A genetic system for detection of protein nuclear import and export

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We have developed a simple genetic assay to detect active nuclear localization (NLS) and export signals (NES) on the basis of their function within yeast cells. The bacterial LexA protein was modified (mLexA) to abolish its intrinsic NLS and fused to the activation domain of the yeast Gal4p (Gal4AD) with or without the SV40 large T-antigen NLS. In the import assay, if a tested protein fused to mLexA-Gal4AD contains a functional NLS, it will enter the cell nucleus and activate the reporter gene expression. In the export assay, if a tested protein fused to mLexA-SV40 NLS-Gal4AD contains a functional NES, it will exit into the cytoplasm, decreasing the reporter gene expression. We tested this system with known NLS and NES and then used it to demonstrate a NES activity of the capsid protein of a plant geminivirus. This approach may help to identify, analyze, and select for proteins containing functional NLS and NES.

Keywords: *Agrobacterium*, Geminivirus, TYLCV, VirD2/VirE2, Nucleotoplasmic shuttle protein

Shuttling of protein molecules between the nucleus and cytoplasm is a process that is central to the regulation of gene expression and underlies many aspects of development, morphogenesis, and signaling pathways in eukaryotic organisms. Furthermore, transport of proteins and protein–nucleic acid complexes into and out of the nucleus is an essential step in many host–pathogen interactions such as viral and bacterial infection. Nuclear traffic occurs exclusively through the nuclear pore complex (NPC). Whereas small molecules (40–60 kDa) diffuse through the NPC, nuclear import of larger molecules is mediated by specific nuclear localization signal (NLS) sequences contained in the transported molecule[1,2].

Once in the nucleus, many proteins are transported back to the cytoplasm as an essential step in their biological function. For example, the Rev protein of human immunodeficiency virus type 1 (HIV-1) exits the nucleus, facilitating export of the unspliced viral RNA (reviewed in ref. 3). Rev nuclear export is mediated by a nuclear export signal (NES), found also in proteins of other viruses[4]. Also, numerous cellular proteins, such as I-κB and MAPKK, contain potential NES sequences that may regulate the biological activity of these proteins by controlling their nuclear export[5].

The relatively small size of the NLS and NES sequences and, more importantly, the lack of clear and consistent consensus motifs in these signals make it difficult to predict their presence in a given protein solely on the basis of analysis of its amino acid sequence. Furthermore, even if a consensus NLS or NES were found, it may not represent a functional signal. For example, β-glucuronidase (GUS), a commonly used reporter enzyme that resides exclusively in the cell cytoplasm[6,7], carries a conserved, albeit nonfunctional, bipartite NLS at its C terminus. Thus, the only practical way to identify active NLS or NES signals is by microinjecting[8–10] or expressing the protein of interest in eukaryotic cells[6,7,11,12], forming heterokaryons[13], or using an in vitro transport system[14–17]. However, these approaches have certain disadvantages. They tend to be labor intensive, and to require protein purification and labeling or a cDNA clone that can be microinjected or transferred into higher eukaryotic cells. Detection frequently relies upon fluorescence or electron microscopy techniques.

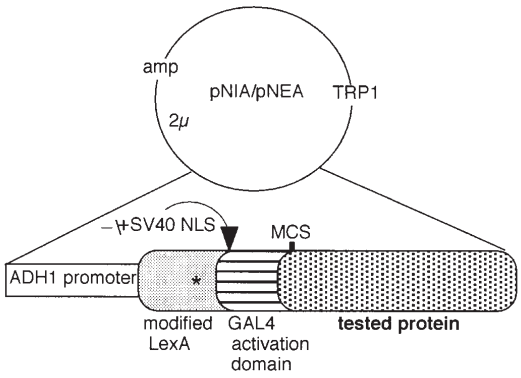
Here, we report a simple functional assay for protein nuclear import and export that circumvents all of the difficulties just mentioned. We have used this approach to demonstrate the nuclear import and export activities of a capsid protein from a plant geminivirus, suggesting a role for the capsid protein in nuclear shuttling of viral genomes during the infection process.

Results

Nuclear import assay. The basic strategy of these experiments is based on expression in yeast cells of a triple-fusion protein comprising bacterial LexA, yeast Gal4p activation domain (Gal4AD), and the tested protein encoded by a cDNA subcloned in-frame downstream of Gal4AD (Fig. 1A). If the tested protein contains a functional NLS, the fusion product will enter the yeast cell nucleus. Following this nuclear import, the LexA domain will target the fusion protein to the LexA operator sites of the reporter *lacZ* gene contained in the L40 yeast strain. Gal4AD then activates the expression of *lacZ*, resulting in β-galactosidase activity. In the absence of a NLS, the fusion protein is unable to reach the cell nucleus and thus activate the reporter gene.

In addition to induction of the β-galactosidase reporter, this one-hybrid system allows direct selection for the nuclear import of the tested protein in the same L40 yeast strain, which contains an integrated copy of the *HIS3* gene with upstream LexA operators. Only cells expressing the NLS-containing fusion protein will grow on a histidine-deficient medium.

Clearly, the success of this approach hinges on the inability of LexA-Gal4AD-tested protein fusion to enter the cell nucleus in the absence of a NLS contained within the tested protein. Thus, neither LexA nor Gal4AD should contain NLS sequences. Although Gal4AD is known to lack NLS[18], LexA, a bacterial protein, is generally thought not to have evolved such a signal. Surprisingly, however, our studies demonstrated that a LexA-Gal4AD fusion containing VirE2, an *Agrobacterium* protein shown to lack a NLS functional in animal



wild-type LexA	(457)	GTT	ACC	GTT	AAG	CGC	CTG	AAA	AAA	CAG	GGC	AAT	(489)
	(153)	V	T	V	K	R	L	K	K	Q	G	N	(163)
						*		*					
	(153)	V	T	V	K	G	L	E	K	Q	G	N	(163)
modified LexA	(489)	GTT	ACC	GTT	AAG	GGC	CTG	GAA	AAA	CAG	GGC	AAT	(489)

Figure 1. A schematic representation of pNIA and pNEA plasmids. (A) Plasmid composition. pNIA expresses a fusion protein consisting of mLexA, Gal4AD, and protein to be tested; pNEA produces a fusion between mLexA, SV40 NLS, Gal4AD, and a tested protein. Asterisk indicates the position of the LexA NLS. MCS indicates multiple cloning sites (*Sma*I, *Bam*HI, *Sal*I, and *Pst*I). The plasmid backbone is derived from pBTM116[33]. (B) LexA NLS and amino acid substitutions (asterisks) which inactivate this signal, producing modified LexA (mLexA). Numbers indicate the position of nucleotides (top) and amino acid residues (bottom) within LexA gene and protein sequences, respectively.

cells[8], induced the β-galactosidase reporter (Fig. 2A, pLGE2) and grew on a histidine dropout medium (Fig. 2B, pLGE2).

VirE2 is a 70 kDa protein; thus, the LexA-Gal4AD-VirE2 fusion is likely to be actively imported into the cell nucleus to allow this gene induction. Because the absence of NLS in LexA was implied from its bacterial origin rather than demonstrated directly, it is possible that it carries a previously unidentified NLS signal. Inspection of the amino acid sequence of LexA identified a short stretch of basic residues (Fig. 1B), which may function as a NLS. We made two amino acid substitutions, R157G and K159E, in this motif (Fig. 1B), resulting in a modified LexA (mLexA) within the hybrid expression vector, designated pNIA (for nuclear import assay). A nuclear import assay demonstrated that mLexA expressed in fusion with Gal4AD and VirE2 from the pNIAE2 construct no longer activated the reporter genes *lacZ* (Fig. 2A) and *HIS3* (Fig. 2B), indicating the lack of nuclear import of the fusion protein.

To exclude the possibility that LexA mutagenesis nonspecifically inactivated this protein, we introduced a short amino acid sequence corresponding to the SV40 large T-antigen NLS between the mLexA and Gal4AD domains of pNIAE2, producing pNIA(+)E2. The fusion protein produced from this construct localized to the cell nucleus, resulting in *lacZ* induction (Fig. 2A) and cell growth in the absence of histidine (Fig. 2B) and indicating mLexA functionality in activation of gene expression. Note that the presence of the tested protein within the pNIA construct is essential for detection of bona fide nuclear import; in the absence of the tested protein, the mLexA-Gal4AD fusion produced from pNIA alone may simply diffuse into the nucleus because of its small size (data not shown).

Next, pNIA was used to test its ability to detect a functional NLS within a known nuclear protein. To this end, a NLS-containing protein, VirD2 of *Agrobacterium*[19,20], was subcloned into pNIA. The resulting mLexA-Gal4AD-VirD2 fusion protein was imported into the cell nucleus as illustrated by activation of the reporter genes *lacZ* (Fig. 2A) and *HIS3* (Fig. 2B). When, as a negative control, a VirD2 cDNA sequence was inserted into pNIA in the antisense orientation, the fusion product did not activate the reporter genes (data not

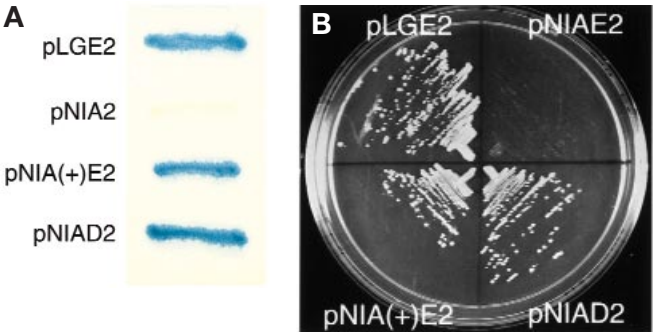


Figure 2. Nuclear import assay. (A) β-galactosidase assay following cell growth on minimal medium without tryptophan. (B) Selection assay by cell growth on minimal medium deficient for both tryptophan and histidine and supplemented with 5 mM of 3-amino-1,2,4-triazole (3AT). pLGE2 expresses VirE2 fused to wild-type LexA and Gal4AD; pNIAE2 expresses VirE2 fused to modified LexA (mLexA) and Gal4AD; pNIA(+)E2 expresses VirE2 fused to mLexA, SV40 NLS, and Gal4AD; pNIAD2 expresses VirD2 fused to mLexA and Gal4AD.

shown). These results demonstrate that pNIA allows specific detection of and selection for proteins containing a NLS sequence functional in yeast cells.

Finally, we tested whether proteins that carry NLS-like sequences but that are known not to be nuclear import substrates activate gene reporters. Two such proteins were chosen, a bacterial enzyme GUS and a cell-to-cell movement protein (MP) of tobacco mosaic virus (TMV). Both proteins contain basic amino acid regions that are similar to the bipartite NLS. Specifically, the bipartite motif, found in >50% of nuclear proteins and in <5% of nonnuclear proteins in the database, comprises two adjacent basic amino acids, a spacer region of any 4 to 37 residues, and a basic cluster in which any three out of the five contiguous amino acids must be basic[11,21]. Although GUS and TMV MP contain this NLS-like motif between their amino acid residues 566–578 and 95–116, respectively, GUS is known to be exclusively cytoplasmic[6,7], whereas MP mediates movement of TMV genomes through plant intercellular connections (the plasmodesmata) and has no nuclear function (reviewed in refs 22, 23). Unlike VirD2, which carries a functional NLS, neither GUS nor TMV MP expressed in the pNIA system induced the reporter genes *lacZ* (Fig. 3) or *HIS3* (data not shown).

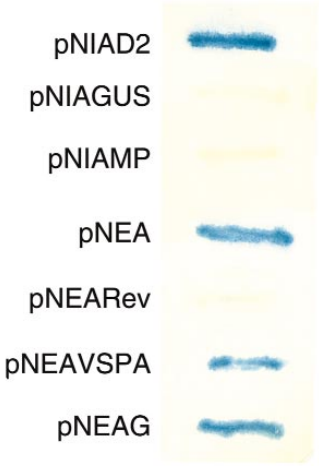


Figure 3. Effects of NLS and NLS-like or NES and NES-like motifs on induction of β-galactosidase expression in cells grown on minimal medium without tryptophan. pNIAD2 and pNEARev express proteins with functional NLS and NES, respectively. pNIAGUS and pNIAMP express proteins that carry NLS-like sequences but are not substrate for nuclear import. pNEAVSPA and pNEAG express proteins that carry NES-like sequences but are not substrate for nuclear export.

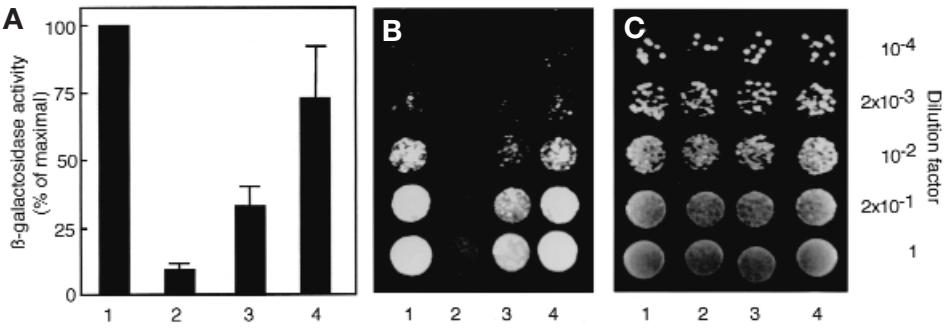


Figure 4. Nuclear export assay. (A) Quantitative β-galactosidase assay in liquid following cell growth in minimal medium without tryptophan. Standard errors are shown based on five independent experiments. β-galactosidase activity is expressed as percentage of maximal enzymatic activity obtained with pNEA alone. (B) Selection assay by cell growth on minimal medium deficient for both tryptophan and histidine and supplemented with 100 mM 3AT. This 3AT concentration was optimal for detecting differences in cell growth between various Rev derivatives. (C) Cell growth on minimal medium deficient for only tryptophan. (1) pNEA alone; (2) pNEARev (NES: LPPLERLTL); (3) pNEAM10 (mutated NES: LPPDLRLTL); (4) pNEARevΔ3 (residual NES: LPPL).

Nuclear export assay. The ability of pNIA to detect protein transport into the nucleus can also be utilized to assay for a reverse protein traffic, that is, nuclear export. To this end, we introduced the SV40 large T-antigen NLS between the mLexA and Gal4AD domains of pNIA, resulting in a nuclear export assay plasmid pNEA (Fig. 1A). In pNEA, fusion to a protein without a NES will result in nuclear import because of the presence of the SV40 NLS. Yeast cells harboring this construct express β-galactosidase and grow in the absence of histidine. Indeed, as mentioned above, subcloning of VirE2 into pNEA (pNEAE2, same as pNIA(+)E2) resulted in a strong β-galactosidase staining (Fig. 2A) and histidine prototrophy (Fig. 2B).

Fusion to a NES-containing protein, on the other hand, is expected to redirect the protein product into the cell cytoplasm, at least partly abolishing the β-galactosidase activity and impeding growth without histidine. We tested this idea using the Rev protein of HIV-1, known to carry a functional NES[5]. Expression of Rev from the pNEA vector dramatically decreased *lacZ* expression (compare pNEA and pNEARev in Fig. 3). Quantification of the β-galactosidase activity showed the reduction to about 12% of that observed with pNEA alone (Fig. 4A), suggesting the predominantly cytoplasmic localization of the fusion product. Residual levels of *lacZ* induction are probably due to a small steady-state pool of Rev protein within the cell nucleus due to its nuclear shuttling activity (reviewed in ref. 3).

That the decrease in *lacZ* induction specifically depends on the Rev NES was demonstrated by mutating or deleting this signal. The M10 NES mutation, which substitutes only two amino acid residues within NES[24], restored β-galactosidase activity to 30% that of the maximum, indicating diminished nuclear export of the mutant fusion protein as compared to the wild-type Rev. A deletion mutation of the Rev NES, RevΔ3, which removes most of the signal sequence[25], increased *lacZ* reporter gene induction to 70–90% of the maximal level (Fig. 4A).

Changes in the degree of *lacZ* gene expression caused by the Rev NES closely paralleled *HIS3* expression. Serial dilutions of yeast cells on the histidine dropout selective medium clearly demonstrated a dramatic reduction in histidine prototrophy. This effect was NES dependent, because both the M10 and RevΔ3 mutations gradually restored growth on the selective medium (Fig. 4B). In the absence of selection, all strains exhibited equal growth (Fig. 4C). These results indicate that the degree of repression of the *lacZ* and *HIS3* reporter genes and, by implication nuclear export, directly reflects the strength of the NES signal, allowing the use of this nuclear export assay to give a quantitative indication of and select for the activity of NES signals in proteins of interest.

As with the pNIA assay, we tested whether pNEA can detect proteins that carry leucine- or isoleucine-rich NES-like motifs[26] but are known not to be involved in nuclear export. To this end, we chose two proteins, an *Agrobacterium* transcriptional activator VirG (reviewed in ref. 27) and soybean vegetative storage protein A (VSPA; reviewed in ref. 28), which contain several leucine/isoleucine-rich regions between positions 187–194 and 155–165, respectively. Consistent with their cellular activities, neither VirG nor VSPA significantly repressed the *lacZ* (Fig. 3, pNEAG and pNEAVSPA, respectively) and *HIS3* reporter genes (data not shown) in the pNEA assay, demonstrating no capacity for nuclear export.

Identification of a functional NES in the capsid protein of a geminivirus. Tomato yellow leaf curl virus (TYLCV) is a monopartite geminivirus containing only one genomic circular ssDNA encapsulated by the viral capsid protein (CP)[29]. Upon infection, TYLCV is imported into the host plant cell nucleus, where DNA replication, transcription, and virus assembly presumably take place[30]. Whereas nuclear import of TYLCV is likely mediated by its NLS-bearing capsid protein[31], the mechanism by which this virus is exported from the nucleus for cell-to-cell movement and spread of infection remains unknown. Here, we used the pNEA-based nuclear export assay and histidine selection to demonstrate that, in addition to its NLS, TYLCV capsid protein contains a NES functional in yeast.

Figure 5A shows that, similarly to Rev, a capsid protein fusion substantially decreased histidine prototrophy, indicating reduction in *HIS3* gene expression and, by implication, the presence of an active NES within the capsid protein. Next, we mapped the capsid protein NES relative to its known NLS sequences, which reside at the N terminus (major NLS) and in the middle part of the protein (augmenting NLS)[31]. The N terminus of the capsid protein promoted efficient expression of the reporter genes (data not shown), suggesting that the capsid protein NES is located within the deleted part of the protein (i.e., residues 38–260). Deletion of amino acid residues from 38 to 113 (CPΔM mutant), on the other hand, did not enhance *HIS3* gene expression (Fig. 5A). This result indicates that the capsid protein NES is not present in the middle portion of the capsid pro-

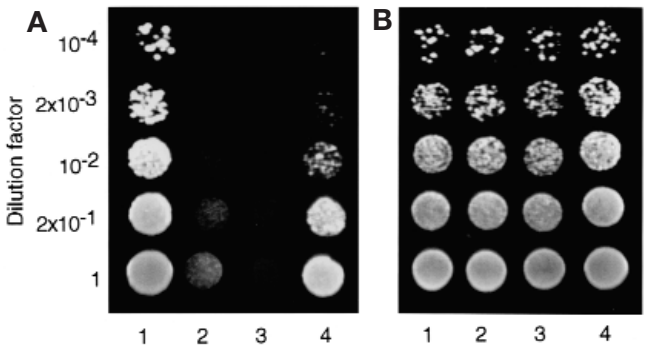


Figure 5. Detection of NES within TYLCV capsid protein. (A) Selection assay by cell growth on minimal medium deficient for both tryptophan and histidine. (B) Cell growth on minimal medium deficient for only tryptophan. (1) pNEA alone; (2) pNEACP; (3) pNEACPΔM; (4) pNEACPΔC.

tein; in fact, deletion of the augmenting middle NLS apparently enhanced nuclear export of the mutant protein as compared to the full-length capsid protein (Fig. 5A). In contrast, removal of the C terminus of the capsid protein (residues 114–260, CPΔC mutant) restored *HIS3* expression (Fig. 5A), suggesting that the deleted C-terminal region contains a functional NES. The differences in colony formation on the selective medium reflected changes in the expression of the *HIS3* reporter, because capsid protein and all of its mutants exhibited equal growth in the absence of histidine selection (Fig. 5B). Thus, capsid protein likely contains two types of spatially distant targeting signals, N-terminal and middle NLSs and a C-terminal NES. Because capsid protein NES functions in the pNEA assay within the context of the full-length capsid protein—that is, in the presence of the N-terminal NLS (see Fig. 5A)—this NES signal may dominate the capsid protein NLS signal.

Discussion

For years, LexA has been used in different variations of the two-hybrid system[32,33]; however, it has remained unclear how LexA fusions were imported into the nucleus to participate in two-hybrid interactions. Here, we have shown that the LexA protein itself contains a functional NLS, inactivation of which did not affect the LexA function in reporter gene induction, indicating that this activity of LexA can be separated from its recognition of the *lexA* operator sequences.

Identification and inactivation of the LexA NLS has allowed the use of this protein to detect functional NLS by activation of yeast nuclear reporter genes. The pNIA assay specifically identifies nuclear targeting activity in individual proteins of interest. For detection of nuclear export, the pNIA system was modified to pNEA. Rapid and simple identification of functional NLS sequences in this system may represent a useful first step in the elucidation of the biological function of protein products of new genes such as those isolated in genome projects.

An advantage of the pNIA- and pNEA-based assays is that they measure the functional rather than merely physical presence of the protein within the cell nucleus. For example, Rev, as a nuclear shuttle protein, must maintain a certain presence in the nucleus. In fact, using immunofluorescence, Rev was initially localized to the nucleus[35], whereas its export activity was first inferred only indirectly from the role in export of the unspliced viral RNA[36]. Our export assay detected both populations of Rev. The Rev nuclear pool was represented by low but statistically significant β-galactosidase activity, whereas its cytoplasmic population manifested as a clear reduction in the reporter gene expression compared to that observed in the absence of Rev. This sensitivity of the assay most likely derives from the requirement for the continuous presence of the triple-fusion molecule within the nucleus to permit binding to the promoter and gene induction. Constant nucleocytoplasmic shuttling may not allow the same protein molecule to stay inside the nucleus long enough to induce high levels of expression of the reporter genes. Thus, the pNEA-based assay identified Rev as a nuclear shuttle protein in a single experimental step.

To our knowledge, there are no previous reports of a simple genetic system to detect NES sequences. The complementary nuclear import assay also represents a significant improvement of the existing genetic techniques. Specifically, a recently reported genetic system for yeast nuclear import did not identify and inactivate the LexA NLS; instead it suppressed it with a strong NES[37]. Thus, this approach detected only NLSs that are stronger than the suppressing NES and was completely unsuited for identification of NES sequences. Finally, our assay utilizes expression of NLS- and NES-containing proteins in yeast cells; however, because nuclear transport machinery is generally well conserved between different organisms (reviewed in ref. 38), the detected signals most likely will be active in other eukaryotic cell types. For example, the *Agrobacterium* VirD2 NLS detected using pNIA normally functions in plants, whereas Rev NES detected with pNEA acts in mammalian cells. It is still possible, however, that some normally nuclear proteins from other organisms may not be imported or exported in our heterologous system. On the other hand, several proteins that carry NLS- and NES-like sequences but that are known not to function as substrates for nuclear import or export did not test positive in pNIA and pNEA systems, suggesting that false positive frequencies are relatively low in these assays.

It is important to note that, whereas our system detects proteins with functional NLSs and NESs, it does not address the molecular pathway by which nuclear import or export of these proteins occur. For example, the similar nuclear proteins are known to be imported into the cell nucleus by different pathways in different organisms. Specifically, nuclear import of a yeast hnRNP-like protein Nab2p NLS is mediated by a karyopherin β2 homolog, Kap104p, in yeast and by a karyopherin β1 protein in isolated nuclei of human cells[39]. Also, yeast and human RNA-binding proteins La appear to utilize different sequence motifs and dissimilar pathways for their nuclear import[40]. Thus, a protein found to move into or out of the nucleus in yeast cells may do so by interacting with a completely different set of cellular import/export factors in its native organism. This limitation of the pNIA/pNEA assay in crossing of the species barrier requires verification of its results in the homologous system native to the tested protein.

Having established the reliability of our nuclear import and export assay, we used this approach to analyze a potential nuclear shuttle protein encoded by a plant geminivirus, TYLCV. Our results suggest that TYLCV capsid protein, which likely mediates viral nuclear entry[31], may also play a role in exporting the TYLCV genome from the host cell nucleus. This finding represents the first indication that a plant viral capsid protein may function as a nucleocytoplasmic shuttle protein. Although this observation illustrates the use of the yeast genetic system for detection of protein nuclear export, the activity of capsid protein NES should be confirmed in vivo, within host plant cells.

Experimental protocol

Yeast strain and DNA constructs. *Saccharomyces cerevisiae* strain L40[33] was used in all experiments using standard protocols[41]. For pNIA and its fusion constructs, the *lexA* gene in pBTM116[33] was fused to Gal4AD, which was PCR-amplified without the adjacent SV40 large T-antigen NLS from pGAD424 (Clontech, Palo Alto, CA), resulting in pLG. Next, the *virE2* gene from pET3b-VirE2[42] was subcloned in-frame into the *Bam*HI site of pLG to produce pLGE2. Using the Transformer Site-Directed Mutagenesis Kit (Clontech), codons CGC and AAA of LexA within pLGE2 were then mutated to GGC and GAA, respectively, generating the substitutions R157G and K159E and converting pLGE2 to pNIAE2. To produce pNIAD2, *virE2* in pNIAE2 was replaced with the *virD2* gene from pGBTD2[17]. To produce pNIAGUS and pNIAMP, PCR-amplified *Escherichia coli gusA*[43] and TMV MP[44] genes were inserted into the *Sma*I and *Bam*HI, and *Bam*HI sites of pNIA, respectively.

For pNEA, we followed the pNIA cloning strategy, except that the SV40 large T-antigen NLS was added to the N terminus of Gal4AD. For pNEARev, pNEARevΔ3, and pNEAM10, the coding sequences for Rev from pDM121, RevΔ3 from pDM121Δ3NI (both obtained from Dr. McDonald, Salk Institute), or Rev M10 mutant from pM10 (ref. 24) (obtained from Dr. Cullen, Duke University Medical Center), were PCR-amplified and inserted into the *Bam*HI, *Sma*I and *Pst*I, or *Bam*HI and *Pst*I sites of pNEA, respectively. For pNEAVirG and pNEAVSPA, the coding sequences for *Agrobacterium virG*[45] and soybean *VSPA* genes[46,47] (obtained from Dr. Staswick, University of Nebraska) were PCR-amplified and subcloned into the *Bam*HI and *Pst*I sites of pNEA.

For pNEACP, the TYLCV capsid protein gene was PCR-amplified from pTYH20[30] and subcloned into the *Bam*HI and *Pst*I sites of pNEA. For pNEACPΔM, pNEACP was digested with *Sty*I and *Cla*I, treated with the Klenow fragment of the *E. coli* DNA polymerase, and self-ligated, preserving

the correct reading frame. Finally, for pNEACPΔC, pNEACP was digested with *Pst*I and *Cla*I, treated with the T4 DNA polymerase followed by the Klenow fragment of the *E. coli* DNA polymerase, and self-ligated. Pfu polymerase (Stratagene, La Jolla, CA) was used for all PCR reactions according to the manufacturer's instructions. All constructs were verified by DNA sequencing.

Analytical methods. For quantitative determination of β-galactosidase activity, the enzymatic assay was performed in liquid as described[48]. Qualitatively, β-galactosidase was assayed on nitrocellulose filters as described[33]. For quantitation of growth, yeast cells were grown in tryptophan dropout minimal medium, harvested, and diluted to the optical density $A_{600} = 0.5$. Serial fivefold dilutions of the resulting cultures were prepared, and a 5 μl sample of each dilution was spotted onto appropriate selective medium plates.

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