; IBB domain, importin β-binding domain.

while only a single isoform of importin β involved in classical NLS-directed nuclear transport has been isolated in most species, multiple isoforms of importin α have been identified in various species (2, 3). Recent studies suggest that such multiplicity of importin α isoforms might contribute to the tissue-specific or temporal regulation of nuclear protein import. For example, at least six different human isoforms of importin α have been identified, and they show significantly different tissue-dependent expression patterns (11, 17–24). They have also been shown to differentially interact with specific NLS sequences (22, 25–28).

As in many vertebrates, multiple isoforms of importin α have been identified from *Arabidopsis* (aIMPα (29), AtKAPα (30), ATHKAP2 (31), and AtIMPα1–4 (32)) and rice (rice importin α1 (33) and rice importin α2 (34)). Intriguingly, *in vitro* binding assays using recombinant plant importin αs have revealed differential recognition of different types of NLS. The aIMPα can bind all three of the typical classes of plant NLSs, namely, the NLS of SV40 large T-antigen (T-NLS, a monopartite type), that of maize transcription factor Opaque 2 (O_2-NLS, a bipartite type), and that of maize transcription factor R (R-NLS, a yeast Matα−2 type) (35). In contrast, rice importin α1 bound to the T- and O2-NLS, but not to the R-NLS (36). Rice importin α1 also mediated nuclear import of fusion proteins containing either T-NLS or O2-NLS, but not R-NLS, in digitonin-permeabilized HeLa cells (36). All these data suggest that multiple isoforms of importin α may serve as a control point for the regulation of nuclear protein import. It is also noteworthy that two closely related importin βs have been identified in rice (37). This is the only report thus far of multiple importin β isoforms identified in a single species. Whether such multiplicity is a common characteristic among plants is not yet clear, as no importin βs have yet been isolated from other plants. At least in rice, however, this may provide an additional point for regulation of nuclear protein import.

In this paper we report the cloning and functional analysis of a novel rice importin α homologue which is in addition to our previously isolated rice importin α1 (33) and α2 (34). Because it shares very high homology (82.8% identity) with the rice importin α1, we have named this novel homologue rice importin α1b and have renamed the rice importin α1 as rice importin α1a. We show that rice importin α1b selectively binds to different types of plant NLSs and mediates the nuclear import of NLS substrates in digitonin-permeabilized HeLa cells. Comparison of the transcript levels of the three isoforms of rice importin α reveals some differential expression, not only in different tissues, but also in response to light. Furthermore, we also demonstrate that the COP1 protein bears a bipartite-type NLS and its nuclear translocation is mediated preferentially by the rice importin α1b.

MATERIALS AND METHODS

cDNA Cloning

A rice expressed sequence tag (EST) clone C3059 (accession number D23592) with high homology to rice importin α1 (33) was obtained from the Rice Genome Project team in the National Institute of Agrobiological Resources, Ministry of Agriculture, Forestry & Fisheries, Japan. We used this EST clone as a probe to screen a cDNA library constructed in λgt11 using poly(A) RNA from the leaves of rice seedlings grown in the light for 14 days, and obtained a positive clone of about 2.4 kilobases in length. However, since this clone did not cover the full-length coding sequence, we carried out the 5′ rapid amplification of cDNA ends using a Marathon cDNA Amplification Kit (CLONTECH, Palo Alto, CA). The 5′-end of cDNA was amplified by PCR using a gene-specific primer, 5′-GGGGATAATCCTCACGTTGGAGGAAGGC-3′, according to the manufacturer's instructions. The products were cloned into pT-Adv vector using the AdvanTAge™ PCR Cloning Kit (CLONTECH) and sequenced. The full-length importin α1b cDNA was used to generated a GST-rice importin α1b construct, as described below.

DNA Constructions

GST-rice Importin α1b—A fragment corresponding to the amino-terminal portion (about 300 base pairs) of rice importin α1b with an artificial *Eco*RI site at the 5′-end was generated by PCR using the 5′-rapid amplification of cDNA ends fragment as template DNA. The forward and reverse primers used were 5′-GGGAATTCATGTCGCT-GCGGCCGAGCGAGCGG-3′ and 5′-GGCAACCCCTCCAACTTCTGCT-GGAGCG-3′, respectively. The PCR fragment contains a *Not*I site near the 3′-end. After double digestion with *Eco*RI and *Not*I, the fragment was inserted between the *Eco*RI and *Not*I sites of pGEX-6p-1 (Amersham Pharmacia Biotech). The rest of the rice importin α1b sequence was digested from the cDNA clone obtained from the rice seedling cDNA library and then cloned into the *Not*I site at the 3′-end of the amino-terminal portion. Both nucleotide sequence and orientation were checked by DNA sequencing.

GST-COP1 NLS-GFPs—Chimeric DNA of GST-COP1 NLS-GFPs were generated essentially as described previously (36). 5′-End oligonucleotide primers corresponding to each putative COP1 NLS and their missense mutants (see Fig. 5*A*) used for PCR are as follows: 1) COP1 bWW, 5′-ACG**GAATTC***AACCAGAGCACCGTGAGCATCGCGCGCAA-AAAACGCATCCATGCGCAGTTCAACGATCTGCAGGAATGCTATCT-GCAGAAACGCCGCCAGCTGGCGGATCAGCCGAACAGC*ATGAGTA-AAGGAGAAGAACTTTTCACTGGAGTT-3′; 2) COP1 bXW, 5′-ACG**GA-ATTC***AACCAGAGCACCGTGAGCATCGCGAACACCACCAACATCC-ATGCGCAGTTCAACGATCTGCAGGAATGCTATCTGCAGAAACGC-CGCCAGCTGGCGGATCAGCCGAACAGC*ATGAGTAAAGGAGAAGA-ACTTTTCACTGGAGTT-3′; 3) COP1 bWX, 5′-ACG**GAATTC***AACCAG-AGCACCGTGAGCATCGCGCGCAAAAAAACGCATCCATGCGCAGTT-CAACGATCTGCAGGAATGCTATCTGCAGACCAACAACCAGCTGG-CGGATCAGCCGAACAGC*ATGAGTAAAGGAGAAGAACTTTTCACT-GGAGTT-3′; 4) COP1 bXX, 5′-ACG**GAATTC***AACCAGAGCACCGTGA-GCATCGCGAACACCACCAACATCCATGCGCAGTTCAACGATCTGC-AGGAATGCTATCTGCAGACCAACAACCAGCTGGCGGATCAGCCG-AACAGC*ATGAGTAAAGGAGAAGAACTTTTCACTGGAGTT-3′; 5) COP1 mW, ACG**GAATTC***AACCTGCTGACCCTGCTGGCGGAACGC-AAACGCAAAAATGGAACAGGAAGAAGCGGAACGC*ATGAGTAAAGG-AGAAGAACTTTTCACTGGAGTT-3′. The NLS-encoding sequences are indicated by italic and the endonuclease restriction sites are indicated by boldface. Underlined are basic amino acid cluster regions. The 3′-end primer used was 5′-ACG**CTCGAG**TTATTTGTAGAGCTCATCCATGC-CATGTGT-3′.

Transient Expression Vector and sGFP-COP1 NLS-sGFP—An oligonucleotide encoding an extra 5 amino acids (Leu-Ile-Gly-Gly-Gly) and incorporating three restriction enzyme sites (*Apa*I, *Xma*I, and *Spe*I) was inserted at the 3′-end of the original sGFP (S65T) gene (38). The resulting plasmid was designated as psGFPcs. On the other hand, a PCR product of sGFP with *Xba*I and *Xma*I restriction sites at the 5′-end, and a *Sac*I site at the 3′-end was produced and subcloned into pBI221 (CLONTECH) using the *Xba*I and *Sac*I sites. Then a fragment encoding sGFP and the *nos* terminator was excised with *Xma*I and *Eco*RI digestion, and inserted between the *Xma*I and *Eco*RI sites of the psGFPcs plasmid. The resulting plasmid was designated pdsGFP. A PCR fragment corresponding to each COP1 NLS sequence with *Apa*I and *Xba*I site at 5′- and 3′-end, respectively, was inserted between two adjacent sGFPs of pdsGFP to generate sGFP-COP1 NLS-sGFP fusions and used for transient expression assays. The sGFP-COP1 NLS-sGFP DNAs were also digested with *Hin*dIII and *Sac*I, and the resulting fragments containing the CaM 35S-promoter and the *nos* terminator flanking the sGFP-COP1 NLS-sGFP were cloned into the pIG121-Hm (39) transformation vector. The sequences of forward and reverse primers used for the PCR are as follows: 1) COP1 bWW, 5′-GGG**GGGCC-C**AATCAGTCAACTGTCTCAATT-3′/5′-CCC**CCCGGG**ACTATTTGGT-TGGTCTGCCAA-3′; 2) COP1 bXW, 5′-GGG**GGGCCC**AATCAGTCAA-CTGTCTCAATTGCTAACACCACCAACATTCATGCTCAGTTC-3′/5′-CCC**CCCGGG**ACTATTTGGTTGGTCTGCCAA-3′; 3) COP1 bWX, 5′-GGG**GGGCCC**AATCAGTCAACTGTCTCAATT-3′/5′-CCC**CCCGGG**-ACTATTTGGTTGGTCTGCCAACTGGTTGTTGGTTTGGAGGTAACA-TTC-3′; 4) COP1 bXW, 5′-GGG**GGGCCC**AATCAGTCAACTGTCTCA-ATTGCTAACACCACCAACATTCATGCTCAGTTC-3′/5′-CCC**CCCGGG**-ACTATTTGGTTGGTCTGCCAACTGGTTGTTGGTTTGGAGGTAAC-ATTC-3′; 5) COP1 mW, 5′-GGG**GGGCCC**AATCTTCTGACACTTCTT-GCG-3′/5′-CCC**CCCGGG**CCTCTCAGCTTCTTCCTGTTC-3′.

Analysis of Transcript Levels of Rice Importin αs—Plant materials and total RNA samples were prepared as previously described (33). The RNA samples were treated with DNase (RT-grade, Nippon Gene, Tokyo) to remove genomic DNA contaminants. Transcript levels of importin αs in the RNA samples were examined with the ABI PRISMS™

7700 Sequence Detection System essentially according to the manufacturer's instruction (PE Applied Biosystems, Foster City, CA) and were normalized with transcript levels of 18 S rRNA. Reverse transcription and amplification of transcripts were carried out using TaqMan™ EZ RT-PCR Kit (PE Applied Biosystems) in the presence of one of the specific primer-probe sets shown below. The sequences of forward and reverse primers and TaqMan probes used for the analyses were as follows: 1) rice importin α1a, 5′-CGATAAGAAGCTCGAAAGCCTT-3′/5′-AAAGCAACTTGCGGAACTGTGT-3′ and 5′-CTGCTATGATTGG-TGGAGTTTATTCGGACG-3′; 2) rice importin α1b, 5′-AATCAGGAGT-GTTCCCAAGGC-3′/5′-ATCCCCAGTGACGATGTTACC-3′ and 5′-TG-GAACTTCTCATGCATCCTTCGGC-3′; 3) rice importin α2, 5′-AGCTC-CAATTTCTGGCAGTGAT-3′/5′-CAACAGCATGCGCAGTCTTTGC-3′ and 5′-TGGTCGATGAGGAGAAAGCATGTCTTG-3′; 4) 18 S rRNA, 5′-GAGAAACGGCTACCACATCCAA-3′/5′-CTAAAGCGCCCGGTATTGT-TAT-3′ and 5′-AAGGCAGCAGGCGCGCAAATTA-3′.

Transient Expression Assays

Onion epidermis was peeled off and placed inside up on plates containing MS medium (4.2 g/liter MS plant salt mixture, 30 g/liter sucrose, 4% Gelrite, pH 5.8) and 2.5 mg/liter amphotericin B antifungal agent (Sigma-Aldrich Co.). Plasmid DNAs (6.75 μg) harboring the fusion genes were precipitated onto 1.6-μm gold particles (0.75 μg) and the particles were resuspended in 60 μl of 100% ethanol. A 10-μl aliquot of the suspension was loaded onto a particle delivery disc and the segments of onion epidermis were bombarded with the particles (PDS-1000/He; Bio-Rad). Bombardment conditions were as recommended by the manufacturer.

Plant Growth Conditions and Arabidopsis Transformation

Arabidopsis thaliana was grown at 22 °C under constant fluorescent illumination of 85 μmol m^{-2} s^{-1}, using Supermix (Sakata Seeds Co., Ltd., Yokohama, Japan) and vermiculite (mixed in 1:1). Seedlings were germinated aseptically on half-concentrated MS medium supplemented with 2% sucrose, 0.3 mg/ml thiamine (HCl), 0.5 mg/ml nicotinic acid, 0.05 mg/ml pyridoxine (HCl) and as required with 20 μg/ml hygromycin. *Arabidopsis* ecotype Columbia was transformed by *Agrobacterium tumefaciens* (EHA 101)-mediated T-DNA transfer using the floral dip procedure (40). Hygromycin-resistant seedlings were selected and allowed to self seed for amplification.

Fluorescence Microscopy

sGFP fusion proteins in the transient expression assays were observed with a microscope (Olympus AX70, Tokyo, Japan) with Nomarski optics or epifluorescence optics. For subcellular localization of sGFP fusion proteins of the transgenic *Arabidopsis*, whole roots were viewed under a stereo fluorescence microscope (Leica MZFL III). Photomicrographs were taken using 35 mm film and the figures were assembled using Adobe Photoshop software (Adobe Systems Inc., San Jose, CA).

Protein Purification

Expression and purification of recombinant proteins and *in vitro* binding and nuclear import assays were carried out as described previously (36). Recombinant GST-NLS-GFPs, rice importin α1a, α2 (36), rice importin β1 (41), and Ran (42, 43) were prepared as described previously.

RESULTS

Cloning of Rice Importin α1b—By searching the rice EST data base, we identified an EST clone (accession number D23592) showing high homology with rice importin α1 previously reported in Ref. 33. Using this clone as a probe, we screened a rice seedling cDNA library and obtained a positive clone. Since DNA sequence analysis indicated that this clone lacked a 5′-segment of the open reading frame, we employed the 5′-rapid amplification of cDNA ends method to isolate a corresponding full-length cDNA clone. The full-length cDNA of the clone is predicted to encode a protein of 534 amino acids with a calculated molecular mass of 58.5 kDa (Fig. 1). The deduced amino acid sequence of the clone showed 82.8% identity with rice importin α1. Therefore, we designated this novel cDNA as rice importin α1b and renamed the rice importin α1 as rice importin α1a. Rice importin α1b also shares significant amino acid homology with other previously identified importin

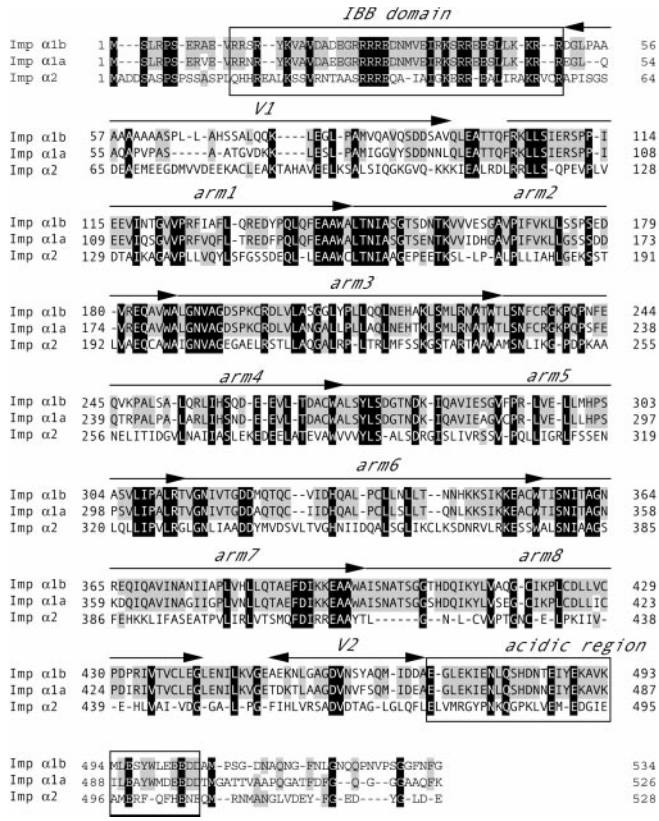

FIG. 1. **Multiple sequence alignment of rice importin αs.** Protein sequences were aligned using GENETYX genetic information processing software (Software Development Co., LTD, Tokyo, Japan). Identical amino acid residues across either all three or two of the clones are indicated by *black* and *gray boxes*, respectively. IBB domain, arm repeats (*arm*), variable region (*V*), and acidic region are indicated in the *upper line*. DDBJ accession numbers of rice importin α1b, α1a, and α2 are AB034311, AB004660, and AB006788 respectively.

αs, such as rice importin α2 (27.5%), aIMPα (59% (29)), AtKAPα (68% (30)), and yeast SRP1 (54% (44)) (Fig. 1). Like the other importin α homologues, rice importin α1b contains the main characteristics of importin α: the importin β-binding domain (IBB domain) at the amino terminus (45, 46), the eight tandem armadillo (arm) repeats of 42 amino acids (47), a COOH-terminal acidic region, and two variable regions flanking the arm repeats region (Fig. 1).

NLS Binding and PTAC Formation by Rice Importin α1b—To address the ability of rice importin α1b to bind NLSs, we performed an *in vitro* binding assay using native gel electrophoresis. On a native gel, complex formation between two proteins gives a new band with a mobility different from that of either of the proteins alone. NLS-GFP fusion proteins, T-, O2-, R-, and Tm-GFP, were used as NLS substrates (36) throughout the functional analysis of rice importin α1b (Fig. 2*A*). Tm-GFP, a point mutant of T-GFP in which the sixth residue, a lysine, of the T-NLS was replaced by a threonine, was used as a negative control.

As shown in Fig. 2*B*, rice importin α1b and each of the NLS-GFPs migrated as single bands (*lanes 1, 3, 5, 7,* and *9*). A mixture of rice importin α1b with T-GFP gave a new major band of retarded mobility, with a little of the unbound protein running as in the control (Fig. 2*B, lane 2*). As was the case with rice importin α1a (36), the complex of O2-GFP and rice importin α1b showed almost indistinguishable mobility relative to O2-GFP alone on the gel (Fig. 2*B, lane 6*). However, complex formation between the two proteins is apparent as all of the rice importin α1b shifted upward, giving a much darker band (Fig. 2*B, lane 6*). In contrast, a mixture of rice importin α1b

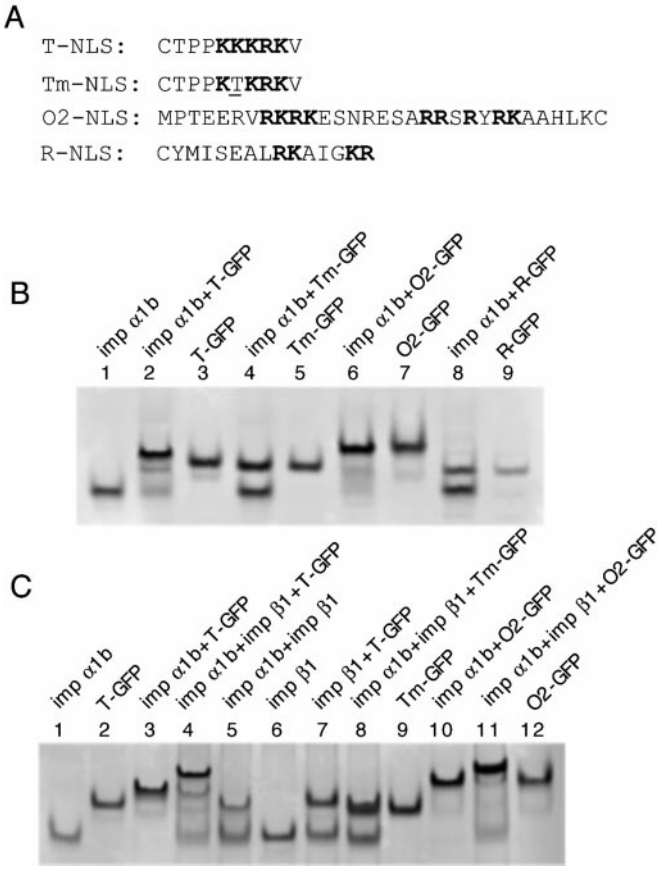

FIG. 2. ***In vitro* protein binding analyses.** *A,* amino acid sequences of SV40 large T-antigen NLS (*T-NLS*), mutant T-NLS (*Tm-NLS*), maize transcription factor opaque 2 NLS (*O2-NLS*), and maize transcription factor R NLS (*R-NLS*) are shown in a single-letter code. Basic amino acid clusters are indicated in *bold face* and amino acid replacement in Tm-NLS is *underlined*. These NLSs were inserted between GST and GFP to generate recombinant GST-NLS-GFP fusion proteins designated T-, Tm-, O2-, and R-GFP, respectively. *B* and *C*, *in vitro* protein binding assays were analyzed by native gel electrophoresis. 20 pmol of each protein was mixed in 15 μl of transport buffer (TB) (20 mM Hepes, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 5 mM sodium acetate, 0.5 mM EGTA, 2 mM dithiothreitol, 1 μg/ml each of aprotinin, leupeptin, and pepstatin A) supplemented with 250 mM sucrose and incubated for 1 h at room temperature. 7.5% polyacrylamide gels were run in the presence of 1 mM dithiothreitol and 1 mM EGTA in both the gels and the running buffer and stained with Coomassie Blue.

with either R-GFP or Tm-GFP gave no new visible bands, with migration of each protein as in the control (Fig. 2*B, lanes 4* and *8*). These results suggest that the rice importin α1b selectively binds to T- and O2-NLS, but not to R-NLS. The binding was NLS-specific as the rice importin α1b did not bind to the Tm-GFP that has been shown to be nonfunctional (Fig. 2*B, lane 4*).

We next examined PTAC formation by rice importin α1b with rice importin β1 and NLS-GFPs (Fig. 2*C*). Each protein migrated as a single band on the gel (Fig. 2*C, lanes 1, 2, 6, 9,* and *12*). A mixture of rice importin α1b with rice importin β1 gave a new major band of retarded mobility, with some unbound proteins migrating at the position of the controls (Fig. 2*C, lane 5*), indicating a direct binding of the two proteins. Addition of either T-GFP (Fig. 2*C, lane 4*) or O2-GFP (Fig. 2*C, lane 11*), but not Tm-GFP (Fig. 2*C, lane 8*), to the mixture resulted in formation of a large complex with lower mobility on the gel relative to the complex formed either between rice importin α1b and β1 (Fig. 2*C, lane 5*) or between rice importin α1b and either T- (Fig. 2*C, lane 3*) or O2-GFP (Fig. 2*C, lane 10*). SDS-polyacrylamide gel electrophoresis of these bands confirmed that the complex contained rice importin α1b and β1

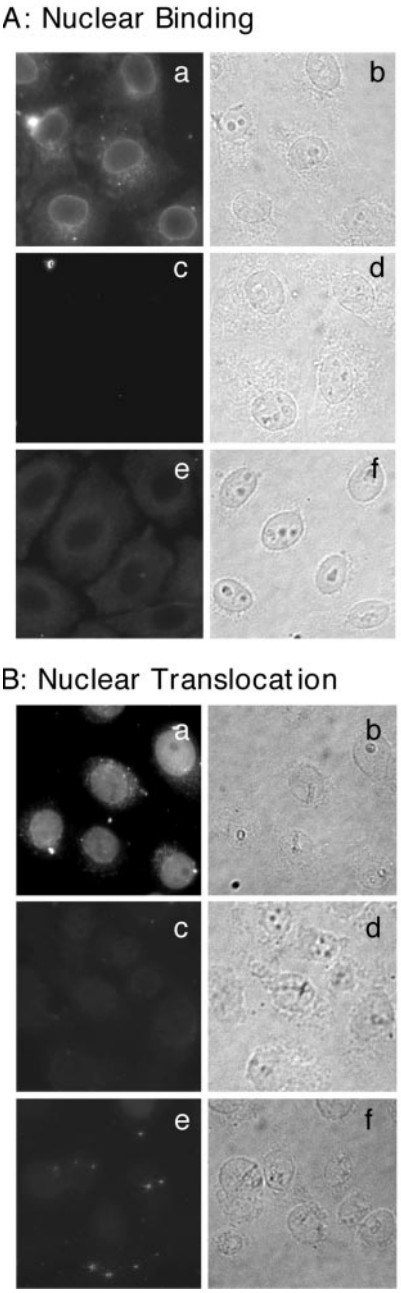

FIG. 3. **The effect of rice importin α1b on the nuclear binding and import of GST-NLS-GFP assayed *in vitro* using digitonin-permeabilized HeLa cells.** *Part A,* for nuclear binding, a 10-μl assay sample was incubated on ice for 20 min. GST-NLS-GFP was 0.2 μg; rice importin β1 was 6 pmol; rice importin α1b was 6 pmol. *Part B*: for nuclear import, a 10-μl assay sample was incubated at 25 °C for 20 min in the presence of mouse Ran-GDP (42 pmol), 1 mM ATP, 20 units/ml creatine phosphokinase, 5 mM creatine phosphate, and 1 mM GTP. GST-NLS-GFP was 0.2 μg; rice importin β1 was 3 pmol; and rice importin α1b was 12 pmol. In both *parts A* and *B*: *a* and *b,* T-GFP + importin β1 + importin α1b; *c* and *d,* T-GFP + importin β1; and *e* and *f,* Tm-GFP + importin β1 + importin α1b. After incubation, cells were fixed with 3.7% formaldehyde in TF. *Panels a, c*, and e, fluorescence images; *panels b, d*, and *f*; Nomarski images.

and either T- or O2-GFP (data not shown).

Activity of Rice Importin α1b in the in Vitro Import Assay using Digitonin-permeabilized HeLa Cells—To assess the functional activity of rice importin α1b in the process of nuclear import of proteins, we performed an *in vitro* nuclear import assay using digitonin-permeabilized HeLa cells. T-GFP and Tm-GFP were used as transport substrates, as positive and negative controls, respectively. The T-NLS containing proteins

have been most commonly used as the transport substrate in nuclear import assays.

As shown in Fig. 3*A*, rice importin α1b, in conjunction with rice importin β1, efficiently accumulated T-GFP at the nuclear rim (Fig. 3*A*, *panel a*). In contrast, rice importin β1 alone was not sufficient to direct the substrate to the nuclear rim (Fig. 3*A, panel c*). Such accumulation did not occur when Tm-GFP was used as substrate (Fig. 3*A*, *panel e*).

Upon addition of mouse Ran-GDP and energy-regenerating mixture, T-GFP was efficiently translocated into the nucleus (Fig. 3*B, panel a*). The translocation of the substrate was rice importin α1b-dependent (Fig. 3*B, panel c*) and NLS-specific (Fig. 3*B*, *panel e*). Omission of Ran-GDP or depletion of ATP by hexokinase from the transport solution abolished translocation of the substrate into the nucleus (data not shown).

Differential Gene Expression of the Three Rice Importin αs—To investigate the expression of the three rice importin αs in different tissues and to examine the effect of light on their expression in green leaves, we performed quantitative analysis of RNA levels using the ABI PRISMS™ 7700 Sequence Detector (PE Applied Biosystems).

Transcripts of the three rice importin αs were detected in all the tissues tested, with minimum levels in green leaves and relatively higher levels in nongreen tissues including roots, etiolated leaves, and calli (Fig. 4). However, the tissue expression patterns for the different importin αs varied significantly. Rice importin α1b and α2 showed maximal transcript levels in roots (Fig. 4, *B* and *C*). In contrast, the highest levels of rice importin α1a were detected in calli (Fig. 4*A*).

We previously reported that transcription of rice importin α1a is down-regulated by light (33). Herein, we examined whether this is also the case with the other two rice importin αs. As shown in Fig. 4, the three rice importin αs displayed varied transcript patterns during dark-adaptation in green leaves. Dark treatment of rice seedlings for 24 h significantly increased the transcript levels of rice importin α1a by about 10-fold (Fig. 4*A*). Rice importin α2 also showed a modest, 5-fold increase in transcript levels after 24 h in the dark (Fig. 4*C*). In comparison, rice importin α1b showed only a minor change in transcript levels during dark-adaptation (Fig. 4*B*). Thus it appears that the rice importin αs express differentially not only in different tissues but also in response to light.

Identification of a Bipartite Type NLS in COP1 Protein—To further investigate any functional differences between the rice importin αs in nuclear import processes, we identified the NLS in the COP1 protein and examined its interaction with rice importin αs.

Analysis of the amino acid sequence of the COP1 protein using PSORT, a computer program for the prediction of protein sorting signals and localization sites in amino acid sequences, on the internet predicted two putative NLS sequences, a monopartite-(mW-COP1 NLS) and a bipartite-type (bWW-COP1 NLS), in the COP1 protein (Fig. 5*A*). To examine their functional NLS activities, we inserted oligonucleotides corresponding to each of these putative NLSs and their missense mutants (Fig. 5*A*) between two adjacent GFPs in a CaMV 35S-promoter-driven plasmid and bombarded into onion epidermis. For convenience herein, we have designated these fusion proteins bWW-, bXW-, bWX-, bXX-, and mW-dsGFP, respectively. As shown in Fig. 5*B*, bWW-dsGFP was localized exclusively in the nucleus (Fig. 5*B, panels a* and *f*). In contrast, neither mW-dsGFP (Fig. 5*B, panels e* and *j*) nor the missense mutants of bWW-dsGFP (Fig. 5*B, panels b-d*, and *g-i*) were nuclear localized. Some GFP signal around the nucleus in Fig. 5*B, panels b-e*, is likely to be due to the fact that the cytoplasm is compacted beneath the plasma membrane and around the nucleus

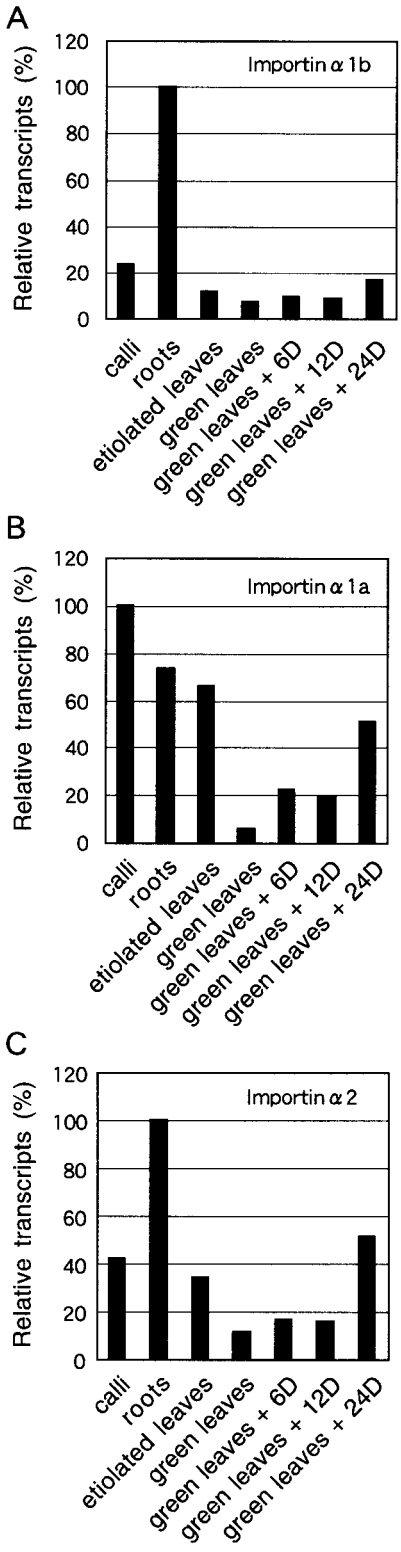

FIG. 4. **Transcript analyses of rice importin αs.** Total RNA samples were prepared from tissues of either dark-(etiolated leaves) or light-grown (green leaves and roots) rice plants (14-days-old). For dark adaptation, the light-grown plants were further grown in complete darkness for the indicated hours (6, 12, and 24 days, respectively). In each panel, the highest transcript levels were regarded as 100% of relative expression.

because of the large vacuole that occupies most of the cell volume in onion epidermal cells. These results suggest that only the bipartite-type NLS, but not the monopartite-type one, in the COP1 protein is functional as a NLS and mutations in

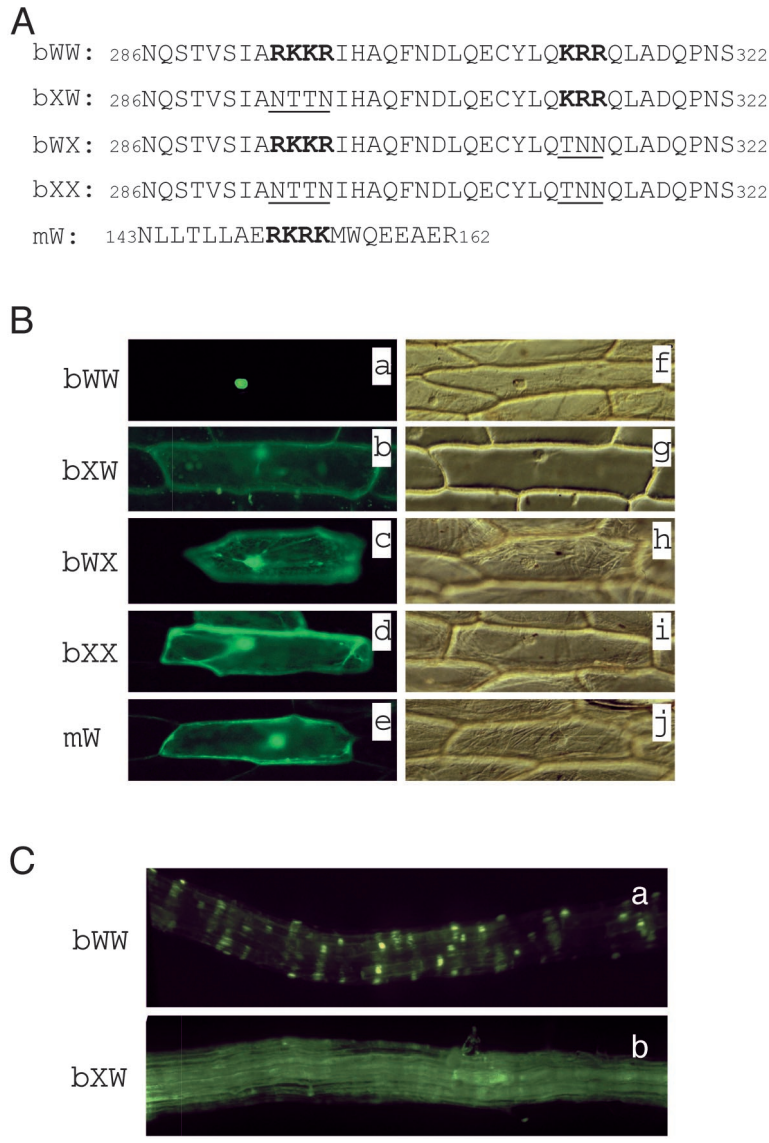

FIG. 5. **Identification of COP1 NLS.** *A*, PSORT program predicted two putative NLS sequences, a monopartite- (mW) and a bipartite-type (bWW), in the COP1 protein. The position and nature of mutations within bWW were indicated (bXW, bWX, and bXX). *B*, DNA constructs encoding COP1 NLS-dsGFPs under CaMV 35S-promoter were bombarded into onion epidermis and their subcellular localization were visualized for sGFP. Only the bWW-dsGFP (*a* and *f*), but not its mutants (*b-d* and *g-i*) and mW-dsGFP (*e* and *j*), was nuclear localized. *Panels a-e,* fluorescence images; *panels f-j,* Nomarski images. *C*, stereofluorescence microscopic images of nuclear localization of bWW-dsGFP (*a*), but not bXW-dsGFP (*b*), in root cells of transgenic *Arabidopsis* plants.

either the basic stretch of amino acids of bWW abolish its NLS activity. These findings were further supported by the observation that the subcellular distribution of bWW- and bXW-dsGFP in transgenic *Arabidopsis* root cells bWW-dsGFP was found to be localized predominantly in the nucleus (Fig. 5*C, panel a*) while its missense mutant bXW-dsGFP was found to be distributed throughout the cytoplasm (Fig. 5*C, panel b*).

This subcellular localization (Fig. 5) and following *in vitro* binding (Fig. 6) and nuclear import (Fig. 7) analyses demonstrate that COP1 protein bears a functional bipartite-type NLS consisting of two stretches of basic amino acids separated by 14 residues. Although the PSORT program also predicted a monopartite-type NLS in COP1 protein, it did not show any NLS activity in our assays. These results are in consistent with the observations of Stacey *et al.* (48), in which they identify a core domain between residues 293 and 392 that is capable of mediating nuclear localization of N-terminal fused GUS or GFP.

In Vitro Binding of COP1 NLSs to Rice Importin αs—In vitro interaction between the COP1 NLS and each of the three rice importin αs was examined using the native gel electrophoresis method. Oligonucleotides corresponding to each of these putative NLSs and their missense mutants (Fig. 5*A*) were inserted between GST and GFP to generate recombinant GST-COP1 NLS-GFP fusion proteins in *Escherichia coli* as described previously (36). For convenience herein, we have designated these fusion proteins bWW-, bXW-, bWX-, bXX-, and mW-GFP, respectively. As shown in Fig. 6, bWW-GFP was specifically bound by rice importin α1a (Fig. 6*A, lane 2*) and α1b (Fig. 6*B, lane 2*), but not by α2 (Fig. 6*C, lane 2*). All the missense mutants of bWW- and mW-GFP were not recognized by any rice importin αs. Addition of rice importin β1 to the mixtures gave rise to the formation of PTAC consisting of bWW-GFP, rice importin β1, and either rice importin α1a (Fig. 6*D, lane 2*) or α1b (Fig. 6*D, lane 5*). No such PTAC formation was observed with rice importin α2 (data not shown).

Preferential Nuclear Import of bWW-GFP by Rice Importin α1b—Because bWW-GFP was recognized *in vitro* by both rice importin α1a (Fig. 6*A*) and α1b (Fig. 6*B*) and further formed PTAC in the presence of rice importin β1 (Fig. 6*D*), we next carried out an *in vitro* nuclear protein import assay. As shown in Fig. 7, both rice importin α1a (Fig. 7*B*) and α1b (Fig. 7*A*), in conjunction with rice importin β1, docked bWW-GFP (Fig. 7A, *panels a* and *b, B, panels a* and *b*), but not its missense mutants and mW-GFP (Fig. 7*A, panels c-j*), to the nuclear envelope. Surprisingly, however, further translocation of the bWW-GFP into the nucleus occurred preferentially in the presence of rice importin α1b (Fig. 7*A, panels k* and *l*). Although nuclear docking occurred with rice importin α1a, almost no (if any) further nuclear translocation proceeded (Fig. 7*B, panels c* and *d*). These results together with those shown in Fig. 6 suggest that

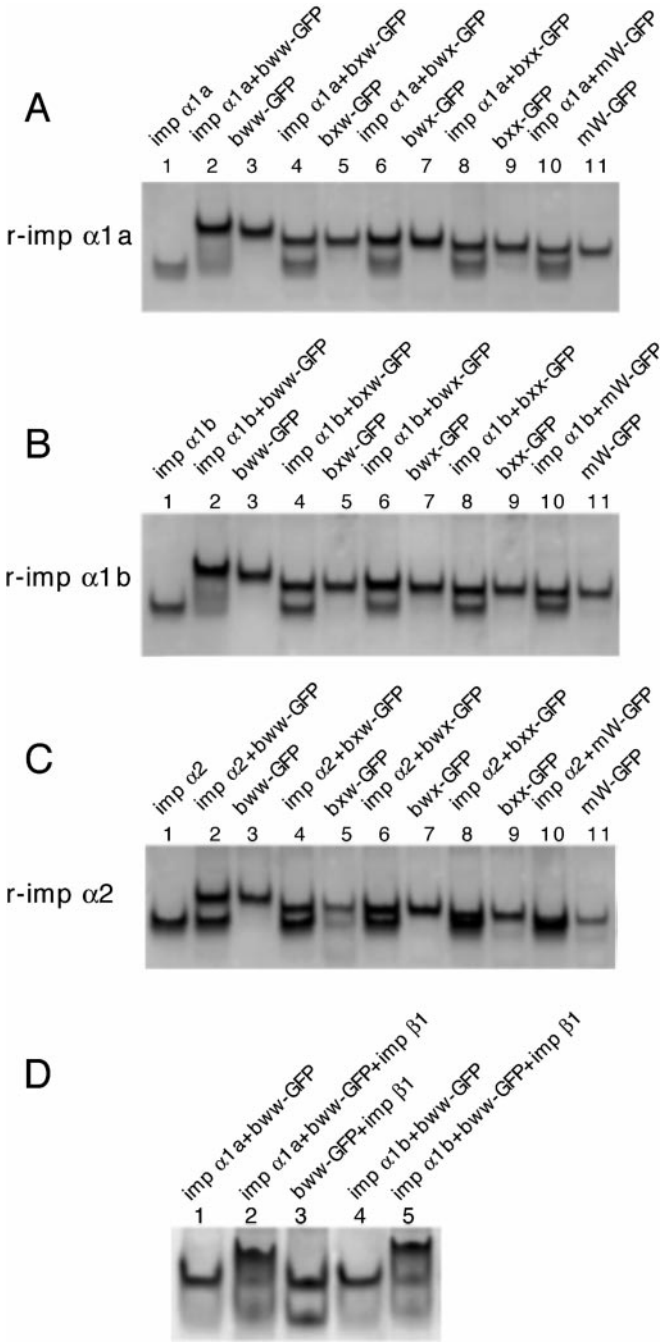

FIG. 6. ***In vitro* interactions between rice importin αs and COP1 NLSs.** Native gel electrophoresis was carried out as described in the legend to Fig. 2.

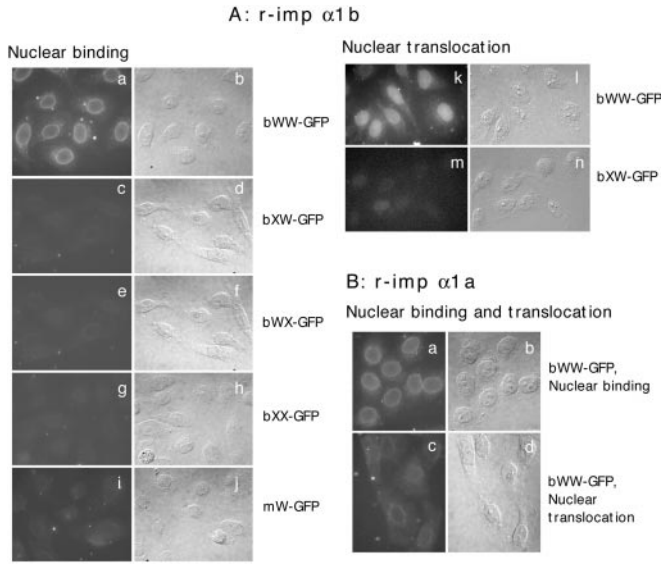

FIG. 7. **Preferential nuclear translocation of bWW-GFP by rice importin α1b.** Nuclear binding and import assays were carried out as described in the legend to Fig. 3.

a functional difference exists between the multiple importin α isoforms in the plant nuclear protein import system.

DISCUSSION

In the present work, we have isolated a novel cDNA for an importin α homologue in rice and named it rice importin α1b. Analysis of its primary structure reveals that rice importin α1b contains the main characteristics of other importin αs, namely an IBB domain, arm repeats, a COOH-terminal acidic region, and variable regions flanking the arm repeats (Fig. 1). The IBB domain is a highly conserved stretch of basic amino acids and is well known to be responsible for the interaction with importin β (20, 49). The putative IBB domain of rice importin α1b displays maximum homology with those of other importin αs. Such high homology of IBB domains among different importin αs may explain why different isoforms of importin α can interact with the same importin β. For instance, all three rice importin αs can bind to rice importin β1 (Fig. 2*B*; Refs. 36 and 41). On the other hand, the region of arm repeats is another well conserved domain and is responsible for NLS binding. Mapping of the NLS-binding sites within importin α molecules has suggested that different NLS types interact with different subsets of arm repeats (17, 50, 51). However, recent studies indicate that NLS binding by importin α also requires the variable regions (51). In the case of the human importin α2 (also termed Rch1, hSRP1α, or pendulin), the first variable region is required for T-NLS-binding, while the second one is required for binding to LEF-1 NLS (51). The variable regions of rice importin α1b show only minimum identities to those of the other importin αs (Fig. 1), and such variability may confer an importin α with its NLS specificity (26). The COOH-terminal acidic region has been demonstrated to be the site for interaction with CAS (51), a nuclear transport factor that exports importin α from the nucleus (52).

In addition to the primary structural data, our *in vitro* assays using recombinant rice importin α1b protein demonstrated that rice importin α1b can bind functional plant NLSs and rice importin β1, forming a stable PTAC (Fig. 2), and mediate nuclear import of NLS-proteins in digitonin-permeabilized HeLa cells (Fig. 3). These data strongly suggest that rice importin α1b functions as a component of the NLS receptor in plant cells.

Both rice importin α1a and α1b proteins showed selective binding to T-NLS and O_2-NLS, but not to R-NLS (36, Fig. 2*A*). This is in contrast to the *Arabidopsis* aIMPα, which binds all the typical plant NLSs (35). Moreover, a different activity was also seen between rice importin α1a and α1b proteins in mediation of nuclear import of COP1 NLS-GFP in the present work (Fig. 7). In fact, preferential affinities between distinct importin αs and different NLS-containing proteins have also been reported in human importin αs. Human DNA helicase Q1 (25) and RCC1 (22) are most efficiently imported into the nucleus by human importin α3, and transcription factor Stat1 is imported into the nucleus by NP1 (human importin α1), but not by Rch1 (human importin α2), in response to interferon-r (27). RanBP3 interacts preferentially with both human importin α3 and α4, but its nuclear import was most efficient in the presence of importin α3 (28). All these data suggest a diverse specificity

among importin αs with respect to NLS recognition, such that each importin α preferentially imports a distinct group of proteins into the nucleus. In addition, the differential expression patterns of rice importin αs (Fig. 4) support the hypothesis that multiple isoforms of importin αs might contribute to spatial and temporal regulation of nuclear protein import.

Although both rice importin α1a and α1b, together with rice importin β1, complexed with COP1 NLS-GFP and docked to the nuclear envelope, translocation of the fusion protein into the nucleus occurred preferentially in the presence of rice importin α1b (Fig. 7). This suggests that the nuclear import of COP1 protein *in vivo* is mediated, at least most efficiently, by the importin α1b and that *in vitro* binding between an importin α and an NLS does not necessarily mean that the nuclear import of the NLS protein is performed by that importin α. Indeed, similar phenomena have been reported in human importin αs. Human RanBP3 NLS shows a binding activity to both human importin α3 and α4, however, its nuclear import was mediated most efficiently by human importin α3 (28). Nachury *et al.* (24) observed a consistently lower nuclear import activity of hSRP1r (human importin α3) for bovine serum albumin-SV40 large T-antigen NLS when compared with that of hSRP1α (human importin α2) or NP1. Taken together, all these data seem to suggest that the nature of the interaction between a distinct importin α and a specific cognate NLS protein is also a critical factor for nuclear protein import process. This idea is also supported by the evidence that β-catenin, that contains arm repeats, alone can dock onto the nuclear envelope and further translocate into the nucleus in a NLS-independent manner without assistance of importin and RanGTPase (53, 54). Thus it is conceivable that arm repeats in importin α may interact with NPC and such interaction could be affected by binding of a specific NLS-protein to this domain.

Acknowledgment—We are grateful to Dr. Patrick J. Hussey (School of Biological Science, Royal Holloway, University of Lodon, Egham, Surrey, United Kingdom) for critical reading of the manuscript.

REFERENCES

1. Imamoto, N., Kamei, Y., and Yoneda, Y. (1998) *Eur. J. Histochem.* **42,** 9–20
2. Mattaj, I. W., and Englmeier, L. (1998) *Annu. Rev. Biochem.* **67,** 265–306
3. Pemberton, L. F., Blobel, G., and Rosenblum, J. S. (1998) *Curr. Opin. Cell Biol.* **10,** 392–399
4. Nagy, F., and Schafer, E. (2000) *EMBO J.* **19,** 157–163
5. von Arnim, V. G., and Deng, X.-W. (1994) *Cell* **79,** 1035–1045
6. Wozniak, R. W., Rout, M. P., and Aitchison, J. D. (1998) *Trends Cell Biol.* **8,** 184–188
7. Dingwall, C., and Laskey, R. A. (1991) *Trends Biochem. Sci.* **16,** 478–481
8. Raikhel, N. (1992) *Plant Physiol.* **100,** 1627–1632
9. Imamoto, N., Shimamoto, T., Kose, T., Takao, T., Tachibana, T., Matsubae, M., Sekimoto, T., Shimamoto, T., and Y. Yoneda, (1995) *FEBS Lett.* **368,** 415–419
10. Imamoto, N., Shimamoto, T., Takao, T., Tachibana, T., Kose, T., Matsubae, M., Sekimoto, T., Shimonishi, Y., and Y., Yoneda. (1995) *EMBO J.* **14,** 3617–3626
11. Görlich, D., Prehn, S., Laskey, R. A., and Hartmann, E. (1994) *Cell* **79,** 767–778
12. Iovine, M. K., Watkins, J. L., and Wente, S. R. (1995) *J. Cell Biol.* **131,** 1699–1713
13. Kraemer, D. M., Strambio-de-Castillia, C., Blobel, G., and Rout, M. P. (1995) *J. Biol. Chem.* **270,** 19017–19021
14. Radu, A., Moore, M. S., and Blobel, G. (1995) *Cell* **81,** 215–222
15. Melchior, F. B., Paschal, J., Evans, J., and Gerace, L. (1993) *J. Cell Biol.* **123,** 1649–1659
16. Moore, M. S., and Blobel, G. (1993) *Nature* **365,** 661–663
17. Cortes, P., Ye, Z.-S., and Baltimore, D. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 7633–7637
18. Cuomo, C. A., Kirch, S. A., Gyuris, J., Brent, R., and Oettinger, M. A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 6156–6160
19. O'Neill, R. E., and Palese, P. (1995) *Virology* **206,** 116–125
20. Weis, K., Mattaj, I. W., and Lamond, A. I. (1995) *Science* **269,** 1049–1053
21. Kohler, M., Ansieau, S., Prehn, S., Leutz, A., Haller, H., and Hartmann, E. (1997) *FEBS Lett.* **417,** 104–108
22. Kohler, M., Speck, C., Christiansen, M., Bischoff, F. R., Prehn, S., Haller, H., Gorloch, D., and Hartmann, E. (1999) *Mol. Cell. Biol.* **19,** 7782–7791
23. Tsuji, L., Takumi, T., Imamoto, N., and Yoneda, Y. (1997) *FEBS Lett.* **416,** 30–34
24. Nachury, M. V., Ryder, U. W., Lamond, A. I., and Weis, K. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 582–587
25. Miyamoto, Y., Imamoto, N., Sekimoto, T., Tachibana, T., Seki, T., Tada, S., Enomoto, T., and Yoneda, Y. (1997) *J. Biol. Chem.* **272,** 26375–26381
26. Nadler, S. G., Tritschler, D., Haffar, O. K., Blake, J., Bruce, A., and Cleaveland, J. S. (1997) *J. Biol. Chem.* **272,** 420–429
27. Sekimoto, T., Imamoto, N., Nakajima, K., Hirano, T., and Yoneda, Y. (1997) *EMBO J.* **16,** 7067–7077
28. Welch, K., Franke, J., Kohler, M., and Macara, I. G. (1999) *Mol. Cell. Biol.* **19,** 8400–8411
29. Hicks, G. R., Smith, H. M. S., Lobreaux, S., and Raikhel, N. V. (1996) *Plant Cell* **8,** 1337–1352
30. Ballas, N., and Citovsky, V. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 10723–10728
31. Németh, K., Salchert, K., Putnoky, P., Bhalerao, R., Koncz-Kálman, Z., Stankovic, Stangeland, B., Bakó, L., Mathur, J., Ökrész, L., Stabel, S., Geigenberger, P., Stitt, M., Reédei, G. P., Schell, J., and Koncz, C. (1998) *Genes Dev.* **12,** 3059–3073
32. Schledz, M., Leclerc, D., Neuhaus, G., and Merkle, T. (1998) *Plant Physiol.* **116,** 868
33. Shoji, K., Iwasaki, T., Matsuki, R., Uchimiya, H., Miyao, M., and Yamamoto, N. (1998) *Gene (Amst.)* **212,** 279–286
34. Iwasaki, T., Matsuki, R., Shoji, K., Sanmiya, K., Miyao, M., and Yamamoto, N. (1998) *FEBS Lett.* **428,** 259–262
35. Smith, H. M. S., Hicks, G. R., and Raikhel, N. V. (1997) *Plant Physiol.* **114,** 411–417
36. Jiang, C. J., Imamoto, N., Matsuki, R., Yoneda, Y., and Yamamoto, N. (1998) *J. Biol. Chem.* **273,** 24083–24087
37. Matsuki, R., Iwasaki, T., Shoji, K., Jiang, C. J., and Yamamoto, Y. (1998) *Plant Cell Physiol.* **39,** 879–884
38. Chiu, W., Niwa, Y., Zeng, W., Hirano, T., Kobayashi, H., and Sheen, J. (1996) *Curr. Biol.* **6,** 325–330
39. Akama, K., Shiraishi, H., Ohta, S., Nakamura, K., Okada, K., and Shimura, Y. (1992) *Plant Cell Rep.* **12,** 7–11
40. Clough, S. J., and Ben, A. F. (1998) *Plant J.* **16,** 735–743
41. Jiang, C. J., Imamoto, N., Matsuki, R., Yoneda, Y., and Yamamoto, N. (1998) *FEBS Lett.* **437,** 127–130
42. Bischoff, F. R., and Ponstingl, H. (1995) *Methods Enzymol.* **257,** 135–144
43. Melchior, F., Sweet, D. J., and Gerace, L. (1995) *Methods Enzymol.* **257,** 279–291
44. Yano, R., Oakes, M., Yamaghishi, M., Dodd, J. A., and Nomura, M. (1992) *Mol. Cell. Biol.* **12,** 5640–5651
45. Görlich, D., Henklein, P., Laskey, R. A., and Hartmann, E. (1996) *EMBO J.* **15,** 1810–1817
46. Weis, K., Ryder, U., and Lamond, A. I. (1996) *EMBO J.* **15,** 1818–1825
47. Peifer, M., Berg, S., and Reynolds, A. B. (1994) *Cell* **76,** 789–791
48. Stacey, M. G., Hicks, S. N., and von Arnim, A. G. (1999) *Plant Cell* **11,** 349–363
49. Görlich, D., N., Pante, Kutay, U., Aebi, U., and Bischoff, F. R. (1996) *EMBO J.* **15,** 5584–5594
50. Conti, E., Uy, M., Leighton, L., Blobel, G., and Kuriyan, J. (1998) *Cell* **94,** 193–204
51. Herold, A., Truant, R., Wiegand, H., and Cullen, B. R. (1998) *J. Cell Biol.* **143,** 309–318
52. Kutay, U., Bischoff, F. R., Kostka, S., Kraft, R., and Görlich, D. (1997) *Cell* **90,** 1061–1071
53. Fagotto, F., Gluck, U., and Gumbiner, B. M. (1998) *Curr. Biol.* **8,** 181–190
54. Yokoya, F., Imamoto, N., Tachibana, T., and Yoneda, Y. (1999) *Mol. Biol. Cell* **10,** 1119–1131

Molecular Cloning of a Novel Importin α Homologue from Rice, by Which Constitutive Photomorphogenic 1 (COP1) Nuclear Localization Signal (NLS)-Protein Is Preferentially Nuclear Imported

Chang-Jie Jiang, Kazuhiro Shoji, Rikyu Matsuki, Akiko Baba, Noritoshi Inagaki, Hiroshi Ban, Toshisuke Iwasaki, Naoko Imamoto, Yoshihiro Yoneda, Xing-Wang Deng and Naoki Yamamoto

J. Biol. Chem. 2001, 276:9322-9329.
doi: 10.1074/jbc.M006430200 originally published online December 20, 2000
