Yeast-Plant Coupled Vector System for Identification of Nuclear Proteins[1][OA]

Adi Zaltsman, Bu-Young Yi, Alexander Krichevsky, Yedidya Gafni, and Vitaly Citovsky*

Department of Biochemistry and Cell Biology, State University of New York, Stony Brook, New York 11794–5215 (A.Z., B.-Y.Y., A.K., V.C.); Department of Environmental Horticulture, University of Seoul, Tongdaemoon, Seoul 130–743, Korea (B.-Y.Y.); and Department of Genetics, Agricultural Research Organization, Volcani Center, Bet Dagan 50250, Israel (Y.G.)

Nuclear proteins are involved in many critical biological processes within plant cells and, therefore, are in the focus of studies that usually begin with demonstrating that the protein of interest indeed exhibits nuclear localization. Thus, studies of plant nuclear proteins would be facilitated by a convenient experimental system for identification of proteins that are actively imported into the cell nucleus and visualization of their nuclear accumulation in vivo. To this end, we developed a system of vectors that allows screening for cDNAs coding for nuclear proteins in a simple genetic assay in yeast cells, and verification of nuclear accumulation in planta following one-step transfer and autofluorescent tagging of the identified clones into a multiple cloning site-compatible and reading frame-compatible plant expression vector. In a recommended third experimental step, the plant expression cassette containing the identified clone can be transferred, also by a one-step cloning, into a binary multigene expression vector for transient or stable coexpression with any other proteins.

Nuclear proteins perform a multitude of diverse cellular functions, from structure (e.g. lamins and histones) to maintenance (e.g. DNA repair and replication) to chromatin dynamics and gene regulation (e.g. chromatin modifiers and transcription factors). Thus, different nuclear proteins represent the focus of numerous studies that often begin with a single observation that a protein of interest indeed resides within the cell nucleus. This first experimental step of protein characterization would be facilitated by a convenient experimental system that allows identification of nuclear proteins and demonstration of their nuclear import in vivo.

Protein nuclear import occurs through the nuclear pore complex. While small proteins (40–60 kD) often diffuse through the nuclear pore complex, nuclear entry of larger molecules and even of some small endogenous proteins, such as histones (Breeuwer and Goldfarb, 1990), is an active process mediated by specific nuclear localization signals (NLSs) contained in the transported molecule (Dingwall, 1991; Garcia-Bustos et al., 1991). That NLSs are relatively short, e.g. 7 and 16 amino acid residues for the SV40 large T antigen NLS and *Xenopus* nucleoplasmin bipartite NLS, respectively (Smith et al., 1985; Goldfarb et al., 1986; Robbins et al., 1991), and often diverse in their amino acid composition (Dingwall, 1991; Garcia-Bustos et al., 1991) makes their identification difficult when based solely on amino acid sequence analysis. Moreover, even apparent consensus NLSs, such as the one present at the C terminus of the cytoplasmic protein GUS (Varagona et al., 1991; Citovsky et al., 1992), may not represent active signals. Thus, the only practical way to identify NLSs that function within living cells is by microinjecting (Kalderon et al., 1984; Goldfarb et al., 1986; Guralnick et al., 1996) or expressing the protein of interest in eukaryotic cells (Varagona et al., 1991; Citovsky et al., 1992; Michael et al., 1995). Although these approaches represent a definitive identification of a specific nuclear protein, they are poorly suitable for large-scale screens for karyophilic proteins.

Here, we describe a system of vectors that allows (1) identification of nuclear proteins from products encoded by large numbers of cDNA clones, or even entire libraries, via a simple genetic assay in yeast cells, and (2) verification of nuclear accumulation in planta following one-step transfer and autofluorescent tagging of the identified clones into a multiple cloning site (MCS)-compatible and open reading frame (ORF)-compatible plant expression vector. In a recommended third experimental step, the plant expression cassette containing the identified clone can be transferred, also by a one-step cloning, into a binary multigene expression vector for transient or stable coexpression—following biolistic delivery or *Agrobacterium*-mediated genetic transformation, respectively—with any other

[1] This work was supported by grants from the National Institutes of Health, National Science Foundation, U.S. Department of Agriculture, and U.S.-Israel Binational Science Foundation to V.C., and from the U.S.-Israel Binational Agricultural Research and Development Fund (BARD) to V.C. and Y.G. A.Z. was supported by Vaadia-BARD Postdoctoral Fellowship from BARD.

* Corresponding author; e-mail vitaly.citovsky@stonybrook.edu.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Vitaly Citovsky (vitaly.citovsky@stonybrook.edu).

[OA] Open Access articles can be viewed online without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.107.105973

proteins, such as subcellular localization markers or potential interactors.

RESULTS

Design of the Assay

We designed a simple two-stage assay for identification of proteins containing an active NLS, with a third recommended stage for transgenic expression of the identified proteins (Fig. 1). In the first stage, a cDNA library is constructed in (or transferred to) a yeast nuclear import assay (NIA) expression vector, pNIA-C, which allows specific induction of a reporter gene if the expressed protein reaches the yeast cell nucleus. For this library construction, the cDNA can be either inserted into one of the pNIA-C vectors, or it can be cloned into the mixture of all three vectors, i.e. pNIA-Ca, pNIA-Cb, and pNIA-Cc (Fig. 2), which allows better representation of all three reading frames in the library. The resulting library is introduced into a yeast strain with an inducible selectable heterotrophic marker; the same strain also carries an inducible β-galactosidase marker that allows verification of the reporter gene induction in the selected colonies. This experimental step allows initial selection of NLS-containing protein products from a cDNA expression library. Alternatively, it is also very useful for rapid and inexpensive testing of individual proteins of interest for the presence of active NLS sequences.

For plant nuclear proteins, it is important to verify their nuclear accumulation directly in planta. Thus, the second stage of our assay is one-step transfer of the selected cDNA clones into a modular plant expression vector, pSAT6-EGFP-C (Fig. 1). Subcellular localization of GFP-tagged proteins transiently expressed from this vector is easily determined directly in living plant tissues by confocal or epifluorescence microscopy. In pNIA-C and pSAT6-C vectors, the proteins are tested as N-terminal fusions with the corresponding reporter protein. Although C-terminal fusions, i.e. fusions between the C terminus of the tested protein and the N terminus of the reporter, can also be used for determination of protein localization (see, for example, Tzfira et al. [2005] for pSAT6-N vector series), NLS sequences are often found within proteins (e.g. Dingwall and Laskey, 1991), rather than at their termini, making the location of the reporter tag less critical for detection of the NLS activity.

Finally, it is often useful to augment the initial identification of a nuclear protein by comparing its localization to that of known subcellular markers or by analyzing potential colocalization with its interactors. Furthermore, it may also be useful to express the identified clones stably, in transgenic plants, for example, for studies of its nuclear import during plant development and morphogenesis, or in response to biotic and abiotic factors. These goals are achieved in the third stage of our assay (Fig. 1), during which the expression cassette is transferred—also by a single-step cloning—to a binary multigene expression vector. This vector, pPZP-RCS2, does not contain promoter and terminator sequences and is specifically designed to accept expression cassettes based on pSAT vectors (or comparable constructs; Goderis et al., 2002; Tzfira et al., 2005), rather than for direct cloning of the tested genes; furthermore, it can also accept up to six additional expression cassettes for other proteins. The

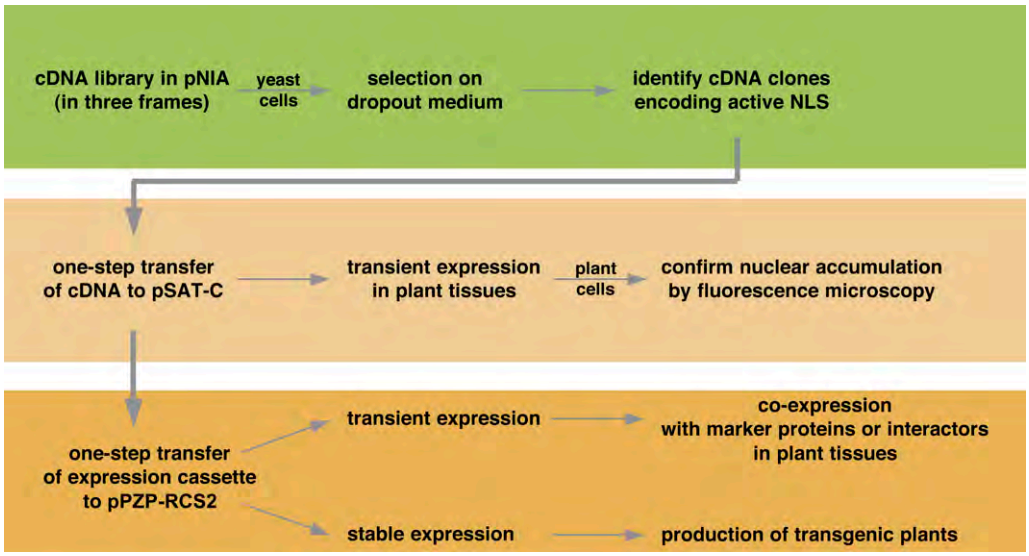

Figure 1. Suggested experimental design for identification of plant nuclear proteins. The assay contains two stages: initial identification of functional NLS by screening of libraries or individual genes/cDNA clones in yeast, followed by verification of nuclear accumulation in planta. The recommended third stage allows coexpression of the tested protein with subcellular localization markers or any other protein(s) of interest as well as production of transgenic plants stably expressing these proteins.

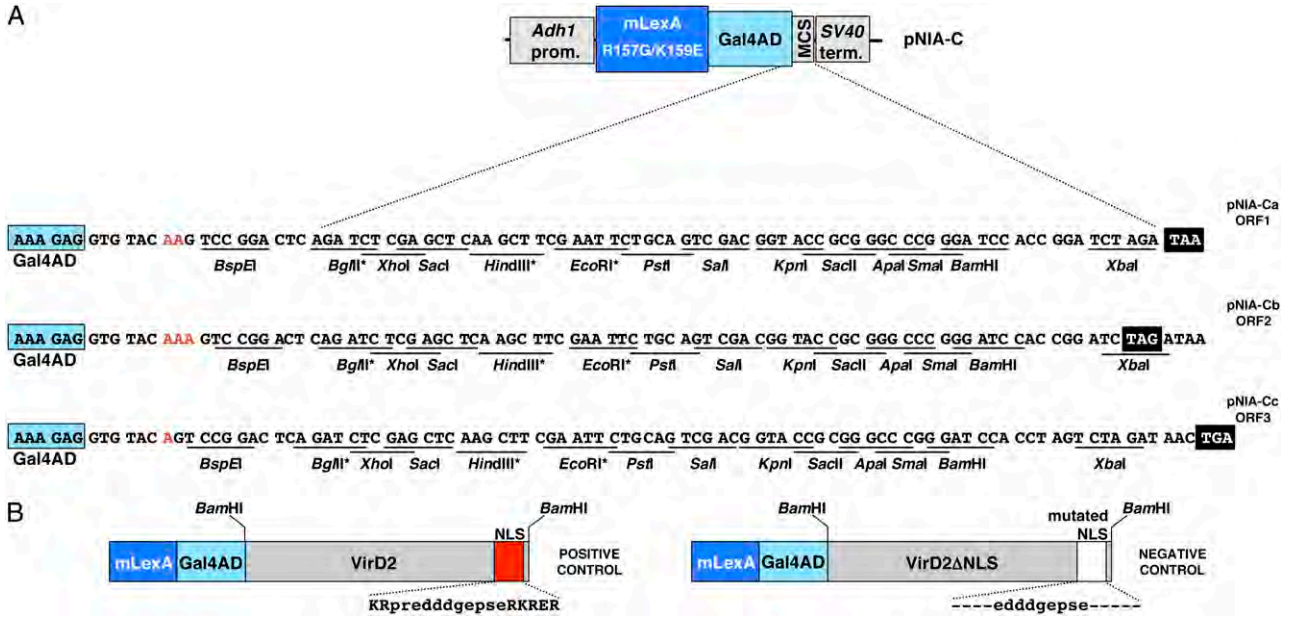

Figure 2. Structural features of the pNIA-C series of vectors. The plasmids produce in-frame fusions of the protein of interest to the C terminus of the mLexA-Gal4AD chimera. A, Three pNIA-C vectors for translational fusions in three reading frames. ORFs of Gal4AD are indicated; mLexA and Gal4AD sequences are indicated by a dark and a light blue box, respectively; two point mutations that produce mLexA are indicated; nucleotide residues inserted to produce three different reading frames are indicated in red; stop codons are indicated by black boxes; MCS restriction sites are indicated; and restriction sites that are not unique are indicated by asterisks. B, Positive and negative controls. mLexA and Gal4AD are indicated by dark and light blue boxes, respectively; VirD2 and VirD2ΔNLS are indicated by gray boxes; and NLS is indicated by a red box, and its sequence is indicated in one-letter amino acid code with the basic residues corresponding to the small and large domains of the bipartite NLS shown in capital letters. In the mutated NLS sequence (white box), the deleted residues are indicated by dashes. prom., Promoter; term., terminator.

resulting binary construct can be used for both transient and stable expression.

Detection of Protein Nuclear Import in Yeast

We produced three vectors, pNIA-Ca, pNIA-Cb, and pNIA-Cc, which constitutively produce translational fusions of the tested protein to the C terminus of a chimeric transcriptional activator mLexA-Gal4AD in all three ORFs (Fig. 2A). mLexA-Gal4AD is derived from our first-generation pNIA vector (Rhee et al., 2000), and it contains two functional components: modified LexA, in which two point mutations, R157G and K159G, abolish its—unexpected for a bacterial protein—NLS (Rhee et al., 2000), and the Gal4 DNA binding domain. In this constellation, mLexA-Gal4AD is capable of inducing a reporter gene from its LexA operator sites; however, due to the absence of a NLS within mLexA-Gal4AD, the reporter gene expression will occur only if an active NLS is present within the tested protein fused to mLexA-Gal4AD (Rhee et al., 2000).

For easy fusion of genes of interest with mLexA-Gal4AD, we constructed an MCS with 12 unique restriction endonuclease recognition sites (Fig. 2A). Importantly, this MCS was engineered to maintain the same reading frames as the pSAT6-EGFP-C vectors for simple, one-step transfer of a target sequence between the yeast and plant expression vectors. In addition, all expression cassettes carried a stop codon after the MCS (as illustrated in Fig. 2A), allowing expression of protein fragments suspected to carry an NLS.

We then constructed two pNIA fusions for use as positive and negative controls in the NIA (Fig. 2B). For the positive control, we used the *Agrobacterium* virulence D2 (VirD2) protein known to be efficiently imported into the nucleus of a wide range of eukaryotic species, plant and nonplant (e.g. Howard et al., 1992; Rossi et al., 1993; Citovsky et al., 1994; Guralnick et al., 1996; Ballas and Citovsky, 1997; Relic et al., 1998; Ziemienowicz et al., 1999, 2001). The functional NLS of VirD2 belongs to the bipartite class of basic NLSs and is located at the C terminus of the protein (Howard et al., 1992; Fig. 2B). For the negative control, we simply utilized a mutant VirD2 in which the small and large basic domains of the bipartite NLS were deleted (Fig. 2B), resulting in its complete inactivation (Howard et al., 1992).

To test the functionality of pNIA plasmids, we used them to assay nuclear import of an Arabidopsis (*Arabidopsis thaliana*) basic Leu zipper domain protein, VIP1, known to reside within the cell nucleus (Tzfira et al., 2001). Furthermore, to illustrate the use of our assay with poorly characterized plant proteins, we selected an Arabidopsis F-box protein, At1g31350,

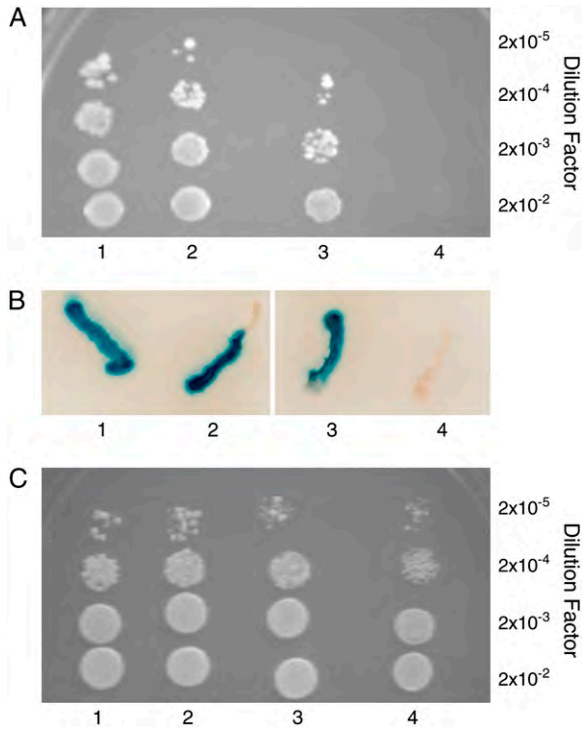

Figure 3. Assay for functional NLS in yeast. A, Cell growth in the absence of His. B, β-Galactosidase assay. C, Cell growth in the presence of His. Lane 1, mLexA-Gal4AD-VIP1; lane 2, mLexA-Gal4AD-At1g31350; lane 3, mLexA-Gal4AD-VirD2; lane 4, mLexA-Gal4AD-VirD2ΔNLS.

with an unknown subcellular localization. Figure 3A shows that mLexA-Gal4AD-VIP1 and mLexA-Gal4AD-At1g31350 promoted cell growth on a His-deficient medium (lanes 1 and 2), indicating nuclear import of the fusion protein that is required for induction of the *HIS3* reporter gene. This was then confirmed by induction of a different reporter gene, *lacZ*, encoding the β-galactosidase activity (Fig. 3B, lanes 1 and 2). As expected, the positive control, mLexA-Gal4AD-VirD2, was imported into the yeast cell nucleus, allowing His prototrophy (Fig. 3A, lane 3) and inducing the *lacZ* gene expression (Fig. 3B, lane 3), whereas the negative control, mLexA-Gal4AD-VirD2ΔNLS, failed to elicit induction of either of the reporter genes (Fig. 3, A [lane 4] and B [lane 4]), indicating the lack of nuclear import that is consistent with the absence of the active NLS in this VirD2 mutant (Howard et al., 1992). Without selection, i.e. in the presence of His, cells harboring all mLexA-Gal4AD fusions displayed vigorous and essentially similar growth (Fig. 3C), indicating that the protein hybrids did not adversely and nonspecifically affect cell physiology.

Detection of Protein Nuclear Import in Planta

Once plant proteins with active NLS sequences are identified in yeast, their nuclear accumulation in plant tissues is tested using three plant constitutive expression vectors, pSAT6-EGFP-Ca, pSAT6-EGFP-Cb, and pSAT6-EGFP-Cc, which allow fusion of the tested proteins to the C terminus of the autofluorescent tag GFP in all three ORFs (Fig. 4A). The MCSs and reading frames of the pSAT6-EGFP-C vectors are identical to those of the corresponding pNIA-C vectors; thus, the tested protein can be transferred from the yeast nuclear localization assay system to the plant nuclear localization detection system by a single cloning step. For positive and negative controls of nuclear accumulation, we inserted VirD2 and VirD2ΔNLS sequences into pSAT6-EGFP-C, producing GFP-VirD2 and GFP-VirD2ΔNLS fusions (Fig. 4B).

In addition to their stand-alone use for transient expression, the pSAT6-EGFP-C vectors can serve as modules for the pPZP-RCS2-based binary plasmids (Goderis et al., 2002; Tzfira et al., 2005) capable of multigene expression and stable expression of integrated transgenes (Fig. 4C). In this approach, expression cassettes from the pSAT6-EGFP-C-based constructs are cloned into the PI-*Psp*I rare-cutting intron-encoded nuclease recognition site of pPZP-RCS2 or its derivatives. For multigene expression, additional expression cassettes from other pSAT vectors, i.e. pSAT1-pSAT5 and pSAT7 (Tzfira et al., 2005), can be inserted into the corresponding sites of intron-encoded nucleases indicated in Figure 4C. For production of transgenic plants, the expression cassettes should be mounted onto pPZP-RCS2 derivatives, i.e. pRCS2-*hpt*, pRCS2-*nptII*, or pRCS2-*bar* (Tzfira et al., 2005), carrying different selection markers (Fig. 4C).

As proof of concept, we transferred VIP1 and At1g31350 from the pNIA-C constructs to pSAT6-EGFP-C and transiently expressed the resulting GFP-VIP1 and GFP-At1g31350 fusions in plant tissues following microbombardment. Figure 5 shows that both fluorescently tagged VIP1 and At1g31350 accumulated in the plant cell nucleus (A and B). Interestingly, another F-box protein, the *Agrobacterium* VirF, which functions in plants, also has been shown to accumulate predominantly in the plant cell nucleus (Tzfira et al., 2004). The nuclear localization pattern, determined by confocal laser scanning microscopy with optical sections through the cell nucleus, matched that of the positive control, GFP-VirD2 (Fig. 5C), whereas the negative control, GFP-VirD2ΔNLS, remained clearly cytoplasmic (Fig. 5D).

Next, we coexpressed our tested and control proteins from a multigene expression vector pPZP-RCS2 with free DsRed2 (Fig. 4C). This reporter, as well as any other small autofluorescent protein, such as cyan fluorescent protein, is very useful for studies of nuclear import because it is known to partition between the cell cytoplasm and the nucleus, conveniently visualizing and identifying both of these cellular compartments (Dietrich and Maiss, 2002; Goodin et al., 2002; Schultheiss et al., 2003; Tzfira et al., 2004; Lacroix et al., 2005; Krichevsky et al., 2007).

Figure 6 shows that GFP-tagged VIP1 (panel A), At1g31350 (panel D), and VirD2 (panel G) accumu-

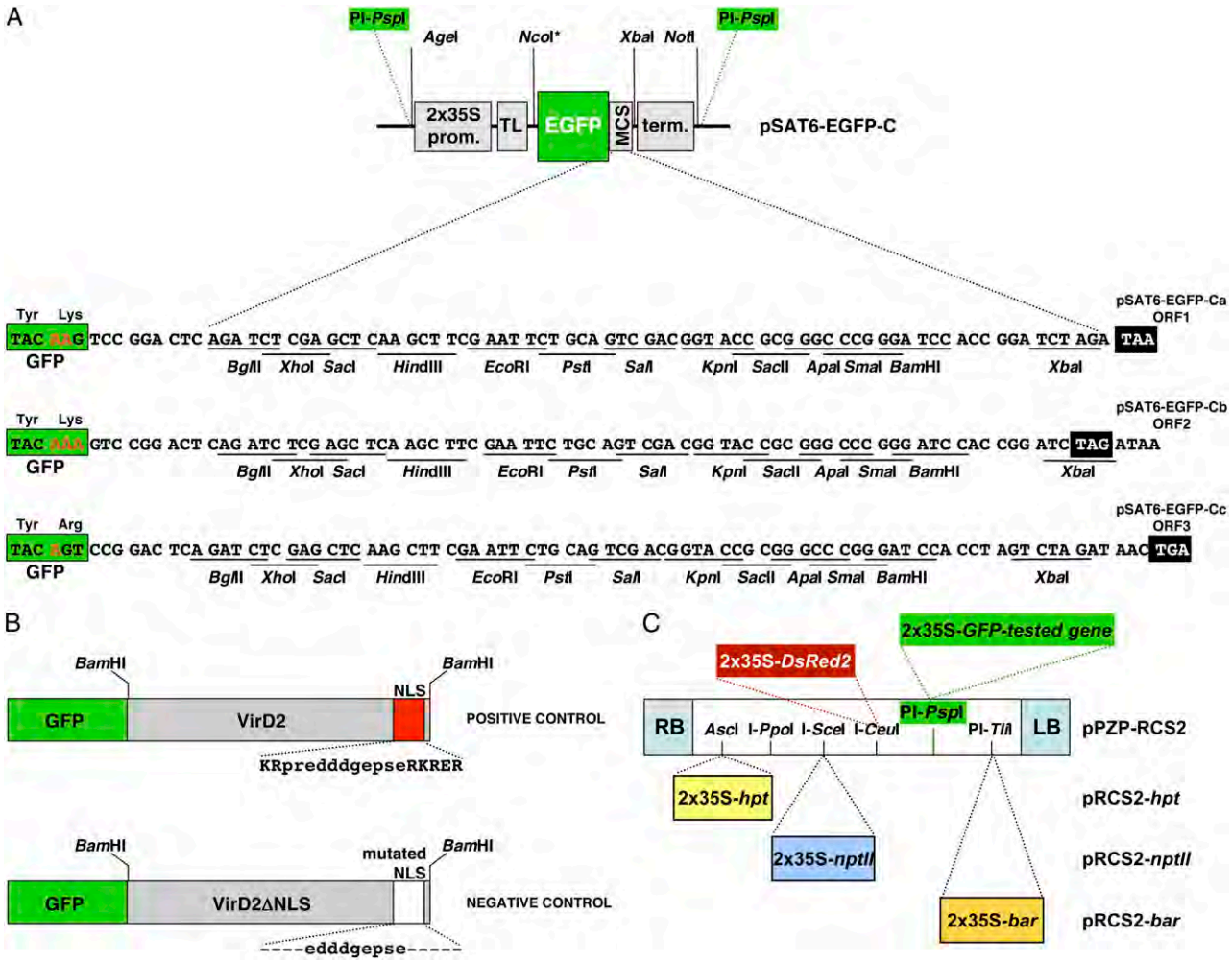

Figure 4. Structural features of the pSAT6-EGFP-C series of vectors. The plasmids produce in-frame fusions of the tested protein to the C terminus of EGFP. A, Three pSAT6-EGFP-C vectors for translational fusions in three reading frames. Expression cassette was inserted as *Age*I-*Not*I fragment into a pSAT6 plasmid (Tzfira et al., 2005), in which the expression cassette is flanked by unique recognition sites of rare-cutting nuclease PI-*Psp*I (highlighted in green). ORFs of EGFP are indicated; EGFP sequences are indicated by green boxes, and nucleotide residues inserted into these sequences to produce three different reading frames are indicated in red; stop codons are indicated by black boxes; MCS restriction sites are indicated; and a restriction site that is not unique is indicated by an asterisk. 2x35S prom., tandem cauliflower mosaic virus (CaMV) 35S promoter; TL, TEV leader; term., CaMV 35S transcriptional terminator. B, GFP-tagged positive and negative controls. GFP is indicated by a green box in each; VirD2 and VirD2ΔNLS are indicated by gray boxes; NLS is indicated by red box, and its sequence is indicated in one-letter amino acid code with the basic residues corresponding to the small and large domains of the bipartite NLS shown in capital letters. In the mutated NLS sequence (white box), the deleted residues are indicated by dashes. C, The region between the right and left T-DNA borders (RB and LB, respectively) of pPZP-pRCS2 binary plasmid is designed to accept expression cassette (indicated by green box) from pSAT6-EGFP-C vectors into its PI-*Psp*I site (highlighted in green). Unique recognition sites for seven rare-cutting nucleases are indicated; as an example, an expression cassette for DsRed2, indicated by a red box, is shown inserted into one of these sites. Modifications of pPZP-pRCS2 that carry expression cassettes for antibiotic resistance genes *hpt*, *nptII*, and *bar* driven by tandem CaMV 35S promoters (indicated by a yellow, a blue, and an orange box, respectively) are suitable for selection of transgenic plants (Tzfira et al., 2005).

lated in the plant cell nucleus. As expected, in the same cells, DsRed2 was found both in the cytoplasm and in the nucleus (Fig. 6, B, E, and H, respectively). Combined images of GFP and DsRed2 fluorescence showed overlapping signal (yellow color) within the cell nucleus, confirming the nuclear localization of these GFP-tagged proteins (Fig. 6, C, F, I, and L). In contrast, GFP-VirD2ΔNLS colocalized mainly with the cytoplasmic DsRed2 (Fig. 6, J–L).

DISCUSSION

Ideally, an assay for identification of nuclear proteins would combine an easy, high-throughput, and,

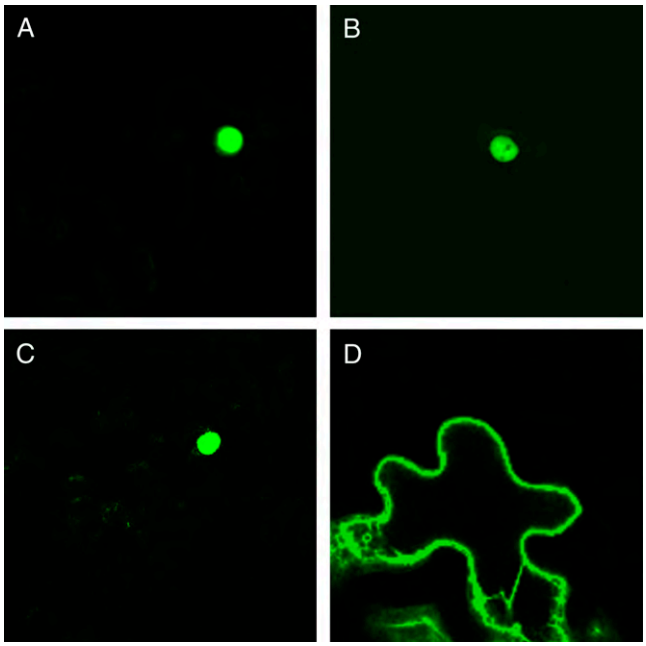

Figure 5. Assay for nuclear import in planta: expression from the pSAT6-EGFP-C vector. A, GFP-VIP1. B, GFP-At1g31350. C, GFP-VirD2. D, GFP-VirD2ΔNLS. GFP signal is in green; plastid autofluorescence was filtered out. Images are projections of several confocal sections.

preferably, selection-based approach with in vivo functionality. In plant biology applications, however, it is impractical to meet these two criteria in a single assay. Instead, we devised a two-step assay that seamlessly couples two well-established systems, pNIA vector-based genetic one-hybrid system that allows selection of nuclear proteins in yeast cells (Rhee et al., 2000) and pSAT vector-based fluorescent protein tagging system for in planta expression (Tzfira et al., 2005).

The first step of our experimental design is based on functional outcome of the nuclear import in which the imported protein activates a nuclear reporter gene. This step therefore is suited for easy selection of proteins with functional NLS sequences from large protein populations, for example, those encoded by cDNA expression libraries. Furthermore, this system is also useful for mutational analysis of nuclear proteins to delineate and characterize the NLS; in this case, large numbers of mutants can be produced directly in the pNIA-C vector and tested for the NLS activity.

The second step of our experimental design directly detects nuclear accumulation of the tested proteins in planta. This step is important for quality control of the nuclear import data and translation of the observations from the heterologous to homologous model system. In addition, instead of detection of nuclear import based on functionality of the imported protein, this assay visualizes the tagged protein within the plant cell. This allows observation of accumulation of the protein within the cell nucleus, confirming the results of the genetic assay, as well as determination of whether this protein is also found in any other cellular compartments. Furthermore, the capability of multiple pSAT vector-derived expression cassettes to be mounted onto a binary vector for coexpression (Tzfira et al., 2005) makes it easy to express the nuclear protein of interest with subcellular localization markers.

To couple both parts of the vector system together, pNIA-C and pSAT6-EGFP-C plasmids were generated with identical MCSs and reading frames, allowing one-step transfer of the tested sequences from one vector to another. For proof of concept, we put through the entire pNIA-C/pSAT6-EGFP-C protocol four unrelated proteins: Arabidopsis nuclear factor VIP1 and Arabidopsis F-box protein with an unknown localization (At1g31350), as tested proteins, and *Agrobacterium* VirD2 protein and its mutant VirD2ΔNLS as positive and negative controls, respectively. All these proteins exhibited their expected functionalities in respect to subcellular localization both in the yeast and in planta assays. Importantly, the transfer of the DNA sequences encoding the tested and control proteins from pNIA-C to pSAT6-EGFP-C and then to pPZP-RCS2 vectors was very simple and efficiently achieved in a single cloning step. Further, the identification of nuclear import was unambiguous in both assays, detected as either vigorous yeast cell growth or substantial intranuclear accumulation of the GFP signal. Identification of the latter pattern was facilitated by coexpression of a small diffusible autofluorescent marker, DsRed2, which clearly delineated the cell nucleus and cytoplasm.

In summary, we described the construction and experimental application of two sets of vectors for identification of functional NLS sequences in yeast and subsequent demonstration of their nuclear import abilities in planta. We hope that these vectors will further facilitate experimentation in plant cell biology that often requires identification of proteins with nuclear import capabilities. Furthermore, in the future, this assay system can be expanded to incorporate pSAT vectors adapted for Gateway cloning and for use with other spectral variants of GFP (Tzfira et al., 2005).

MATERIALS AND METHODS

Construction of pNIA-C and pSAT6-EGFP-C Vectors

Standard DNA amplification and cloning methods were used to construct all vectors. pEGFP-C1 (CLONTECH) was used as the source of the MCS and SV40 terminator, which were amplified by PCR using 5′AACCGGTGTACAAGTCCGGACTCAGATCTCG3′, 5′AACCGGTGTACAAAGTCCGGACTCAGATCTCG3′, and 5′AACCGGTGTACAGTCCGGACTCAGATCTCG3′ as forward primers for each of the three reading frames, and 5′TTGCCGGCGGCCGCTTACAATTTACGCGT3′ as reverse primer. Each of the amplification products was digested with *Age*I and *Nae*I and inserted into the *Xma*I-*Nae*I sites of pNIA (Rhee et al., 2000), resulting in pNIA-Ca, pNIA-Cb, and pNIA-Cc (GenBank accession nos. EF587311, EF587312, and EF587313, respectively). The MCS-terminator cassette was then excised from each of the pNIA-C vectors by digesting them with *Bsr*GI and *Not*I and inserted into the same site of pSAT6-EGFP-C1 (Tzfira et al., 2005), producing pSAT6-EGFP-Ca, pSAT6-EGFP-Cb, and pSAT6-EGFP-Cc (GenBank accession nos. EF587314,

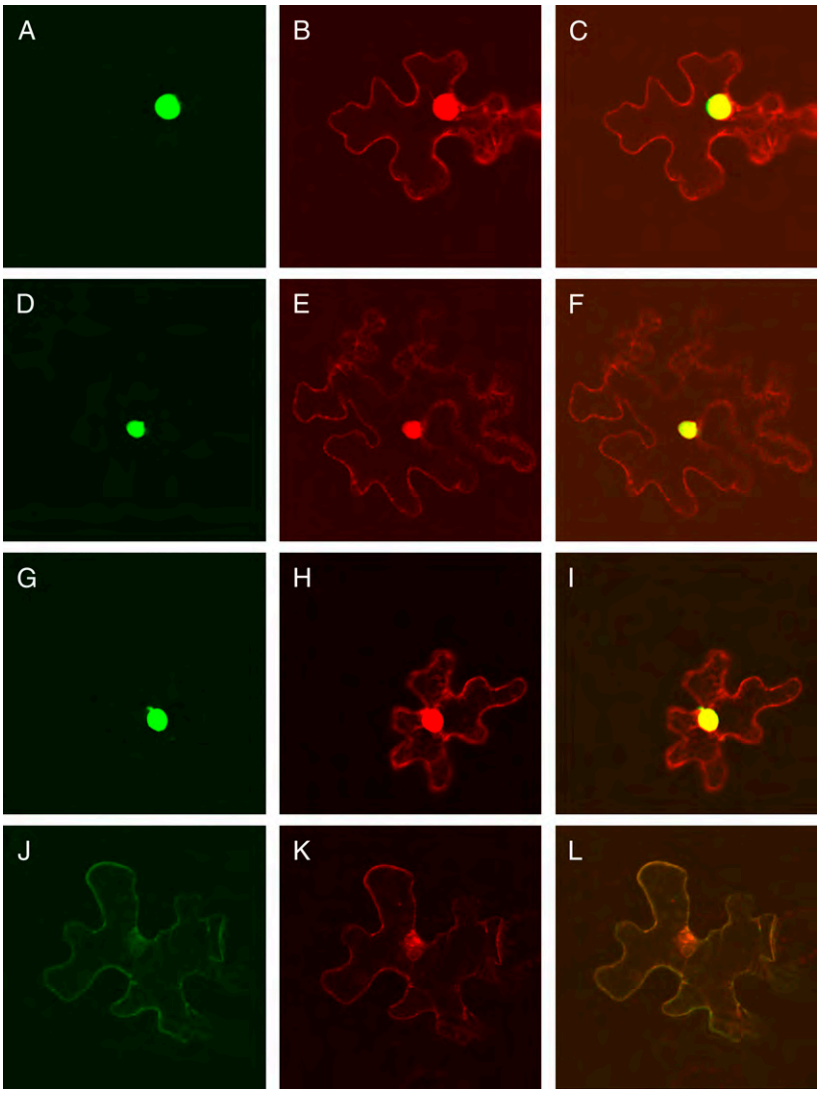

Figure 6. Assay for nuclear import in planta: multigene expression from the pPZP-pRCS2 vector. A to C, GFP-VIP1 + free DsRed2. D to F, GFP-At1g31350 + free DsRed2. G to I, GFP-VirD2 + free DsRed2. J to L, GFP-VirD2ΔNLS + free DsRed2. A, D, G, and J show GFP signal in green; B, E, H, and K show DsRed2 signal in red; and C, F, I, and L show merged GFP and DsRed2 images with colocalizing signals in yellow. Plastid autofluorescence was filtered out. Images are projections of several confocal sections.

EF587315, and EF587316, respectively). VIP1 cDNA (Tzfira et al., 2001) was cloned as a PCR-amplified fragment into the *Sal*I-*Bam*HI sites of pNIA-Cb and then transferred into the *Sal*I-*Bam*HI sites of pSAT6-EGFP-Cb. Similar cloning strategy was employed for At1g31350 and VirD2 (or VirD2ΔNLS), except that they were cloned into the *Xho*I-*Pst*I and *Bam*HI sites of the expression vectors, respectively. Note that while we demonstrate nuclear import using the pNIA-Cb and pSAT6-EGFP-Cb constructs, the functionality of vectors expressing in all three reading frames has been tested (data not shown).

For coexpression of a tested GFP-tagged protein with free DsRed2 from a single plasmid, we first produced pSAT5-DsRed2 by cloning the DsRed2 expression cassette as a *Age*I-*Not*I fragment from pSAT6-DsRed2-C1 (Tzfira et al., 2005) into a pSAT5-based vector (Tzfira et al., 2005), producing pSAT5-DsRed2-C1. The DsRed2 expression cassette was then removed by digestion with I-*Ceu*I and inserted into the I-*Ceu*I site of pPZP-RCS2, producing pRCS2-DsRed2 (Tzfira et al., 2005). Finally, the EGFP-tested protein expression cassette was removed from the pSAT6-EGFP-Cb vector by digestion with PI-*Psp*I and inserted into the PI-*Psp*I site of pRCS2-DsRed2 (Tzfira et al., 2005). pRCS2-*hpt*, pRCS2-*nptII*, and pRCS2-*bar* have been described before (Tzfira et al., 2005). All constructs were verified by DNA sequencing.

Nuclear Import in Yeast Cells

For yeast nuclear import, the *Saccharomyces cerevisiae* strain L40 cells (Hollenberg et al., 1995) were transformed with the indicated pNIA-C constructs and grown for 2 d at 30°C on a Leu- and Trp-deficient medium in the presence or absence of His; His prototrophy indicated functional nuclear import (Rhee et al., 2000). Alternatively, cells were grown on a Leu- and Trp-deficient medium, and the β-galactosidase activity was assayed on nitrocellulose filters (Hollenberg et al., 1995).

Nuclear Import in Plant Tissues

DNA (50 μg) was adsorbed onto 10 mg of 1-μm gold particles (Bio-Rad) and bombarded at 150 to 200 psi into the leaf epidermis of greenhouse-grown *Nicotiana benthamiana* plants using a Helios gene gun (PDS-1000/He; Bio-Rad), followed by incubation for 24 h at 25°C. For detection of fluorescently tagged proteins, the bombarded tissues were directly viewed under a Zeiss LSM 5 Pascal confocal laser scanning microscope. Experiments were repeated at least four times, with 10 to 20 expressing cells examined in each experiment.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers EF587311 (pNIA-Ca), EF587312 (pNIA-Cb), EF587313 (pNIA-Cc), EF587314 (pSAT6-EGFP-Ca), EF587315 (pSAT6-EGFP-Cb), and EF587316 (pSAT6-EGFP-Cc).

ACKNOWLEDGMENT

We thank Dr. Tzvi Tzfira for stimulating discussions.

Received July 26, 2007; accepted August 13, 2007; published August 17, 2007.

LITERATURE CITED

Ballas N, Citovsky V (1997) Nuclear localization signal binding protein from *Arabidopsis* mediates nuclear import of *Agrobacterium* VirD2 protein. Proc Natl Acad Sci USA **94:** 10723–10728

Breeuwer M, Goldfarb DG (1990) Facilitated nuclear transport of histone H1 and other small nucleophilic proteins. Cell **60:** 999–1008

Citovsky V, Warnick D, Zambryski PC (1994) Nuclear import of *Agrobacterium* VirD2 and VirE2 proteins in maize and tobacco. Proc Natl Acad Sci USA **91:** 3210–3214

Citovsky V, Zupan J, Warnick D, Zambryski PC (1992) Nuclear localization of *Agrobacterium* VirE2 protein in plant cells. Science **256:** 1802–1805

Dietrich C, Maiss E (2002) Red fluorescent protein DsRed from *Discosoma* sp. as a reporter protein in higher plants. Biotechniques **32:** 286–293

Dingwall C (1991) Transport across the nuclear envelope: enigmas and explanations. Bioessays **13:** 213–218

Dingwall C, Laskey RA (1991) Nuclear targeting sequences—a consensus? Trends Biochem Sci **16:** 478–481

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

Goderis IJ, De Bolle MF, Francois IE, Wouters PF, Broekaert WF, Cammue BP (2002) A set of modular plant transformation vectors allowing flexible insertion of up to six expression units. Plant Mol Biol **50:** 17–27

Goldfarb DS, Gariepy J, Schoolnik G, Kornberg RD (1986) Synthetic peptides as nuclear localization signals. Nature **322:** 641–644

Goodin MM, Dietzgen RG, Schichnes D, Ruzin S, Jackson AO (2002) pGD vectors: versatile tools for the expression of green and red fluorescent protein fusions in agroinfiltrated plant leaves. Plant J **31:** 375–383

Guralnick B, Thomsen G, Citovsky V (1996) Transport of DNA into the nuclei of *Xenopus* oocytes by a modified VirE2 protein of *Agrobacterium*. Plant Cell **8:** 363–373

Hollenberg SM, Sternglanz R, Cheng PF, Weintraub H (1995) Identification of a new family of tissue-specific basic helix-loop-helix proteins with a two-hybrid system. Mol Cell Biol **15:** 3813–3822

Howard E, Zupan J, Citovsky V, Zambryski PC (1992) The VirD2 protein of *A. tumefaciens* contains a C-terminal bipartite nuclear localization signal: implications for nuclear uptake of DNA in plant cells. Cell **68:** 109–118

Kalderon D, Roberts BL, Richardson WD, Smith AE (1984) A short amino acid sequence able to specify nuclear location. Cell **39:** 499–509

Krichevsky A, Gutgarts H, Kozlovsky SV, Tzfira T, Sutton A, Sternglanz R, Mandel G, Citovsky V (2007) AtCZS, an *Arabidopsis* C2H2 zinc finger-SET histone methyltransferase is a plant-specific chromatin modifier. Dev Biol **303:** 259–269

Lacroix B, Vaidya M, Tzfira T, Citovsky V (2005) The VirE3 protein of *Agrobacterium* mimics a host cell function required for plant genetic transformation. EMBO J **24:** 428–437

Michael WM, Choi M, Dreyfuss G (1995) A nuclear export signal in hnRNP A1: a signal-mediated temperature-dependent nuclear protein export pathway. Cell **83:** 415–422

Relic B, Andjelkovic M, Rossi L, Nagamine Y, Hohn B (1998) Interaction of the DNA modifying proteins VirD1 and VirD2 of *Agrobacterium tumefaciens*: analysis by subcellular localization in mammalian cells. Proc Natl Acad Sci USA **95:** 9105–9110

Rhee Y, Gurel F, Gafni Y, Dingwall C, Citovsky V (2000) A genetic system for detection of protein nuclear import and export. Nat Biotechnol **18:** 433–437

Robbins J, Dilworth SM, Laskey RA, Dingwall C (1991) Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. Cell **64:** 615–623

Rossi L, Hohn B, Tinland B (1993) The VirD2 protein of *Agrobacterium tumefaciens* carries nuclear localization signals important for transfer of T-DNA to plant. Mol Gen Genet **239:** 345–353

Schultheiss H, Dechert C, Kogel KH, Hückelhoven R (2003) Functional analysis of barley RAC/ROP G-protein family members in susceptibility to the powdery mildew fungus. Plant J **36:** 589–601

Smith AE, Kalderon D, Roberts BL, Colledge WD, Edge M, Gillett P, Markham AF, Paucha E, Richardson WD (1985) The nuclear location signal. Proc R Soc Lond B Biol Sci **226:** 43–58

Tzfira T, Tian GW, Lacroix B, Vyas S, Li J, Leitner-Dagan Y, Krichevsky A, Taylor T, Vainstein A, Citovsky V (2005) pSAT vectors: a modular series of plasmids for fluorescent protein tagging and expression of multiple genes in plants. Plant Mol Biol **57:** 503–516

Tzfira T, Vaidya M, Citovsky V (2001) VIP1, an *Arabidopsis* protein that interacts with *Agrobacterium* VirE2, is involved in VirE2 nuclear import and *Agrobacterium* infectivity. EMBO J **20:** 3596–3607

Tzfira T, Vaidya M, Citovsky V (2004) Involvement of targeted proteolysis in plant genetic transformation by *Agrobacterium*. Nature **431:** 87–92

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

Ziemienowicz A, Görlich D, Lanka E, Hohn B, Rossi L (1999) Import of DNA into mammalian nuclei by proteins originating from a plant pathogenic bacterium. Proc Natl Acad Sci USA **96:** 3729–3733

Ziemienowicz A, Merkle T, Schoumacher F, Hohn B, Rossi L (2001) Import of *Agrobacterium* T-DNA into plant nuclei: two distinct functions of VirD2 and VirE2 proteins. Plant Cell **13:** 369–384