

Protein nucleocytoplasmic transport and its light regulation in plants

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Light exerts a great influence on gene expression, physiology and development pattern in higher plants. Protein factors involved in these processes, such as the photoreceptor, phytochrome B, a key regulatory protein, COP1, and some bZIP transcription factors have been identified and their light-regulated movement between the cytoplasm and the nucleus has been demonstrated. These findings imply that nucleocytoplasmic transport plays a crucial role in light regulation in higher plants. This review summarizes recent investigations into plant nuclear transport systems and specific cases where nucleocytoplasmic transport is subject to light regulation.

Introduction

While animals can search for food and move away from a variety of stressful environments, the immobile higher plants must respond and adapt to changing environmental conditions. Among a variety of environmental stimuli that affect plants, light exerts the most prominent influence on their development (Kendricks & Kronenberg 1994). For example, changes in day length provide the most reliable information on seasonal changes in plants. The most extensively studied case is the light control of seedling development (von Arnim & Deng 1996). While seedlings grown in darkness follow a pathway called skotomorphogenesis, light-grown seedlings pursue a contrasting development pathway named photomorphogenesis. Those two development paths are accompanied by remarkable distinctions in gene expression pattern, plastid development, and the development of photosynthetic activity. Light signals are perceived by multiple photoreceptors, including the phytochrome and cryptochrome families (Quail 1994; Cashmore *et al.* 1999). Recent molecular and genetic studies have begun to reveal several important insights into the phytochrome signalling cascade (Barnes *et al.* 1997; Ni *et al.* 1998;

Yeh & Lagarias 1998; Frankhauser *et al.* 1999; Hoecker *et al.* 1999).

Interestingly, the accumulated evidence clearly suggested a critical role for the regulated nuclear localization of proteins in light signalling. For example, a photoreceptor, phytochrome B, is localized in nuclei in the light, but is reduced in nuclei during dark adaptation (Sakamoto & Nagatani 1996; Kircher *et al.* 1999b; Yamaguchi *et al.* 1999). A photomorphogenic regulator, COP1, accumulates in the nuclei to inhibit photomorphogenesis in darkness, and disappears from nuclei in the light (von Arnim & Deng 1994; Stacey *et al.* 1999). Some bZIP transcription factors such as GBF2 and CPRF2, which can bind to a *cis*-element G-box that is commonly present in the light-responsive promoters, are localized to the nucleus in the light condition but not in darkness (Harter *et al.* 1994; Terzaghi *et al.* 1997; Kircher *et al.* 1999a).

Nucleocytoplasmic transport system has been extensively investigated in this decade (Imamoto *et al.* 1998; Mattaj & Englmeier 1998; Pemberton *et al.* 1998). In addition to this, much has been learned about nuclear import systems in plant cells. The nuclear localization signals (NLS) of plant nuclear proteins (Raikhel 1992) and nuclear import-related proteins have been characterized, and an *in vitro* nuclear import system has been developed (Hicks *et al.* 1996; Merkle *et al.*

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1996). Here we have attempted to summarize recent advances in understanding nucleocytoplasmic transport system of plant cells and how light may exert its regulatory effect.

Nuclear import system

An overview

Nucleocytoplasmic transport systems and related factors have been extensively investigated in this decade, and a variety of protein factors have been identified from yeast to mammals (for reviews see Imamoto *et al.* 1998; Mattaj & Englmeier 1998; Pemberton *et al.* 1998). Thus, the general mechanisms of these systems have been elucidated (Fig. 1). Our knowledge on those of plants were relatively limited, although the plant nucleocytoplasmic translocation system in general is similar to that of other systems (for review see Merkle & Nagy 1997). Nucleocytoplasmic transport takes place through the nuclear pore complex (NPC). 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, transcription and transcriptional regulation, must enter the nucleus. Multiple nucleocytoplasmic transport pathways have been identified, each likely responsible for the translocation of a distinct

group of proteins (Pemberton *et al.* 1998; Wozniak *et al.* 1998). Among them, the best characterized is the import of proteins containing a classical nuclear localization signal (NLS) containing a short stretch of basic amino acids

The NLS-containing proteins are initially recognized in the cytoplasm by an NLS-receptor complex, a heterodimer consisting of importin α and importin β subunits. Importin α binds the NLS specifically, forming a stable nuclear pore-targeting complex (PTAC), whereas importin β mediates the docking of the PTAC to the cytoplasmic face of the NPC. Translocation of the docked PTAC into the nucleus is an energy-dependent process, mediated by the small GTPase, Ran, along with a homodimeric factor known as p10 or NTF2. The nucleotide binding affinity of Ran is regulated by two different proteins, the chromatin-bound exchange factor, RCC1, which generates Ran-GTP in the nucleus, and the cytoplasmic GTPase activating protein, RanGAP1, which depletes Ran-GTP from the cytoplasm. Ran-GTP binding to importin β has been shown to release importin α -substrate complexes into the nucleus.

NLS of plant nuclear proteins

Most nuclear proteins contain an NLS, which consisted of a short stretch of basic amino acids (Raikhel 1992; Hicks & Raikhel 1995). In studies on plant NLSs, chimeric genes encoding either NLS-GUS or

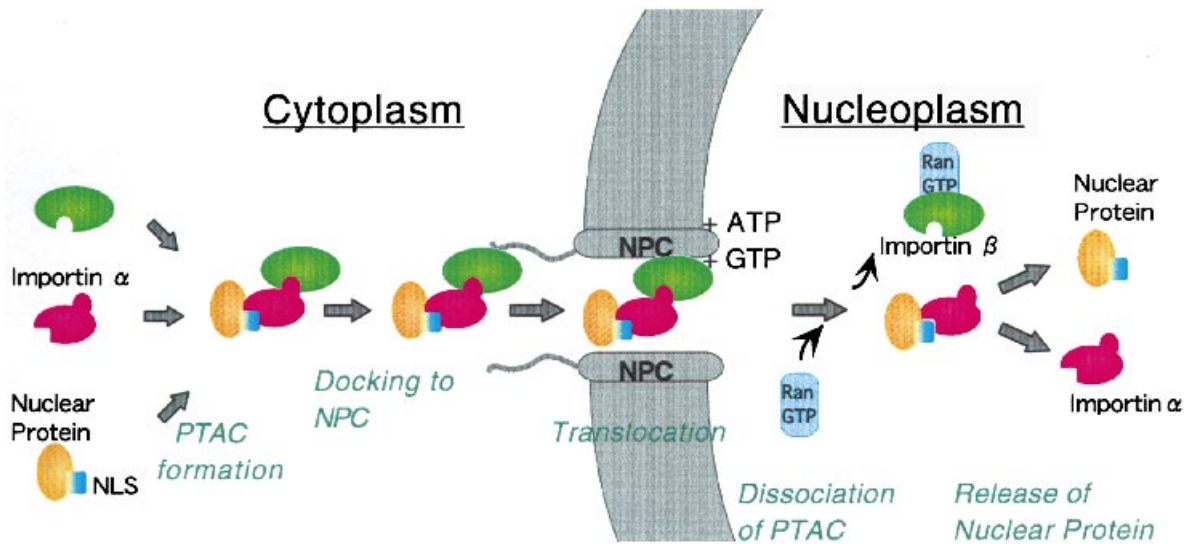


Figure 1 A schematic representation of nuclear protein import. Abbreviations are; PTAC, nuclear pore-targeting complex; NPC, nuclear pore complex; NLS, nuclear localization signal; For details, see text.

NLS-GFP were introduced transiently in onion epidermal cells with a particle gun or stably into transgenic plants, and NLS activities were estimated by the nuclear localization of GUS or GFP reporter. The NLS of plant nuclear proteins have been classified into three types. The best characterized monopartite NLS are similar to the NLS of SV40 T antigen. This type of NLS has been found in plant nuclear proteins and has been shown to function in nuclear import processes. Bipartite NLSs consist of two clusters of basic amino acids separated by 10 or more amino acids. This type was originally found in a *Xenopus* nucleoplasmin and was later found in a maize Opaque-2 transcription factor (called O2-NLS) and many plant proteins. Finally, the yeast Mat α 2 NLS typifies a third class of NLS that is unusual in possessing both hydrophobic and basic amino acids. It has been reported that the Mat α 2 NLS does not function in mammals. However, R-NLS, which shows some similarity to Mat α 2 NLS, was found in maize transcription factor R. Although the basic mechanisms of nuclear import appear to be conserved among eukaryotes, the presence of R-NLS might indicate a plant cell-specific character in the nuclear import system.

Importin α and importin β

Both importin α and importin β have been identified in a wide range of species, including vertebrates, yeast and plants. Multiple isoforms of importin α have been identified in most species (Mattaj & Englmeier 1998; Pemberton *et al.* 1998). Recent studies have suggested that the multiplicity of importin α isoforms might contribute to the tissue-specific or temporal regulation of nuclear protein import (Table 1). For example, more than five isoforms of importin α have been identified in humans, and they exhibit distinct tissue-specific expression patterns (Cortes *et al.* 1994; Cuomo *et al.* 1994; Görlich *et al.* 1994; O'Neill & Palese 1995; Weis *et al.* 1995; Köhler *et al.* 1997; Tsuji *et al.* 1997; Nachury *et al.* 1998). These isoforms have also been shown to interact with specific NLS sequences (Miyamoto *et al.* 1997; Nadler *et al.* 1997; Sekimoto *et al.* 1996; Nachury *et al.* 1998).

In plants, cDNAs have been cloned for importin α homologues. As in many vertebrates, multiple isoforms of importin α have been identified in rice and *Arabidopsis*. The rice importins include importin α 1a (Shoji *et al.* 1998), α 1b (C.-J. Jiang, K. Shoji,

Table 1 A possible functional differentiation among multiple forms of importin α deduced from sequence-specific interaction with NLS proteins and differential expression

Importin α	Substrate specificity	Differential expression (Light responsiveness and tissue specificity)	Reference
<i>Rice (Oryza sativa)</i>			
Importin α 1a	T-, O2-NLS†	Down-regulation by light Higher in calli and roots but expressed in all the tissues	Jiang <i>et al.</i> (1998a) Shoji <i>et al.</i> (1998)
Importin α 1b	T-, O2-NLS†	Higher in root but expressed in all tissues	Jiang <i>et al.</i> (submitted)
Importin α 2	T-NLS†	n.d.	Iwasaki <i>et al.</i> (1998) Unpublished data
<i>Arabidopsis thaliana</i>			
aI MPa	T-, O2- and R-NLS		Smith <i>et al.</i> (1997) Hicks <i>et al.</i> (1996)
At KAP α *	VirD2 protein	n.d.	Ballas & Citovsky (1997)
ATHKAP α *	pr1 protein	n.d.	Németh <i>et al.</i> (1998)
HSRP1/NPI1	Stat1, NF κ B	Brain, Cerebellum	Sekimoto <i>et al.</i> (1997)
Rch1	ICP8	Thymus, Spleen	Nadler <i>et al.</i> (1997)
Qip1	Human DNA helicase Q1	Testis, Ovary, Small intestine, Pancreas	Miyamoto <i>et al.</i> (1997)
hSRP1r		Skeletal muscle, Testis, Colon	Nachury <i>et al.</i> (1998)

* The importin α s were identified as proteins that interacted with the protein listed in the 'Substrate' column from the yeast two hybrid system.

† NLS-specific binding and nuclear import activities were determined by an *in vitro* binding assay and by a permeabilized HeLa cell nuclear import assay, respectively.

R. Matsuki, N. Inagaki, H. Bau, T. Iwasaki, N. Imamoto, Y. Yoneda & N. Yamamoto, unpublished data), and $\alpha 2$ (Iwasaki *et al.* 1998) and *Arabidopsis* importins include $\alpha 1$ MPa (Hicks *et al.* 1996), AtKAP α (Ballas & Citovsky 1997) and ATHKAP2 (Németh *et al.* 1998). Two putative functional domains in the primary structures of importin α were revealed, one is an importin β binding domain (IBB), and the other is the armadillo repeat. Armadillo repeats of 42 amino acids were originally identified in the fruit fly armadillo protein, and have been thought to be a prominent feature of importin α . Recently, the region consisting of armadillo repeats was found to be involved in the binding to NLS signals (Cortes *et al.* 1994; Sekimoto *et al.* 1997; Conti *et al.* 1998).

Much effort has been made to determine whether different importin α isoforms have a specificity for different NLS substrates (Table 1). Three representative plant NLSs, T-, O2-, or R-NLS as described above, were inserted between GST and GFP. The resultant GST-NLS-GFP fusion protein was used as an artificial nuclear import substrate in an *in vitro* binding assay (Jiang *et al.* 1998a), in which recombinant rice importin α proteins were mixed with the NLS proteins, and protein-protein interactions were observed using native gel electrophoresis. Intriguingly, the gel results indicated that different types of importin α recognized different types of NLS. For instance, rice importins $\alpha 1$ bound to T- and O2-NLS, but not to R-NLS (Jiang *et al.* 1998a), while rice importin $\alpha 2$ showed binding affinity for T-NLS (Iwasaki *et al.* 1998), but not for O2- or R-NLS (unpublished data). These data suggest that the multiple isoforms of importin α serve as a control point for the regulation of nuclear protein import in plant cells as well as in animal cells (Table 1).

In contrast, an *Arabidopsis* importin α , $\alpha 1$ MPa, was shown to bind all three of the typical classes of plant NLSs (Smith *et al.* 1997). In addition to the $\alpha 1$ MPa, which was shown to encode an amino acid sequence similar to known importin α , two other members of importin α , AtKAP α and ATHKAP2, were isolated from *Arabidopsis* as interacting protein with VirD2 and a regulatory WD protein, PRL1, respectively (Ballas & Citovsky 1997; Németh *et al.* 1998). Thus there are at least three importin α genes in the *Arabidopsis* genome, and they may have differential functionality.

As suggested from the conserved IBB sequences in the N-terminal region, rice importin $\alpha 1$ a showed an ability to form a PTAC with importin β and T-NLS fusion protein. Furthermore, we have demonstrated that they were able to mediate the targeting of PTAC to nuclear envelope in the presence of importin β in

digitonin-permeabilized HeLa cells. Finally, they also mediated the nuclear import of the fusion proteins containing either T-NLS or O2-NLS, but not R-NLS, in digitonin-permeabilized HeLa cells (Jiang *et al.* 1998a). These data indicate that basic functional domains appear to be conserved among a large number of importin α molecules from a variety of organisms, while divergences in functional specificity such as NLS-selective binding activities were also a common theme.

The functional role of importin β has been elucidated in mammals and yeast, but no knowledge on plant importin β has been obtained. Recently, the two cDNA-containing β -like sequences from the rice plant were identified (Matsuki *et al.* 1998) and an initial characterization of one of them (rice importin $\beta 1$) was carried out using a recombinant protein produced in *E. coli* (Jiang *et al.* 1998b). As was assumed from previous studies of importin β from mammals and yeast, its biochemical activities were examined *in vitro* with respect to: (i) PTAC assembling activity, (ii) targeting activity to the nuclear envelope, (iii) interaction with Ran, and (iv) nuclear import activity. Importin β assembled a PTAC with importin α and NLS protein. The PTAC assembling activity of importin β was indicated by native gel electrophoresis by the appearance of a new band composed of rice importin β , rice importin $\alpha 1$ a and NLS protein. Following its assembly, the PTAC is targeted to the nuclear membrane by virtue of the importin β , which probably interacts with nucleoporins at the NPC. Fluorescent dye-labelled importin β was applied to evacuated tobacco protoplasts, and the accumulation of importin β was observed as nuclear rim, indicating the its targeting activity. The cargo of PTAC, NLS protein, must be released from the PTAC in the nucleus after transport through the NPC. Therefore the interaction between importin β and Ran-GTP was also examined on native gel electrophoresis using mouse Ran. Rice importin β interacted with mouse Ran-GTP, whereas the importin α/β heterodimer was dissociated in the presence of Ran-GTP, indicating the functional interaction of importin β with GTPase Ran. Finally, rice importin β also mediated the nuclear import of NLS protein, in digitonin-permeabilized HeLa cells (Jiang *et al.* 1998b).

It is also noteworthy that two closely related importin β s, importin $\beta 1$ and $\beta 2$, were identified in a single plant species, rice (Matsuki *et al.* 1998). 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 known, as no importin β s have yet been isolated from other plants. At least in rice, however, this may provide an additional point for the regulation of nuclear protein importation.

Other factors involved in plant nuclear import

Ran cDNA was isolated from tobacco, tomato, *Arabidopsis* and *Vicia faba* based on their highly conserved similarity (Merkle & Nagy 1997). Nuclear import activity in evacuated protoplasts was blocked by nonhydrolysable GTP analogs (Hicks *et al.* 1996; Merkle *et al.* 1996). Since GTP hydrolysis is essential for Ran function in other systems (Görlich 1997), this inhibition suggests that the role of Ran homologues is conserved in nuclear import in plant cells. Functional complementation studies in heterologous systems using a tobacco Ran cDNA suggested that plant Ran may play the same role in nuclear import in plant cells as those in mammalian and yeast cells (Merkle *et al.* 1994). However, the direct evidence from studies in plant cells is not yet available.

Like other regulatory GTPases, Ran has a low intrinsic GTPase activity. It interacts with GTPase-activating protein, RanGAP, and Ran-binding protein, RanBP1, to achieve GTPase activity in humans. It also needs the cofactor RCC1 to exchange the formed GDP for GTP (Mattaj & Englmeier 1998). Although there is no evidence that the plant homologues of these proteins are involved in nuclear import in plant cells, RanBP1 homologues were isolated from *Arabidopsis* by a two-hybrid system using Ran as 'bait' (Haizel *et al.* 1997). The specific binding of RanBP1 to Ran-GTP suggests that the function of RanBP1 is conserved in plant cells, and consequently the mechanisms regulating the levels of Ran-GTP and Ran-GDP and the direction of nuclear transport are also conserved. The RanGAP and RCC1 homologues in plants have yet to be identified.

Nuclear transport assay system in plants

Adam *et al.* (1990) described an excellent *in vitro* nuclear transport assay system using mammalian cells. While the treatment of cultured cells with digitonin permeabilizes the plasma membranes to macromolecules, the nuclear envelopes remain intact. These semi-intact cells show nuclear import activity in the presence of cytosolic factors, GTP and ATP, and NLS proteins are accumulated in the nuclei. Using this system, a variety of cytosolic factors involving nuclear import activity have been investigated and the biochemical processes

have been elucidated (Adam *et al.* 1991). Although this system has been employed to study the activity of rice importin α and β as described above, the development of a similar *in vitro* system from plant cells would be essential. As some characteristics of the plant nuclear transport system have previously been found to be specific to plant cells and distinct from the nuclear transport system of animal cells (see below). Consequently, in principle, the characterization of a variety of nuclear import factors should be best carried out in an *in vitro* assay system prepared from plant cells.

An *in vitro* nuclear transport assay system from tobacco cultured cells has been described by two groups (Hicks *et al.* 1996; Merkle *et al.* 1996). Tobacco protoplasts were evacuated by Percoll density gradient centrifugation and permeabilized by either reduction of osmotic concentration (Hicks *et al.* 1996) or treatment with Triton-X 100 (Merkle *et al.* 1996). Conjugated proteins harbouring O2-NLS or T-NLS were imported into the nuclei in an NLS-dependent manner. While cytosolic factors are necessary for nuclear import in the mammalian cell system, the plant cell system did not require the cytoplasmic factors, indicating that the soluble nuclear transport factors such as importin α were retained in permeabilized protoplasts. This difference between mammalian and plant systems might be attributed to the nature of individual nuclear import factors themselves and/or the susceptibility of plant membrane to some detergents. Accordingly, the plant cell-derived import assay system could not be used in the study of soluble nuclear import factors. To elucidate the mechanism of plant nucleocytoplasmic transport, in particular the fine mechanism regulating nucleocytoplasmic transport in signal transduction, a suitable plant assay system needs to be developed.

Distinct features of plant nuclear import

Determination of the subcellular localization of importin α and related factors suggests that plants may have some distinct features in their nuclear transport system. Importin α has been thought to localize predominantly in the cytoplasm (Imamoto *et al.* 1995; Görlich *et al.* 1996; Weis *et al.* 1996), but plant importin α appears to be tightly associated with the nucleus. Importin α is concentrated at the nuclear envelope in tobacco protoplasts (Smith *et al.* 1997) and retained in permeabilized protoplasts (Hicks *et al.* 1996). Incomplete inhibitory effects of GTP- γ -S or ATP- γ -S suggested that GTP and ATP persist in sufficient amounts (Merkle *et al.* 1996). These findings also

suggest that Ran homologues are removed completely from the permeabilized protoplasts.

The finding that the nuclear import system does not depend on importin α might be attributable to the plant-specific subcellular localization of importin α . Hicks *et al.* (1996) initially indicated that importin α was localized in the cytoplasm and the nucleus using cell fractionation followed by a Western blot analysis. Moreover, the nuclear localization of importin α , in particular its association with the nuclear envelope, was demonstrated in a dicotyledonous plant, *Arabidopsis*, by immunolocalization (Smith *et al.* 1997). Recently, the subcellular localization in a monocotyledonous plant, rice, by the use of GFP-importin α and immunostaining was independently performed (A. Baba, C.-J. Jiang, N. Inagaki, R. Matsuki & N. Yamamoto, unpublished data). Both experiments indicated that rice importin $\alpha 1$ was predominantly localized in the nucleus. These discoveries offered a reasonable explanation of why cytosolic factors are not required for *in vitro* nuclear import in permeabilized plant cells. The localization of importin $\alpha 1$ in the nucleus could be interpreted as a plant cell-specific property of the nuclear import system.

In addition, a substantial amount of importin α is localized in the cytoplasm, as was reported previously (Hicks *et al.* 1996). In *Arabidopsis* cells, most of the cytosolic importin α was colocalized with microtubules and microfilaments in a NLS protein-dependent manner (Smith & Raikhel 1999). Thus, the involvement of the cytoskeleton has also been suggested to be involved in the process of nuclear import of NLS proteins. Furthermore, depolymerization of microfilaments seemed to cause an accumulation of importin α inside the nucleus, indicating that the microfilaments are involved in the retention of importin α in the cytoplasm.

WGA (Wheat germ agglutinin) has been routinely used to demonstrate the active import of nuclear protein via the NPC, since it binds specifically to N-acetylglucosamine of NPC glycosylated proteins and blocks the nuclear import of NLS proteins in animal systems, but not in yeast (Hicks & Raikhel 1995). However, when WGA was added to an *in vitro* assay system using plant protoplasts, the nuclear import activity was not inhibited, even though WGA was bound to the nuclear envelope (Hicks *et al.* 1996; Merkle *et al.* 1996). The lack of an inhibitory effect of WGA might be also interpreted as a plant-specific feature.

The lack of WGA inhibition using rice importin $\alpha 1a$ (Shoji *et al.* 1998) in digitonin-permeabilized HeLa cells was also demonstrated (Fig. 2). Interestingly,

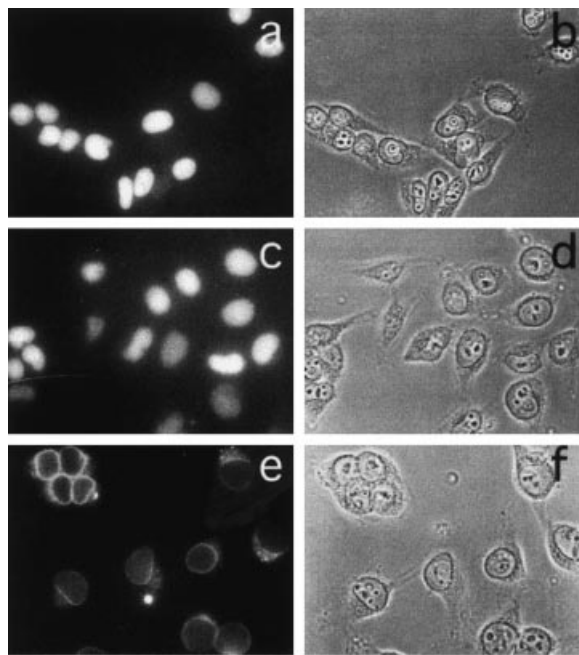


Figure 2 Substitution of mouse importin α with rice importin α completely abolished the inhibitory effect of wheat germ agglutinin (WGA) on nuclear protein import. Effect of WGA on nuclear import was examined in digitonin-permeabilized HeLa cells, in which GST-T-NLS-GFP fusion protein, importin α from rice (Shoji *et al.* 1998) or from mouse (Imamoto *et al.* 1995) and other necessary factors were added and incubated at 25 °C for 20 min, as described previously (Jiang *et al.* 1998a). To examine the effect of WGA, permeabilized cells were treated with 0.5 mg/mL WGA for 10 min prior to addition of the nuclear import assay mixture. While GST-T-NLS-GFP fusion protein was imported into nuclei in the presence of mouse importin α (panels a and b), the import was blocked in the cells treated previously with WGA (panels e and f) indicating accumulation of GST-T-NLS-GFP around the nuclear rim. However, when mouse importin α was substituted with rice importin α (panels c and d), the inhibitory effect of WGA was abolished. Panels a, c and e, fluorescence images; panels b, d and f, phase contrast images.

a simple substitution of mouse importin α (Imamoto *et al.* 1995) with rice importin α in an *in vitro* system almost completely cancelled the inhibitory effect of WGA on the nuclear import of NLS protein. For example, the treatment of the cells with WGA blocked the nuclear import of GST-T-NLS-GFP fusion protein. However, replacement of mouse importin α with rice importin α almost completely cancelled this inhibitory effect of WGA. This finding suggests a possibility that WGA not only binds to the NPC but may also interact directly with vertebrate importin α , whereby

it exerts its inhibitory effect on nuclear protein import in vertebrate cells. This may also account for the inability of WGA to inhibit plant nuclear protein import. Further studies of the interaction between WGA and importin α are clearly needed.

Nuclear localization regulated by light

Arabidopsis COP1, a negative regulator and the nucleocytoplasmic transport

Dark-grown (skotomorphogenic) seedlings are characterized by elongated and closed cotyledons on an apical hook, whereas photomorphogenic seedlings have short hypocotyl and expanded green cotyledons. Loss-of-function mutations in the *Arabidopsis* *COP1* gene cause the plants to display a constitutive photomorphogenic (COP) phenotype regardless of their light environment (Deng *et al.* 1991). This phenotype indicates that the gene product, COP1, acts as a negative regulator of photomorphogenesis, plastid development and the expression of light responsive genes (Deng & Quail 1992; Deng *et al.* 1992). A GUS-COP1 fusion gene was stably introduced into transgenic *Arabidopsis* to examine its subcellular localization in different light conditions (von Arnim & Deng 1994; von Arnim *et al.* 1997). GUS-COP1 accumulated in the hypocotyl cell nuclei of dark-grown seedlings, but the accumulation was depleted in the light. The fact that the GUS-COP1 fusion protein rescued the defects of the *cop1* mutations indicated that the GUS-COP1 localization pattern most likely mimics that of COP1 and is biologically relevant. The light-triggered nuclear depletion of COP1 suggests that COP1 represses the expression of photomorphogenesis-related genes in the nucleus in darkness and that the light inactivation of COP1 correlates with its nuclear depletion. Therefore the cellular localization of COP1 plays a critical role in mediating light control of *Arabidopsis* seedling development. Environmental light is received by multiple photoreceptors in plants. In particular, far red, red and blue light are primarily perceived by phytochrome A, phytochrome B and the cryptochromes, respectively. Through a semiquantitative analysis of the nuclear abundance of GUS-COP1 in *Arabidopsis* plants that were either defective or over-expressing individual photoreceptors, it was suggested that multiple photoreceptors can independently modulate COP1 nuclear localization (Osterlund & Deng 1998). On the other hand, all other *COP1*-like pleiotropic photomorphogenic genes, many of which encode subunits of the

highly conserved COP9 signalosome (Wei *et al.* 1994; Wei & Deng 1999), are required for the proper nuclear localization of COP1 in darkness and might be involved in the nuclear retention of COP1 (Chamovitz *et al.* 1996).

COP1 has three structural modules: an N-terminal RING-finger, followed by a coiled-coil region, and then a C-terminal WD-40 repeat domain (Deng *et al.* 1992; McNellis *et al.* 1994). The functional roles of the individual domains have recently been analysed (Torii *et al.* 1998; Stacey *et al.* 1999). COP1 functions as a homodimer and the coiled-coil domain contributes to the dimerization. The WD-40 repeat domain functions as an autonomous repressor domain. A bipartite NLS was identified between coiled-coil and WD-40 domains, which itself functions in a light-independent manner (Stacey *et al.* 1999). A novel and distinct cytoplasmic localization signal (CLS), bordering the RING and coiled-coil domains, which was able to redirect the fusion protein to the cytoplasm, seemed to mediate the nuclear exclusion. The light-regulation of the nucleocytoplasmic localization of COP1 can be reconstituted by the N-terminal half of COP1 that contains both the NLS and CLS. Therefore, both NLS and CLS, in the context of the COP1 N-terminal region, are required for the light regulated nucleocytoplasmic partitioning of COP1. As illustrated in Fig. 3, recent studies suggested that in darkness, COP1 act within the nucleus to directly interact and negatively regulate transcription factors responsible for promoting the light activation of gene expression and photomorphogenesis (Ang *et al.* 1998; Yamamoto *et al.* 1998).

Light-mediated nuclear import of bZIP transcription factors

Transcription factors have to exert their effect at the site of transcription within the nucleus. In many instances the cellular localization of a transcription factor serves as a convenient point of regulation. In plants, some bZIP proteins from parsley and *Arabidopsis* have been shown to be localized in the cytoplasm (Harter *et al.* 1994; Terzaghi *et al.* 1997; Kircher *et al.* 1999a). The G-box and the GT-box were identified as light-responsive *cis*-elements on the promoter region of a number of light-responsive genes (Thompson & White 1991). Harter *et al.* (1994) examined the subcellular distribution of binding activity to these *cis*-elements by a gel mobility shift assay. Whereas almost all GT box-binding activity was found in the nucleus, G-box

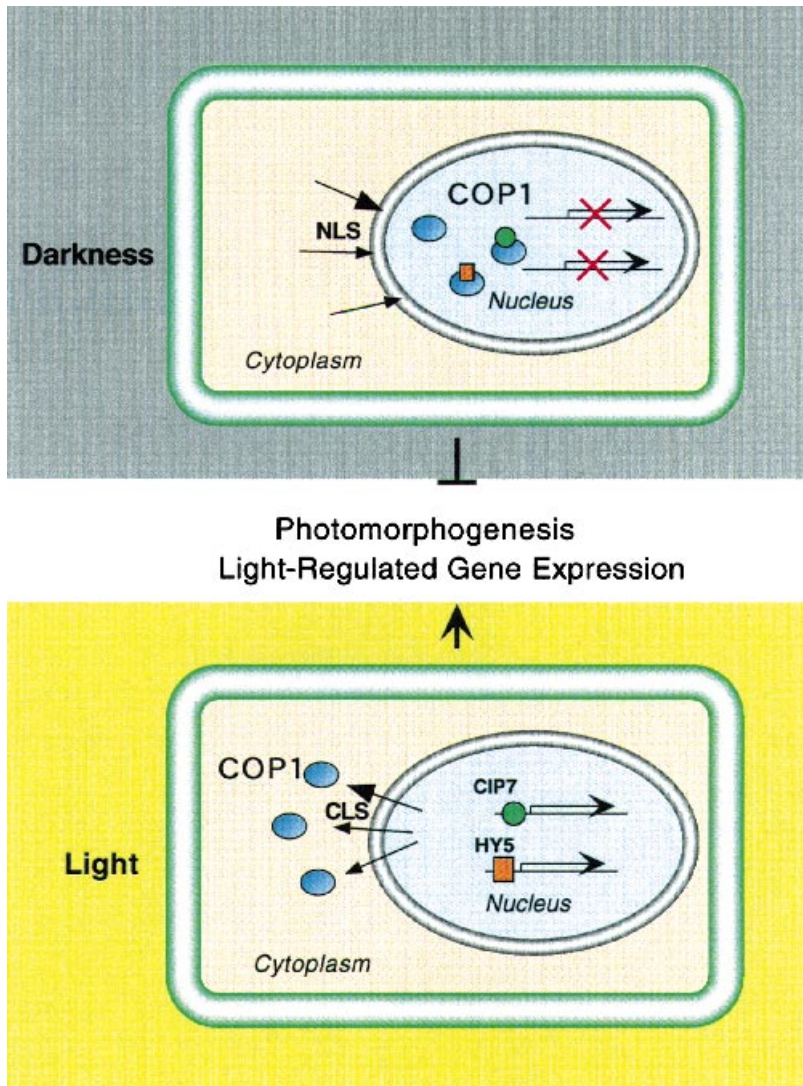


Figure 3 A working model illustrating light-regulated nucleocytoplasmic localization of COP1, which regulates photomorphogenic development including light-responsive gene expression. Note the arrows illustrate the direction of COP1 partitioning, not necessarily the physical movement of the protein. NLS, nuclear localization signal; CLS, cytoplasmic localization signal.

binding activity was observed in both nuclear and cytoplasmic compartments. Later, they demonstrated that a member of the parsley bZIP transcription factors, CPRF2, is imported into the nuclei in response to light, whereas it is localized in the cytoplasm of cells cultured in darkness (Kircher *et al.* 1999a). It was suggested that phytochromes A and B trigger nuclear import (Kircher *et al.* 1999a). Thus, the expression of a series of light-responsive genes could be regulated by the light-mediated subcellular localization of transcription factors.

Arabidopsis GBF (G-box binding factor), which also belongs to the bZIP class of transcription factors and is composed of four family members (GBF1–4), has been examined similarly with respect to subcellular

localization (Terzaghi *et al.* 1997). Subcellular localization was determined immunologically. Over 90% of GBF was detected in the cytoplasmic fraction, suggesting the cytoplasmic localization of a majority of GBF. However, the localization of individual members of the GBF family was examined in a transient expression assay of GUS or GFP fusion genes using protoplasts. Each member of the GBF family showed a specific pattern. Remarkably, a substantial amount of GUS-GBF2 was nuclear localized in cells cultured under blue light, while only reduced amount was found in those cultured in darkness. These results again indicate that nucleocytoplasmic localization of transcription factors plays a crucial roles in light regulation of plant gene expression and development.

Light-activated nuclear accumulation of phytochrome B

Nucleocytoplasmic transport of regulatory proteins and transcription factors appears to play an important role in the steps of the light signal transduction cascade emanating from light perception of photoreceptors. Interestingly, light perception itself seems also alter the nucleocytoplasmic transport of photoreceptors themselves (Sakamoto & Nagatani 1996; Kircher *et al.* 1999b; Yamaguchi *et al.* 1999).

Phytochromes are better characterized group of plant photoreceptors, and are encoded by five genes (A to E) in *Arabidopsis*. The nuclear localization of phytochrome B was shown by the activities of GUS fused to C-terminal fragments of phytochrome B several years ago (Sakamoto & Nagatani 1996). In addition, the results of an immunoblot analysis also indicated that a substantial amount of phytochrome B was localized in the nuclei of light-grown transgenic plants, whereas nuclear phytochrome B was significantly reduced during dark adaptation. Recently, a fusion protein consisting of full-length phytochrome B and GFP was introduced in the *phyB* mutant of *Arabidopsis* and the subcellular localization was re-examined (Yamaguchi *et al.* 1999). The PHYB-GFP was demonstrated to be biologically active by the observation of phenotypes of transgenic lines. As shown in the previous study, the fusion protein composed of phytochrome B and GFP was localized in the nucleus in the light and disappeared in darkness. The regulation of subcellular localization has not been elucidated yet but the putative NLSs have been found (Sakamoto & Nagatani 1996). Most recently, both phytochrome A and phytochrome B were demonstrated to be nuclear localized under light conditions in transgenic plants (Kircher *et al.* 1999b). This light-activated nuclear import of phytochrome has significant implications in phytochrome signal transduction. For example, a nuclear localized phytochrome would be able to directly regulate the transcription factors. Indeed, a helix-loop-helix class of transcription factors was recently identified as phytochrome-interacting factor 3 (PIF3) in *Arabidopsis* by the use of the yeast two-hybrid system using the C-terminal domain of phytochrome B as bait (Ni *et al.* 1998).

Other mechanisms regulating nucleocytoplasmic transport

Cytosolic retention forces

During the light-regulated nuclear localization of COP1, phytochrome B, and bZIP factors, cytoplasmic

and nuclear retention forces may be involved in the subcellular localization of these molecules. Recently, a cytoplasmic retention domain has been identified on the context of COP1 as CLS (Stacey *et al.* 1999). In the search for COP1 interactive proteins, CIP1 protein was isolated and shown to be localized in the cytoplasm in an association of the cytoskeleton (Matsui *et al.* 1995). CIP1 protein might be involved in the cytoplasmic retention of COP1.

The cytoplasmic localization of CPRF2 might be due to two cytoplasmic retention domains. A deletion analysis demonstrated that CPRF2 contains two cytoplasm-retention domains, named domains 1 and 2 (Kircher *et al.* 1999a). Cytoplasmic retention domain 1 is located in the N-terminal region. It exhibits a sequence similarity and structural homology to an α -helical domain of heat shock factor 2, which could mediate cytoplasmic retention under nonstress conditions. Kircher *et al.* (1999a) proposed that a putative phosphorylation site within the cytoplasmic retention domain 2 may be subjected to possible modification by light-mediated phosphorylation, which abolishes the masking of the NLS and results in the nuclear import of CPRF2. Recently, a recombinant phytochrome was found to exhibit serine/threonine kinase activity (Yeh & Lagarias 1998). It might therefore be assumed that the kinase activity of phytochromes may cause the phosphorylation-directed nuclear import of some nuclear proteins.

Phosphorylation and dephosphorylation

While the specificity for nuclear import of a substrate is determined by the NLS, it is likely that the rate of import is regulated by many different parameters. As mentioned above, phosphorylation is putatively involved in nucleocytoplasmic transport. Phosphorylation or dephosphorylation of proteins around the NLS regulates the nuclear import in mammals (Hübner *et al.* 1997). A similar example in plants is the maize Rab17 protein, which was identified from embryos as an abscisic acid-responsive protein (Jensen *et al.* 1998). GUS-Rab17 fusion protein is preferentially localized to the nuclei of onion epidermal cells. However, nuclear localization was reduced when a mutation was introduced in the consensus site for casein kinase 2 recognition, while a mutation in the putative NLS did not modify the distribution pattern. This example suggested that nuclear import could be regulated by phosphorylation as in the case of yeast and mammalian cells, although importin α/β might not be involved in the process.

Co-localization

Arabidopsis transcription factors APETALA3 (AP3) and PISTILLA (PI) are homeotic proteins that specify floral organ identities. McGonigle *et al.* (1996) examined the localization of AP3-GUS and PI-GUS fusion proteins in onion epidermal cells. Though AP3-GUS or PI-GUS alone was localized in the cytoplasm, the co-expression of both genes resulted in nuclear localization of GUS activity. The results suggest that the interaction of two or more proteins might cause a conformational change via the formation of a complex, and resulting in the unmasking of an NLS or a novel NLS contributed by more than one partner.

Prenylation

Another factor affecting nucleocytoplasmic localization may be prenylation. When CaM53, a type of *Petunia* calmodulin, is prenylated, it is associated with the plasma membrane. However, a nonprenylated mutant CaM53 was localized in the nucleus in transgenic plants (Rodriguez-Concepcion *et al.* 1999). Thus, prenylation of CaM53 appears to regulate its nucleocytoplasmic localization.

Perspectives

Nucleocytoplasmic transport is an important activity in all eukaryotes, and this mechanism has been studied extensively in yeast and mammalian cells. As shown in this review, similar efforts have recently been focused on plants. Remarkable conservation of nucleocytoplasmic transport mechanisms has been observed between the plant and animal kingdoms. However, some plant-specific characters have been demonstrated in import machinery itself, such the preferential nuclear localization of importin α and the absence of WGA inhibition. In addition, light-regulated nucleocytoplasmic localization has been demonstrated in the process of photomorphogenesis and light-regulated gene expression in plants. These findings suggest that other plant-specific characters involving the responsiveness to light or changes in other environmental conditions have yet to be discovered.

Recently, a yeast homologue of a human nuclear protein (La) was identified. The yeast homologue functions as an RNA-folding protein (Rosenblum *et al.* 1998). Even though the human and yeast proteins have a sequence homology, the nuclear localization signal regions are different. While the nuclear import of human La is mediated by an importin α/β complex,

the transport factor that mediates the import of the yeast homologue is a member of the importin β family (Kap108p). It is noteworthy that the primary structures of La and the yeast homologue are conserved, but the functions of these proteins have diverged during evolution. This example suggests that other divergences in the function of nuclear import proteins may be found between plants and other organisms. Therefore, it is critical to continue the effort to understand plant-specific nucleocytoplasmic transport and its regulation.

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