The Plant Journal (2007) **50**, 149–158 doi: 10.1111/j.1365-313X.2007.03029.x

TECHNICAL ADVANCE

A functional genetic assay for nuclear trafficking in plants

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Received 26 September 2006; revised 17 November 2006; accepted 21 November 2006.

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Summary

The receptor importin-α mediates the nuclear import of functionally diverse cargo proteins that contain arginine/lysine-rich nuclear localization signals (NLSs). Functional homologs of importin-α have been characterized in a wide range of species including yeast, human and plants. However, the differential cargo selectivity of plant importin-α homologs has not been established. To advance nuclear import studies conducted in plant cells, we have developed a method that allows importin-α-dependent nuclear import to be assayed in *Nicotiana benthamiana*. We employed virus-induced gene silencing (VIGS) to knock down the expression of two importin-α homologs, *NbImpα1* and *NbImpα2*, which we identified from *N. benthamiana*. Agro-infiltration was then used to transiently express the NLS-containing proteins *Arabidopsis thaliana* fibrillarin 1 (AtFib1) and the Nuk6, Nuk7 and Nuk12 candidate effector proteins of the oomycete plant pathogen *Phytophthora infestans*. In this manner, we demonstrate importin-α-dependent nuclear import of Nuk6 and Nuk7. In contrast, the nuclear import of Nuk12 and AtFib1 was unaffected in cells of *NbImpα*-silenced plants. These data suggest that *P. infestans* Nuk6 and Nuk7 proteins are dependent on one or more α-importins for nuclear import. Our VIGS-based assay represents a powerful new technique to study mechanisms underlying the transport of proteins from cytoplasm to nucleus in plants.

Keywords: importin-α, plant pathogen, nuclear import, VIGS, agro-infiltration.

Introduction

Transport of macromolecules between the nucleus and cytoplasm is an energy- and signal-dependent process (Gorlich and Mattaj, 1996; Newmeyer, 1993; Newmeyer and Forbes, 1988; Nigg, 1997; Richardson *et al.*, 1988). Proteins that target cell nuclei frequently carry signals recognized by cell components of the nuclear import pathway. The first-described and best-characterized type of signal is the family of related short basic sequences collectively referred to as nuclear localization signals (NLSs) (Dingwall and Laskey, 1991). Most NLSs may be classified into one of two categories. One category comprises the monopartite NLSs composed of a continuous stretch of basic amino acids as identified in the simian virus 40 (SV40) large T-antigen protein (Kalderon *et al.*, 1984a,b). The second category comprises the bipartite NLSs composed of two sets of two to three positively charged amino acids separated by a 10-amino acid linker region, such as the NLS of nucleoplasmin (Dingwall *et al.*, 1982; Robbins *et al.*, 1991). For nuclear import to occur, importin-α binds to the NLS of proteins (Adam *et al.*, 1995; Gorlich *et al.*, 1995) and importin-β mediates docking of the proteins to the cytoplasmic side of the nuclear pore complex (NPC).

Subsequently, proteins are transported along with importin-α into the nucleoplasm with the aid of other import factors, such as GTPase Ran and NTF2 (Gorlich and Mattaj, 1996; Nigg, 1997).

Many plant pathogens deliver effector molecules inside host cells to manipulate cellular and molecular processes and establish pathogenicity. There are several examples of plant pathogen proteins that function inside host cells where they recruit the host nuclear import machinery for nuclear transport (Ballas and Citovsky, 1997; Tzfira and Citovsky, 2002). Examples include transport of viral and bacterial proteins into the host nucleus. Plant viral proteins such as cucumber mosaic virus (CMV) 2b (Lucy *et al.*, 2000), tobacco etch virus (TEV) NIb (Li *et al.*, 1997), beet necrotic yellow vein virus (BNYVV) p25 (Vetter *et al.*, 2004) and various proteins of the rhabdoviruses Sonchus yellow net virus (SYNV) and maize fine streak virus (MFSV; Goodin *et al.*, 2002; Tsai *et al.*, 2005) have been shown to be transported to the nuclei of infected cells. Similarly, the bacterial protein AvrBs3 of *Xanthomonas vesicatoria* contains two functional NLSs and has been shown to interact with importin-α (Van den Ackerveken and Bonas, 1997). These findings suggest that pathogen effectors utilize the host cell nuclear transport machinery, including α-importins, to target plant cell nuclei. On the other hand, protein import into the nucleus plays an important role in plant defense against pathogens (Kinkema *et al.*, 2000; Mou *et al.*, 2003; Palma *et al.*, 2005; Zhang and Li, 2005). Recently, the Arabidopsis importin-α MOS6 (AtImpα3) was shown to contribute to basic defense against the oomycete *Hyaloperonospora parasitica* (Palma *et al.*, 2005).

As far as we know, studies of importin-α interactions with substrates in plants have been performed *in vitro* or in yeast, and studies to unravel the functions of plant α -importins *in planta* are lacking. In animal systems, functional analyses of importin genes have only become possible recently with the advent of RNAi knockdown methods (Quensel *et al.*, 2004). Virus-induced gene silencing (VIGS), based on a tobacco rattle virus (TRV)-based vector (Baulcombe, 1999), has been used successfully for generating transient loss-of-function assays to understand the functions of specific genes, as a more rapid alternative to knockout approaches, which are based on stable transformations (Baulcombe, 1999; Lu *et al.*, 2003). In VIGS, plants are infected with a recombinant TRV carrying a partial sequence of a host gene. When the virus spreads systemically and expresses this partial sequence, the endogenous gene transcripts are degraded by post-transcriptional gene silencing (PTGS; Baulcombe, 1999; Ratcliff *et al.*, 2001). One major advantage of VIGS is the ability to silence essential genes that cannot be assayed using stable knockout mutants. In this study, we took advantage of the VIGS technology to develop a straightforward functional genetic assay for α -importins. This is important because, so far, techniques for studying the transport of NLS-containing proteins in plants have been limited to interaction assays in yeast two-hybrid and co-immunoprecipitation experiments.

Results

Two importin-α homologs identified from Nicotiana benthamiana

The *N. benthamiana* EST database at the Institute for Genome Research (TIGR) was searched for sequences with similarities to tomato α-importins using BLASTX. This resulted in the identification of two cDNA clones that were further sequenced by primer walking. The fully sequenced cDNAs showed high similarities to members of the importin-α gene family, and were subsequently named *NbImpα1* and *NbImpα2*. The full-length open reading frame (ORF) of *NbImpα1* was 1599 bp, corresponding to a predicted translation product of 533 amino acids, and the ORF of *NbImpα2* was 1590 bp, corresponding to a 530 amino acid protein. NbIMPα1 and NbIMPα2 shared 81% identity and 90% similarity in their amino acid sequences. The identity values for NbIMPα1 compared with other α-importins ranged from 64% to 92%, and the similarity values ranged from 75% to 95%. For NbIMPα2, the identity values ranged from 62% to 95%, and the similarity values ranged from 75% to 95%. Both NbIMPα1 and NbIMPα2 contained highly conserved domains including an N-terminal importin-binding domain (IBB domain) (Gorlich *et al.*, 1996), a C-terminal acidic region, eight tandem armadillo (arm) repeats of 42 amino acids (Goldfarb *et al.*, 2004), and two variable regions flanking the arm repeats region.

Sequence alignment and phylogenetic analyses of NbIMPα1 and NbIMPα2 with plant, animal and fungal importin-α revealed that the two *N. benthamiana* α-importins fall into group 1α, based on the classification of Goldfarb *et al.* (2004). Except for rice (*Oryza sativa*) Impα2, all other known plant α-importins fall into this family subgroup (Palma *et al.*, 2005). To further investigate the phylogenetic relationships between the *N. benthamiana* α-importins and other plant importins, we constructed a neighbor-joining tree of 18 protein sequences, including the eight Arabidopsis α-importins AtImpα1–8 (Figure 1). NbIMPα1 and NbIMPα2 fell into distinct clusters in the tree, and were most similar to importins from the solanaceous plants tomato (*Solanum lycopersicum*) and pepper (*Capsicum annuum*). NbIMPα1 was most similar to tomato IMPα1, and NbIMPα2 clustered with a tomato sequence (TC155302) and pepper IMPα2. We could not unambiguously identify the orthologs of the NbIMPα in Arabidopsis because the bootstrap values were not significant (Figure 1).

In order to determine the number of copies of the cloned *NbImpα1* and *NbImpα2* sequences that occur in the *N. benthamiana* genome, Southern blot hybridizations were performed. *Bst*NI-, *Dra*I-, *Eco*RI-, *Eco*RV-, *Sca*I-, *Hin*dIII- and

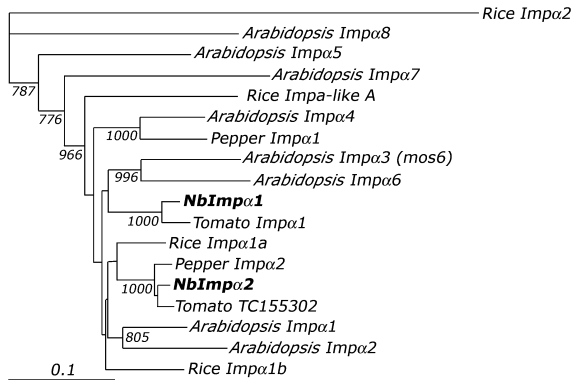

Figure 1. Phylogenetic tree of plant importin-α proteins.
The phylogenetic tree was produced using the neighbor-joining method, based on the conserved overlapping portion of the amino acid sequences. Alternative topologies were viewed with the program TreeView PPC 1.6.6 (page, 1996). Bootstrap values based on 1000 replications with values higher than 700 are indicated on the branches. The scale bars indicated 10% weighted sequence divergence. GenBank accession numbers: Arabidopsis Impα1, NP_850524; Arabidopsis Impα2, NP_567485; Arabidopsis Impα3 (mos6), NP_192124; Arabidopsis Impα4, NP_849623; Arabidopsis Impα5, NP_199742; Arabidopsis Impα6, NP_973743; Arabidopsis Impα7, NP_187223; Arabidopsis Impα8, NP_200013; rice Impα2, NP_909338; rice Impα-like A, NP_908847; rice Impα1a, NP_912763; rice Impα1b, NP_910164; pepper Impα1, AAK38726; pepper Impα2, AAK38727; tomato Impα1, AAC23722; NbImpα1, EF137253; NbImpα2, EF137254. The tomato TC155302 sequence was obtained from the TIGR tomato database (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=tomato).

*Xba*I-digested genomic DNA of *N. benthamiana* was hybridized with probes corresponding to the *NbImpα1* and *NbImpα2* cDNA fragments cloned in pTV00. Two *Dra*I, *Eco*RI, *Eco*RV and *Hin*dIII fragments were detected for both cDNAs, whereas no *Bst*NI fragments and three *Xba*I fragments for *NbImpα1* and two *Xba*I fragments for *NbImpα2* were detected (Figure 2). The hybridizing fragment sizes for *NbImpα1* and *NbImpα2* were clearly different in length, indicating that the probes did not cross-hybridize. This is consistent with the observation that *NbImpα1* and *NbImpα2* are different genes (Figure 1). As there are no *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII and *Xba*I restriction sites in the cloned *NbImpα1* and *NbImpα2* fragments , this result indicates that there are at least two copies of the *NbImpα1* gene and two copies of the *NbImpα2* gene in the *N. benthamiana* genome. These two copies could correspond to two *NbImpα1* and *NbImpα2* alleles polymorphic for flanking restriction sites, or could correspond to conserved repeated sequences of *NbImpα1* and *NbImpα2*.

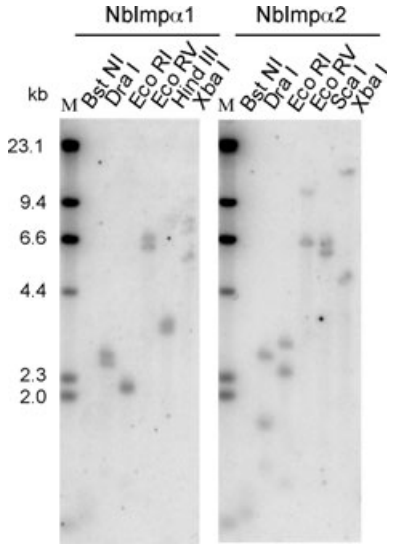

Figure 2. Southern blot analysis of *Nicotiana benthamiana* genomic DNA.
Genomic DNA (10 μg per lane) was digested with various restriction enzymes as indicated above each lane, and membranes were hybridized with probes corresponding to the NbImpα1 or NbImpα2 cDNA fragments cloned into the pTV00 silencing vectors. The fragment sizes of the marker (M) for both blots are indicated in kb on the left.

NbIMPα1 and NbIMPα2 target plant cell nuclei

To investigate the localization of NbIMPα proteins in *N. benthamiana*, the corresponding gene sequences were cloned into the pGD binary vector that allows fusion of NbIMPα proteins at the N-terminus to the red fluorescent protein, DsRed2 (Goodin *et al.*, 2002). *Agrobacterium tumefaciens* strains carrying pGDR:NbIMPα1 and pGDR:NbIMPα2 were infiltrated into young and fully expanded leaves of *N. benthamiana*. As a negative control, *A. tumefaciens* carrying the empty vector (pGDR) was used. Two days after infiltration, leaf sections of infiltrated areas were examined by confocal microscopy to determine the localization of the fusion proteins. Images revealed that pGDR:NbIMPα1 and pGDR:NbIMPα2 accumulated in the nuclei of plant epidermal cells (Figure 3). In contrast, DsRed2 alone (derived from empty pGDR) was evenly distributed between the nuclei and the cytoplasm (Figure 3). Thus, NbIMPα1 and NbIMPα2 targeted plant cell nuclei. However, pGDR:NbIMPα1 and pGDR:NbIMPα2 have slightly different distributions in the nucleus (Figure 3).

Silencing of NbImpα1 *and* NbImpα2 *genes using the TRV VIGS vector*

The two *NbImpα* genes in *N. benthamiana* were silenced with the TRV-based VIGS system. Primers were designed to amplify a 0.5 kb 5′ fragment of *NbImpα1* and *NbImpα2* cDNAs. The PCR products were cloned into the pTV00 vector

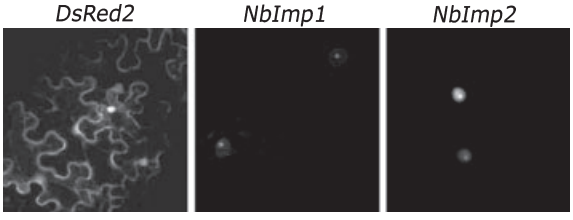

Figure 3. Nuclear accumulation of NbIMPα1 and NbIMPα2 fused to DsRed2 in *Nicotiana benthamiana leaves.*

containing RNA2 of the TRV genome (Ratcliff *et al.*, 2001). Young *N. benthamiana* plants (five-leaf stage) were infiltrated with mixtures of *Agrobacterium* strains carrying pBINTRA6 (RNA1 of the TRV genome) combined with one of the following plasmid constructs: pTV00 (RNA2 of the TRV genome), pTV00:PDS (phytoene desaturase gene), pTV00:*NbImpα1* or pTV00:*NbImpα2*. Construct pTV00:PDS was used as a positive control to monitor and visualize the onset of gene silencing, because silencing of PDS induces photobleaching of green leaves (Kumagai *et al.*, 1995; Ratcliff *et al.*, 2001; Ruiz *et al.*, 1998). We used the empty pTV00 vector as a negative control to monitor phenotypes resulting from TRV infection. At approximately 3 weeks after infiltration, young leaves of PDS-silenced plants showed complete bleaching. At this time, silencing of the *NbImpα* genes was verified by collection of leaf samples from all infiltrated plants and subsequent total RNA extraction and RT-PCR. Two plants for each construct were assayed. To ensure that only the endogenous gene was being amplified, RT-PCR primers were designed to anneal externally to the DNA fragment cloned in pTV00. The RT-PCR results showed significantly lower levels of amplification for *NbImpα* transcripts in the pTV00:*NbImpα1*- and pTV00:*NbImpα2*-treated leaves than in the control samples, whereas the amplification levels of the tomato tubulin transcripts were similar in all samples (Figure 4). Therefore, these results suggest that the *NbImpα* genes were silenced in *N. benthamiana* leaves.

Identification of Phytophthora infestans *NLS-containing proteins*

As part of an independent project, we mined the unigene set of the oomycete plant pathogen *P. infestans* for proteins that carry a signal peptide and NLS and may localize inside host cell nuclei. A total of 18 000 unigenes (EST consensus sequences) of *P. infestans* were used for the identification of proteins containing signal peptides as described earlier (Torto *et al.*, 2003) and of NLS using PredictNLS software (Cokol *et al.*, 2000). The ESTs were translated into three forward reading frames, and the corresponding translations were compiled in FASTA format (Pearson and Lipman, 1988). The PredictNLS program was applied to identify proteins containing NLSs among these sequences. Three candidate proteins, named Nuk6, Nuk7 and Nuk12, that contain monopartite NLSs, were identified (Figure 5). DNA sequencing of the corresponding full-length cDNAs revealed intact ORFs, confirming that the three proteins carry both a signal peptide and monopartite NLSs (Figure 5). To generate YFP fusions of these proteins at the N-terminus, corresponding genes were cloned into pGD binary vectors (Goodin *et al.*, 2002). *A. tumefaciens* strains carrying pGDY:Nuk6, pGDY:Nuk7, pGDY:Nuk12 and AtFib1:GFP constructs were infiltrated into *N. benthamiana* plants. All three fusion proteins were found to accumulate in plant nuclei (Figure 6, column 'healthy'), similarly to the AtFib1:GFP construct (Tsai *et al.*, 2005) (Figure 6). We also constructed an YFP fusion to the SV40 NLS motif (pGDY:SV40NLS) that is known to target proteins to nuclei in an importin-α-dependent manner (Harreman *et al.*, 2004), and, as expected, this protein also targets the nuclei of *N. benthamiana* cells (Figure 6).

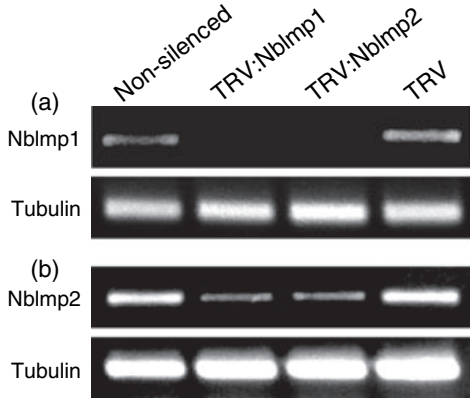

Figure 4. RT-PCR analysis confirming the silencing of *Nicotiana benthamiana NbImpα* genes.
(a) Products from RT-PCR with 24 cycles; (b) products from Rt-PCR with 30 cycles. Products derived from non-silenced plants, *NbImpα1*-silenced plants, *NbImpα2*-silenced plants and non-silenced plants infected with TRV alone are shown. Gene-specific primers that amplify both *NbImpα1* and *NbImpα2* were used in the PCR. As a control for RNA amount, the mRNA levels of tubulin were examined.

AtFib1
MRPPVTGGRGGGGF**RGGRDGGGR**GFG**GGRSFGGG**RSGDRGRSGPRGRGRGAPRGRGGPP
RGGMKGGSKVIVEPHRHAGVFIAKGKEDALVTKNLVPGEAVYNEKRISVQNEDGTKVEY
RVWNPFRSKLAAAILGGVDNIWIKPGAKVLYLGAASGTTVSHVSDLVGPEGCVYAVEFS
HRSGRDLVNMAKKRTNVIPIIEDARHPAKYRMLVGMVDVIFSDVAQPDQARILALNASF
FLKTGGHFVISIKANCIDSTVAAEAVFQSEVKKLQQEQFKPAEQVTLEPFERDHACVVG
GYRMPKKQKTPAS

Nuk12
MRRKVRRPVVLFWSSTSLISWMNAKERRGEIISRRKISTSCSFRPKRLRTKS**TRRRKS**C
VRSSETPRVTTSTMK

Nuk6
MKLLQVITFVSAAVSLSTSAYGHSSNALAGETAASVVESSADPTADQRSRRTSIRADIN
TYPYGAKKSDVAVDDGDAPRRFRRRLTLKEEEEIEEDLEKVEKKKKAAKKRVDEATEEI
EEGLENSNDDEVDDGLKEKREAEKDVEDAEVEEKVDEDLEEIQVEEEEVEKMEEDEEKAS
S**KKSKKAKTDDEDTDDDTEDDDDDQEAQTTKKSSKKTK**SDEETDEDDNGDGDDEEAPAK
KSKKSSTKSKTDEDEDEEDEAQRRRLTESDEKELEEDMKQVKKKEKAAKKKMEEATEEI
EEGLENSNDDEVDEGLEEKLEAEKEEEEAEVEEKMDEDLEKIQKKEAAEKQKSADDEEE
DEEEDEDEDETPAKKKSSKSATKSKSVDDDEERDDDKTFKKSSKKSKADEEAEEEAEEE
LEEELEEIEEEIEEEEEAEKAKKAKKSTHKSKTDEEEEEDDKTRRRHLRGLSTINEQEV
ETDMDKAKKAEKEAKAKEAEADEDIQEGLENSNDDEVDEAMATKLAAEKKKK

Nuk7
MFPRVRLALLLLSALTACVLADYGPRDSVTILTDKNFEKEVLQSPDYWLVEFYAPWCGH
CKQLEPQYKAAAKKLKKHARLGAVDATVHQQLAHKYQIKGYPTIKEFGAKKKRPQDYRG
GRTTREIVQYVKNSPEAKKLGASGGNVATLEYDKVHAFLSKDLPSAIFFGTKKKGKKSS
KVPAWLGNVAKSFMEGTT**KKRKK**QPTVQLAFVPASDDKVASHFGLSEDQLPTVIYVYPA
SQKYVVSDVSKLNEAAAKKFIDDALANTETAEKDESLPNVPLFPSPEVAKKKPVVALKE
LDAATARECAAKRGKMCVVVAREDTELIRSLAKKYRRDPFTFLSSKPDAQAFHVLTEFV
GEISAEVIVVKPGRKVKYSALSGANDESDISEFLDKLIGGSSPFSVPSGGLEAFEAAMS
ASSDDAVKHEEL

Figure 5. Sequences of *Arabidopsis thaliana* Fib1 (with the bipartite nucleolar localization signal in bold) and of the *Phytophthora infestans* proteins Nuk6, Nuk7 and Nuk12 (with the monopartite NLSs in bold).

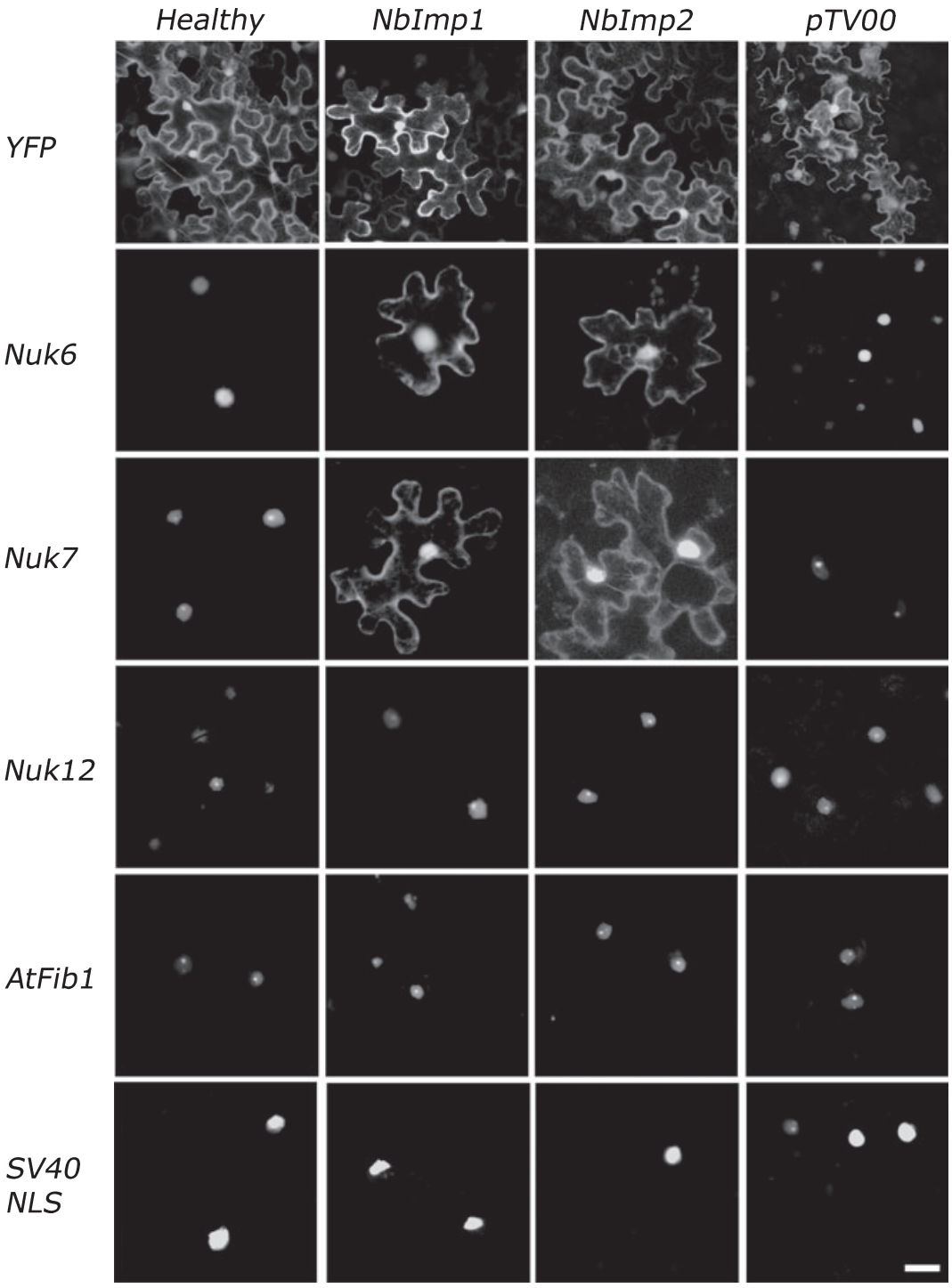

Figure 6. Confocal laser scanning microscopy images showing the nuclear import of NLS-containing proteins in *Nicotiana benthamiana*.
Nuclear import of Nuk6 and Nuk7 is importin-α-dependent, resulting in a shift from nuclear localization to an even distribution between the nucleus and the cytoplasm. In contrast, the nuclear import of AtFib1, Nuk12 and SV40NLS is independent of NbIMPα1 and NbIMPα2, resulting in their presence in nuclei of silenced plants. Bar = 20 μm. All micrographs were taken at identical magnifications.

Silencing of NbImpα *reduces nuclear import of some NLS-containing proteins*

To study the dependency of the nuclear accumulation of NLS proteins on NbIMPα, we used a VIGS-based functional genetic assay (Baulcombe, 1999). The *NbImpα*-silenced plants described above were infiltrated with *A. tumefaciens* carrying pGDY:NLS protein constructs. At 2 days post-infiltration, leaves were examined for localizations by confocal microscopy. In this assay, we examined whether AtFib1 of

Arabidopsis and *P. infestans* proteins require NbIMPα for import into nuclei. Our results showed that Nuk6 and Nuk7 protein fusions shifted to an even distribution between nucleus and cytoplasm in silenced plants. Thus, silencing of *NbImpα1* and *NbImpα2* or similar α-importins affected the localization patterns of Nuk6 and Nuk7. This result suggests dependency of these proteins on importin-α for their import into nuclei (Figure 6). In contrast, the cellular distributions of Nuk12, AtFib1 and SV40NLS in non-silenced leaves and in leaves silenced for the *NbImpα* genes were similar (Figure 6). Leaves from healthy and empty TRV-infiltrated plants showed accumulation of the NLS proteins in the nuclei of plant epidermal cells. From these results, it was apparent that at least some of the proteins (Nuk6 and Nuk7) are dependent on NbIMPα1 and NbIMPα2 or close homologs thereof for their nuclear transport, and that some (Nuk12 and AtFib1) can target nuclei independently of these α-importins.

Discussion

In eukaryotes, proteins can be targeted to the nucleus via an importin-α-mediated mechanism that requires a specific NLS in the protein. In this study, we describe a method for functional studies of nuclear import in plants. We identified and functionally assayed two distinct α-importins, NbIMPα1 and NbIMPα2, in *N. benthamiana.* The Southern blot data indicated that there are at least four copies of the two importin-α isoforms in the *N. benthamiana* genome. However, the exact number of importin-α genes is unknown, and it is possible that more isoforms exist in this plant. *A. thaliana* contains nine genes for α-importins (Ballas and Citovsky, 1997; Smith *et al.*, 1997), rice contains at least four (Iwasaki *et al.*, 1998; Jiang *et al.*, 2001; Matsuki *et al.*, 1998) and chili pepper (*Capsicum frutescens*) contains two (Szurek *et al.*, 2001). Several α-importins have also been reported in vertebrates. However, in the case of yeast (*Saccharomyces cerevisiae*), importin-α (SRP1) is encoded by a single gene (Gorlich, 1998). The availability of additional sequence data for *N. benthamiana* will enable the identification of other α-importins and the design of gene-specific silencing constructs.

Sequence and phylogenetic analysis showed that *N. benthamiana* NbIMPα1 and NbIMPα2 are similar to other members of the importin-α protein family, particularly α-importins from tomato and pepper. NbIMPα1 and NbIMPα2 contain a highly conserved region of eight continuous repeats of the 'arm' motif, which is typical for α-importins and functions as a binding site for NLS sequences. Although there are differences in the first variable region flanking the arm repeats of NbIMPα1 and NbIMPα2, most of the amino acid sequences of NbIMPα1 and 2 are similar in containing the IBB domain, arm repeats and a C-terminal acidic region. This result was expected because α-importins are conserved among plants, animals and fungi (Jiang *et al.*, 1998; Matsuki *et al.*, 1998). However, the occurrence of several importin-α isoforms in animals and plants has raised questions about the specificity and efficiency in transport of different NLS proteins (Hicks *et al.*, 1995; Tzfira and Citovsky, 2002). Indeed, *A. thaliana* AtImpα1 can bind all three types of NLSs, including the monopartite NLS of SV40 T-antigen, the bipartite NLS of maize (*Zea mays*) transcription factor Opaque 2 (O2), and the Mat 2-type NLS of maize transcription factor R (Smith *et al.*, 1997). Selective binding and nuclear import of rice α-importins 1a and 1b to plant NLS was also demonstrated using *in vitro* assays (Jiang *et al.*, 1998, 2001). Similarly differential substrate specificities were reported for various isoforms of human importin-α (Fagerlund *et al.*, 2005; Kohler *et al.*, 1999; Quensel *et al.*, 2004).

This study demonstrates *in vivo* that proteins are dependent on importin-α for import into plant cell nuclei, and that importin-α isoforms have distinct preferences for different NLS-carrying proteins. Inoculation of *N. benthamiana* with binary plasmid constructs of recombinant TRV carrying *NbImpα* sequences resulted in silencing of *NbImpα1* and *NbImpα2* that peaked at about 3 weeks after infiltration. The nuclear targeting of *P. infestans* Nuk6 and Nuk7, which have monopartite NLSs, was inhibited in silenced plants. In contrast, the nuclear import of *P. infestans* Nuk12, which also has a monopartite NLS, of *Arabidopsis thaliana* fibrillarin 1 (AtFib1), a known nucleolar marker protein that contains a bipartite NLS (Barneche *et al.*, 2000), and the monopartite SV40 NLS of the YFP–SV40NLS fusion is unaffected in these silenced plants. Based on the Southern blot data, it is likely that the expression of the *NbImpα1* and *NbImpα2* isoforms were silenced as well. Further, because successful silencing of target genes can be achieved with complementary sequences of only 25 nucleotides in length, it is possible that other *N. benthamiana* importin-α genes were targeted for silencing in this study but were not detected in the Southern blot experiment. However, it is likely that not all *N. benthamiana* importin-α genes were silenced, as nuclear import of the YFP–SV40NLS fusion was not inhibited in the silenced plants, and it has been shown that the SV40 NLS depends on importin-α for nuclear import (Harreman *et al.*, 2004). The most plausible explanation for this result is that the nuclear import of the YFP–SV40NLS fusion depends on other *N. benthamiana* importin-α isoforms than NbIMPα1 and NbIMPα2 and close homologs. Once all the importin-α isoforms of *N. benthamiana* have been identified, VIGS of corresponding importin-α genes will allow *in vivo* dissection of their roles in specific transport of various NLS-containing and other nuclear-targeted proteins of plants and plant pathogens. Indeed, VIGS offers the unique advantage of specifically targeting a chosen gene (Baulcombe, 1999; Liu *et al.*, 2002; Ratcliff *et al.*, 2001). As VIGS is a homology-dependent process, careful selection of a unique region of the target sequence can ensure the silencing of the expression of a specific gene family member. Nevertheless, we can conclude from our data that

P. infestans Nuk6 and Nuk7 are dependent on α-importins for nuclear import.

Several plant pathogens are known to transport effector molecules inside host cells to establish pathogenicity. These pathogen-encoded proteins contain functional NLSs and have been shown to interact with importin-α (e.g. Vanden Ackerveken and Bonas, 1997). Although NLS-containing proteins from plant viruses (Carrington *et al.*, 1991; Goodin *et al.*, 2002; Li *et al.*, 1997; Lucy *et al.*, 2000; Tsai *et al.*, 2005; Vetter *et al.*, 2004) and bacteria (Van den Ackerveken and Bonas, 1997) have been studied, there are no reports on the import of proteins from the oomycete *Phytophthora*. This study shows that proteins containing a monopartite NLS of the oomycete *P. infestans* can target plant cell nuclei, and that two of the three nuclear-targeted proteins function in an importin-α-dependent manner. This finding is interesting, particularly in relation to a recent report demonstrating that a mutation in the *MOS6* gene, which encodes one of the nine α-importins (AtImpα3) in Arabidopsis, enhanced susceptibility to another oomycete plant pathogen *Hyaloperonospora parasitica* (Palma *et al.*, 2005). This suggests that nucleo-cytoplasmic trafficking, which is central to many virus–plant interactions, also plays a significant role in defense against oomycete pathogens (Palma *et al.*, 2005).

In summary, we describe a powerful functional genetic assay for determination of nucleo-cytoplasmic trafficking that should prove particularly useful for the genetic dissection of nuclear import pathways in plants. We also report an *in vivo* demonstration that some proteins are dependent on importin-α for import into plant cell nuclei. This study is a prelude to gaining a better understanding of the mechanisms involved in nuclear import and signaling events that involve transcriptional control of genes in plants.

Experimental procedures

Gene identification and sequence analyses

N. benthamiana EST clones, NBMC392 (GenBank accession number CK291685) and NBMBW57 (accession number CK290682), encoding importin-α genes, were obtained from the Institute for Genomic Research (http://www.tigr.org). To obtain the full-length coding sequences of importin-α genes, the cDNA clones were sequenced by primer walking using an Applied Biosystems 3730 DNA analyzer (http://www.appliedbiosystems.com/). Sequences were assembled and annotated using Sequencher™ 4.1 (Gene Codes Corp, www.genecodes.com). Similarity searches were performed locally on an Apple Macintosh OSX workstation using BLAST (Altschul *et al.*, 1997). The *NbImpα1* and *NbImpα2* complete nucleotide and amino acid sequences were deposited in GenBank with the accession numbers EF137253 (*NbImpα1*) and EF137254 (*NbImpα2*). The amino acid sequences of NbIMPα1 and NbIMPα2 were searched against the Pfam database (Bateman *et al.*, 2002) to identify various domains. Amino acid sequences of importin-α homologs from other organisms were retrieved from the Arabidopsis Information Resource (TAIR) and GenBank. Amino acid sequence alignment and phylogenetic analyses were conducted using the Clustal W algorithm (Thompson *et al.*, 1994).

cDNA cloning

To clone *N. benthamiana NbImpα1* and *NbImpα2* in the TRV vector pTV00, N-terminal fragments of approximately 0.5 kb length from the above cDNA clones were PCR-amplified using high-fidelity Pfu DNA polymerase (Stratagene; http://www.stratagene.com/) using NbImpα1F (GCGGGATCCAGAGAAGAGAGTTTGCTCAAGAAGCGT) and NbImpα1R (GCGGGGCCCAAAGGTAAGAAAGTGCCCAGCATGCATCTGT) primers that contain *Bam*HI and *Apa*I recognition sites (underlined). PCR-amplified products were digested and ligated into the pTV00 vector (http://www.jic.bbsrc.ac.uk/Sainsbury-Lab/dcb/Services/vigsprotocol.htm). The *P. infestans* NLS-containing proteins Nuk6, Nuk7 and Nuk12, and *N. benthamiana NbImpα1* and *NbImpα2* were PCR-amplified as described above, and cloned into pGD binary vectors (Goodin *et al.*, 2002) using the primer pairs Nuk6-pGDY-F (GCGctcgagccCACTCATCAAACGCTTTAGCCGGTGAAACT) and Nuk6-pGDY-R (GCGggatccCGTTGCTGTTCTCCAGCCCCTCCTCGATCTCTTCC), Nuk7-pGDY-F(GCGaagcttccGATTATGGGCCGAGAGACTCCGTGACGATCCTT) and Nuk7-pGDY-R (GCGggatccAGCACACTCGCGAGCAGTTGCTGCGTCCAGCTC), Nuk12-pGDY-F (GCGaagcttccAAAGAACGGCGTGGGGAGATCATTTCA) and Nuk12-pGDY-R (GCGggatccGGTCGTGACTCTCGGCGTTTCGCTGCTCCT), NbIMPα1-pGDR-F (GCGctcgagccATGTCGCTGAGGCCGAATTCGAGAAC) and NbIMPα1-pGDR-R (GCGggatccGGGGACACACTCCAGCTTCAATAACAGC), and NbIMPα2-pGDR-F (GCGctcgagccATGTCTCTGAGACCAAGTGCTAGGACGG) and NbIMPα2-pGDR-R (GCGggatccAGGAGACGGATGCATGAGGAGCTCAACCA) with restriction sites used for cloning of PCR products printed in lower case. DNA was manipulated as described previously (Sambrook and Russell, 2001), and the recombinant plasmids were transformed into the electro-competent *A. tumefaciens* strain GV3101. Recombinant clones were tested for the identity of the inserts by PCR amplification using the above-listed gene-specific primers and DNA sequencing.

Construction of the vector for production of YFP–SV40NLS

The region corresponding to three tandem repeats of the SV40 large T-antigen NLS was amplified from pECFP-Nuc (BD Biosciences, www.bdbiosciences.com) using the primers ECFP-F (AGAAGAACGGCATCAAGGC) and NLS-pGDY-R (TGATCAGTTATCTAGATCCG). The PCR product was digested with *Xho*I and *Bam*HI and then ligated into the binary vector pGDY. The recombinant plasmid was transformed into electro-competent *A. tumefaciens* strain GV3101. Successful cloning was verified by DNA sequencing.

Southern blot analysis

Genomic DNA was extracted from leaf tissue of *N. benthamiana* according to the method described by Fulton *et al.* (1995) scaled up to large preparation. Briefly, leaf tissues were ground with DNA extraction buffer (Fulton *et al.*, 1995). Nuclei were spun down at 560 ***g*** for 15 min. The resulting pellet was resuspended in a 5:5:2 ratio of DNA extraction buffer, nuclei lysis buffer (Fulton *et al.*, 1995) and 5% sarkosyl), and then incubated at 65°C for 30 min. Phase separation was performed by adding chloroform:isoamyl alcohol (24:1), and centrifuging at 560 ***g*** for 15 min after vigorous mixing. DNA was precipitated by adding 1 volume of isopropanol to the aqueous phase samples. The precipitated DNA were allowed to dry

at room temperature and resuspended in Tris-EDTA. Genomic DNA (10 μg per lane) was digested with following restriction enzymes: *Bst*NI, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Sca*I or *Xba*I, and then separated by electrophoresis on a 0.8% agarose gel. The separated DNA was transferred from gels to nylon membranes (Hybond-XL, Amersham Biosciences; http://www5.amershambiosciences.com/) in 0.8 N NaOH solution, and subsequently hybridized to the NbImpα1 and NbImpα2 probes. The cDNA probes for *NbImpα1* (694 bp) and *NbImpα2* (694 bp) corresponding to the cloned fragments in the pTV00 silencing constructs were labeled with ^{32}P-dCTP (Perkin-Elmer www.perkinelmer.com) using Ready-To-Go DNA labeling beads (GE Healthcare www.gehealthcare.com) according to the manufacturer's instructions. Southern blots were pre-hybridized in modified Church buffer (0.5 M sodium phosphate, pH 7.0, 1 mM EDTA, 7% SDS) at 65°C for 16–18 h. Next, the blots were hybridized with heat-denatured, radiolabeled probes in modified Church buffer at 65°C for another 16–18 h. For low-stringency washes, the blots were washed for 30 min each in 2× SSC and 0.1% SDS, and 1× SSC and 0.1% SDS at 65°C sequentially. For high-stringency washes, the blots were washed for 15 min each in 0.5× SSC and 0.1% SDS, and 0.25× SSC and 0.1% SDS at 65°C, after the low-stringency washes. The blots were exposed to a storage phosphor screen (Molecular Dynamics www.bioscience.org) for 24 h. Images were captured and analyzed using a Storm 840 gel and blot imaging system and ImageQuant software (Molecular Dynamics), converted to TIFF format for export, and processed in Adobe Photoshop CS2 (Adobe Systems Inc., www.adobe.com).

TRV-induced gene silencing

A. tumefaciens strain GV3101 carrying the binary TRV RNA1 construct pBINTRA6 and the TRV RNA2 vector pTV00 (Ratcliff *et al.*, 2001), or pTV00:PDS, pTV00:*NbImpα1* or pTV00:*NbImpα2*, were prepared for agro-infiltration assays as described previously (Van der Hoorn *et al.*, 2000). The bacterial suspensions were mixed in a 1:1 ratio (RNA1:RNA2) in MES induction buffer (10 mM $MgCl_2$, 10 mM MES, pH 5.6) containing 150 μM acetosyringone. The cultures were maintained at room temperature for 2–3 h. Infiltrations were performed by gently pressing a 1 ml disposable syringe to the abaxial surface of fully expanded leaves of 3-week-old *N. benthamiana* plants, followed by gentle release of the bacterial suspension in the syringe until the leaves had a water-soaked appearance. This typically required 1–4 infiltrations per leaf. Following agro-infiltration, plants were maintained in a greenhouse at 22°C with a 16 h photoperiod. Leaves were collected for total RNA isolation 3 weeks after agro-infiltration with the TRV strains.

RT-PCR analyses

For RT-PCR, total RNA was isolated from the 4th leaf above the infiltrated leaves in silenced and non-silenced *N. benthamiana* plants using Trizol reagent (Invitrogen, www.invitrogen.com) according to the manufacturer's instructions. First-strand cDNAs were synthesized from 4.5 μg of total RNA using a universal polyT primer and ThermoScript reverse transcriptase from the ThermoScript RT-PCR system (Gibco-BRL). Reactions were performed according to the manufacturer's instructions. PCR amplifications were carried out with equal amounts of cDNAs using the primer pairs NbIMPα1-pGDR-F and NbIMPα1-pGDR-R for NbImpα1 and NbIMPα2-pGDR-F and NbIMPα2-pGDR-R for NbImpα2 (see above for sequences). These primers anneal outside the region targeted for silencing to ensure that the endogenous gene is tested. The integrity of mRNA and cDNA was controlled using primers Totub-F1 (ATCGCATCCGAAAGCTTGCAG) and Totub-R1 (ACATCAACATTCAGAGCTCCATC) specific for the constitutively expressed tomato tubulin factor 1 gene. PCR was performed for 24 and 30 cycles.

Agro-infiltrations for in planta *cellular localization of NbIMPα and NLS proteins*

Recombinant *A. tumefaciens* strains containing various pGD binary plasmids were prepared for agro-infiltration as described previously (Van der Hoorn *et al.*, 2000; Goodin *et al.*, 2002). Transformed GV3101 bacterial suspensions were adjusted to an OD_{600} of 0.6 in MES buffer. Acetosyringone was added to a final concentration of 150 μM, and the suspensions were maintained at room temperature for 2–3 h. For co-infiltration of various *Agrobacterium* transformants, equal volumes of each suspension were mixed prior to infiltration. For *in planta* localization analysis, wild-type and importin-silenced *N. benthamiana* plants of the same age were used. The leaves to be infiltrated were chosen to be at similar growth stage as those of PDS-silenced leaves showing a photobleaching phenotype. Leaves were examined by confocal laser scanning microscopy between 40 and 90 h post-infiltration. Experiments were repeated three times, and each construct was evaluated on 10 plants. Leaves of all the plants showed the localization.

Confocal laser scanning microscopy

Leaves were collected from infiltrated plants and examined under a Leica TCS SP spectral confocal laser scanning microscope (Leica Microsystems, www.leica-microsystems.com) equipped with the following lasers: argon (450 and 488 nm), krypton (568 nm) and helium–neon (630 nm). YFP and GFP were excited at 488 nm, and DsRed2 was excited at 568 nm. The emitted fluorescence was filtered through the trichronic 488/543/633, and detected at the following wavelengths: 500–530 nm for GFP, 510–550 nm for YFP, 575–620 nm for DsRed2 and 650–700 nm for chloroplast fluorescence. Images were captured using Leica TCSNT software, and processed using Adobe Photoshop 5.0.

Acknowledgements

This work was funded by NSF Plant Genome Research Program grant DBI-0211659. Salaries and research support were provided, in part, by state and federal funds allocated to the Ohio Agricultural Research and Development Center, Ohio State University. We thank J. Moyseenko and Dr E. van der Knaap for help with the Southern blot analysis. Further, we thank D. Baulcombe (Sainsbury Laboratory, Norwich, UK) for providing TRV vectors and R. Buell for the *N. benthamiana* cDNAs.

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Accession numbers for sequences: NbImpα1, EF137253; NbImpα2, EF137254; Nuk6, EF137255; Nuk7, EF137256; and Nuk12, EF137257

