

VIP1, an *Arabidopsis* protein that interacts with *Agrobacterium* VirE2, is involved in VirE2 nuclear import and *Agrobacterium* infectivity

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T-DNA nuclear import is a central event in genetic transformation of plant cells by *Agrobacterium*. This event is thought to be mediated by two bacterial proteins, VirD2 and VirE2, which are associated with the transported T-DNA molecule. While VirD2 is imported into the nuclei of plant, animal and yeast cells, nuclear uptake of VirE2 occurs most efficiently in plant cells. To understand better the mechanism of VirE2 action, a cellular interactor of VirE2 was identified and its encoding gene cloned from *Arabidopsis*. The identified plant protein, designated VIP1, specifically bound VirE2 and allowed its nuclear import in non-plant systems. In plants, VIP1 was required for VirE2 nuclear import and *Agrobacterium* tumorigenicity, participating in early stages of T-DNA expression.

Keywords: *Agrobacterium*/nuclear import/T-DNA/tumorigenicity/VirE2

Introduction

Agrobacterium infection, the only known case of inter-kingdom DNA transfer (Stachel and Zambryski, 1989), elicits neoplastic growths on many plant species. This genetic transformation is achieved by transporting a single-stranded copy (T-strand) of the bacterial transferred DNA (T-DNA) from the tumor-inducing (Ti) plasmid into the plant cell nucleus followed by its integration into the host genome (reviewed by Gelvin, 2000; Tzfira *et al.*, 2000; Zupan *et al.*, 2000). The wild-type T-DNA carries genes involved in the synthesis of plant growth regulators and tumor-specific compounds, opines. Production of growth regulators in the transformed cell induces the formation of tumors, which then synthesize opines, a major carbon and nitrogen source for *Agrobacterium*. Thus, *Agrobacterium* are usually classified based on the type of opines specified by their T-DNA, the most common strains being nopaline- or octopine-specific. In addition to the T-DNA contents, nopaline and octopine *Agrobacterium* differ from each other in the composition and nucleotide sequence of the virulence (*vir*) region of their Ti-plasmids, which encodes the protein machinery of the T-DNA transfer (reviewed in Hooykaas and Beijersbergen, 1994).

While only the wild-type T-DNA contains tumor-inducing genes, any DNA placed between the T-DNA borders will be transported into the plant cell nucleus

(reviewed by Zambryski, 1992). This lack of sequence specificity implies that a T-DNA molecule itself does not encode specific signals for nuclear import and integration. Instead, these functions are probably performed by two *Agrobacterium* virulence proteins, VirD2 and VirE2, which are thought to associate directly with the T-strand, forming a transport (T) complex (Zupan and Zambryski, 1997). In the T-complex, one molecule of VirD2 is covalently attached to the 5' end of the T-strand, while VirE2, a single-stranded (ss) DNA-binding protein (SSB), is presumed to coat the rest of the ssDNA molecule cooperatively (Gietl *et al.*, 1987; Christie *et al.*, 1988; Citovsky *et al.*, 1988; Das, 1988; Sen *et al.*, 1989) and package it into a rigid coiled structure (Citovsky *et al.*, 1997). The need for active nuclear uptake is evident from the calculated diameter of VirE2–ssDNA complexes (12.6 nm; Citovsky *et al.*, 1997), which exceeds the diffusion limit of the nuclear pore (9 nm; reviewed by Rout and Went, 1994). Presumably, the T-complex nuclear import is mediated by VirD2 and VirE2 proteins, which localize to the plant cell nucleus (Herrera-Estrella *et al.*, 1990; Citovsky *et al.*, 1992, 1994; Howard *et al.*, 1992; Shurvinton *et al.*, 1992; Koukolikova-Nicola *et al.*, 1993; Rossi *et al.*, 1993; Zupan *et al.*, 1996). Whereas VirD2 and VirE2 accumulate in the cell nucleus even in plant species that are recalcitrant to *Agrobacterium*-induced tumor formation (Citovsky *et al.*, 1994), they probably employ different pathways for nuclear import. VirD2 is imported by a mechanism conserved between animal, yeast and plant cells (Herrera-Estrella *et al.*, 1990; Howard *et al.*, 1992; Koukolikova-Nicola *et al.*, 1993; Rossi *et al.*, 1993; Citovsky *et al.*, 1994; Guralnick *et al.*, 1996; Ziemienowicz *et al.*, 1999; Rhee *et al.*, 2000), while the nuclear import of VirE2 is plant specific in living cells (Citovsky *et al.*, 1992, 1994; Guralnick *et al.*, 1996; Rhee *et al.*, 2000). Consistent with this idea, VirE2 is not recognized by the *Arabidopsis* karyopherin α protein, AtKAP α , which has been shown to mediate nuclear import of VirD2 (Ballas and Citovsky, 1997). Interestingly, VirE2 nuclear localizing ability is sufficient for transport of ssDNA into the plant cell nucleus even in the absence of VirD2 (Zupan *et al.*, 1996) or for genetic transformation of plant cells by an *Agrobacterium* mutant strain lacking the VirD2 nuclear localization signal (NLS) (Gelvin, 1998).

To understand better the molecular mechanism by which VirE2 functions during the *Agrobacterium*–plant cell T-DNA transfer, it would be useful to identify and characterize plant cellular components that specifically associate with VirE2. Here, we used the yeast two-hybrid protein–protein interaction system (Fields and Song, 1989; Hollenberg *et al.*, 1995) to identify and isolate a VirE2-interacting protein, designated VIP1, from *Arabidopsis thaliana*. VIP1 allowed VirE2 to be imported into the

nuclei of living yeast and mammalian cells and was required for VirE2 nuclear import and *Agrobacterium*-induced tumor formation in tobacco plants, participating in early stages of T-DNA expression.

Results

Identification of VIP1

We used the yeast two-hybrid screen (Fields and Song, 1989; Hollenberg *et al.*, 1995) with an *Arabidopsis* cDNA library and the *Agrobacterium* VirE2 protein as bait. Screening of $\sim 3 \times 10^6$ transformants resulted in identification and isolation of several independent cDNA clones producing VirE2 interactors. Two of these clones encoded the same cDNA, designated VIP1 (VirE2-interacting protein 1). The largest clone, representing the full-length cDNA of VIP1, was characterized in detail.

The interaction of VIP1 with VirE2 was specific because it did not occur with DNA topoisomerase I and lamin C, known as non-specific activators in the two-hybrid system best suited to eliminate false-positive interactions (Bartel *et al.*, 1993; Park and Sternglanz, 1998). Figure 1A shows that co-expression of VIP1 and VirE2, but not of topoisomerase I or lamin C, activated the *HIS3* reporter gene. Furthermore, VIP1 did not interact with VirD2 (data not shown), which is thought to function differently from VirE2 during the T-DNA nuclear import (Guralnick *et al.*, 1996). In control experiments, under the non-selective conditions, all combinations of the tested proteins resulted in efficient cell growth (Figure 1B).

In an independent approach, VIP1–VirE2 binding was examined directly using a renatured blot overlay assay for protein–protein interactions (Dorokhov *et al.*, 1999; Chen *et al.*, 2000). In this approach, VIP1 and a negative control protein, VirD2, are electrophoresed (Figure 1C, lanes 1 and 2), immobilized on a PVDF membrane by electroblotting, reacted with purified VirE2, and VirE2 binding is detected using anti-VirE2 antibodies. Figure 1C shows that VirE2 specifically interacted with immobilized VIP1 (lane 3) but not with VirD2 (lane 4). Furthermore, when the blot was probed with unspecific ligands, i.e. purified VirD2 (lanes 5 and 6) or cell–cell movement protein (MP) of tobacco mosaic virus (TMV) (lanes 7 and 8), no binding to VIP1 was observed. These results strengthened the notion that VirE2 specifically recognizes and binds VIP1.

Sequence analysis of the VIP1 cDNA showed that it contained a single open reading frame (ORF) encoding a protein of 261 amino acid residues (see DDBJ/EMBL/GenBank accession Nos AF225983 for VIP1 cDNA and AC009526 for the genomic sequence containing the *VIP1* gene). The deduced amino acid sequence of VIP1 contained a conserved stretch of basic amino acids (basic domain) abutting a heptad leucine repeat (leucine zipper), two structural features characteristic of the basic-zipper (bZIP) proteins (Figure 2). Because plant bZIP proteins are known to localize to the cell nucleus (van der Krol and Chua, 1991), we hypothesized that binding of VIP1 to VirE2 may function to facilitate nuclear import of VirE2. Indeed, the basic domain of the VIP1 bZIP motif contained a consensus sequence for the bipartite NLS (Dingwall and Laskey, 1991) (Figure 2). Interestingly, VIP1 exhibited a modest homology to bZIP proteins from various plant species, such as *Arabidopsis*, tomato, *Paulownia*

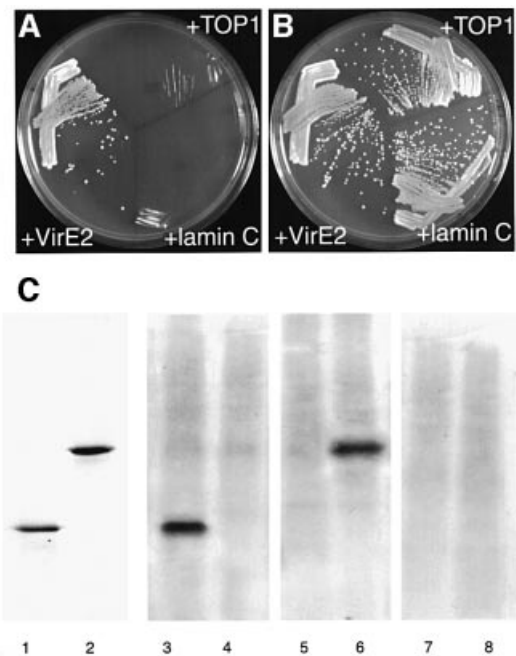


Fig. 1. Specific interaction between VIP1 and VirE2 in the two-hybrid system and *in vitro*. (A) Growth in the absence of histidine, tryptophan and leucine. (B) Growth in the absence of tryptophan and leucine. VIP1 was expressed from pGAD424 whereas VirE2 and negative control interactors lamin C and topoisomerase I (TOP I) were expressed from pBTM16. Growth in histidine-deficient medium represents selective conditions for protein–protein interactions. (C) VirE2 binding to immobilized VIP1 *in vitro*. VIP1 (lanes 1, 3, 5 and 7) and VirD2 (lanes 2, 4, 6 and 8) were electrophoresed, blotted onto a membrane, incubated with VirE2 (lanes 3 and 4), VirD2 (lanes 5 and 6) or TMV MP (lanes 7 and 8) and probed with anti-VirE2, anti-VirD2 or anti-TMV MP antibodies, respectively. Lanes 1 and 2, Coomassie blue staining of VIP1 and VirD2, respectively, after electrophoresis; lanes 3–8, autoradiographs of the binding assays. Protein molecular mass standards are indicated on the left in kDa.

kawakamii, rice and tobacco (Figure 2), whereas no animal or yeast bZIP homologs of VIP1 were found. This finding supports the notion that VIP1 may, at least partly, be responsible for the plant-specific nuclear import of VirE2. To test this hypothesis, we examined whether expression of VIP1 reconstructs nuclear import of VirE2 in non-plant systems.

VIP1 facilitates transport of VirE2 into the nuclei of yeast and mammalian cells

The ability of VIP1 to transport VirE2 into the yeast cell nucleus was examined using a recently developed genetic assay for functional nuclear import (Rhee *et al.*, 2000). In this approach, a gene encoding the bacterial LexA protein was modified (mLexA), abolishing its intrinsic nuclear targeting activity, and fused to a sequence coding for the activation domain of the yeast Gal4p (Gal4AD). If a protein of interest fused to mLexA-Gal4AD (nuclear import assay hybrid, NIA) enters the yeast cell nucleus, it activates the expression of the reporter *HIS3* gene, resulting in cell growth on a histidine-deficient medium (Rhee *et al.*, 2000).

First, VirE2 and VIP1 were tested for their own nuclear import capacity. Figure 3 shows that NIA-VirE2 expressed alone did not promote cell growth, indicating the lack of

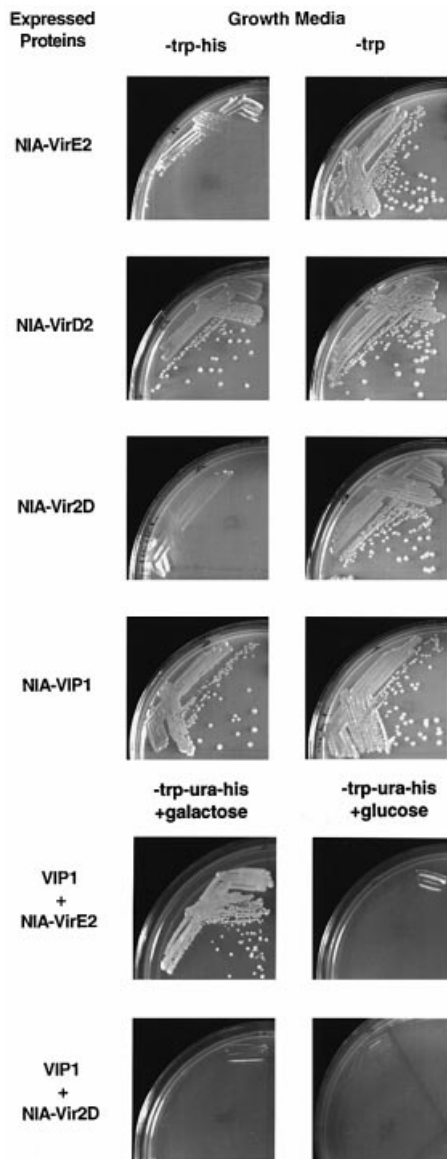


Fig. 3. A functional genetic assay for VIP1-mediated VirE2 nuclear import in yeast cells. Yeast cells expressing the indicated proteins were grown under selective (histidine and tryptophan double-dropout medium) or non-selective conditions (tryptophan single-dropout medium) for nuclear import. For co-expression with VIP1, yeast cells expressing the indicated combinations of tested proteins were grown in a histidine, tryptophan and uracil triple-dropout medium supplemented either with galactose or glucose to induce or repress the VIP1 expression, respectively.

plasmids does not generate or sustain protein concentrations necessary for the complete nuclear import of VirE2. Alternatively, in plants, VIP1 may be augmented by other cellular factors absent from the heterologous mammalian system.

VIP1 antisense plants are resistant to *Agrobacterium*-induced tumor formation

To study the biological role of VIP1 in *Agrobacterium* infection *in planta*, we generated transgenic tobacco plants expressing the VIP1 cDNA in the antisense orientation. A total of 10 independently transformed lines were produced and analyzed as described below. Five lines were not

altered in their susceptibility to *Agrobacterium* infection, whereas the other five lines became largely resistant (data not shown). Here, we describe a detailed analysis of two of these resistant antisense lines, which were first examined for the presence of sense and antisense VIP1 RNA using quantitative RT-PCR and strand-specific oligonucleotide primers (Ni *et al.*, 1998). Figure 5A shows that control, wild-type tobacco plants produced sense (lane 1), but not antisense (lane 2), VIP1 RNA, demonstrating the presence of VIP1 in tobacco (see also Figure 2). Two independent lines of VIP1 antisense plants, which exhibited resistance to *Agrobacterium* (see below), produced the antisense VIP1 RNA (Figure 5A, lanes 4 and 6) and significantly reduced amounts of the sense VIP1 RNA (Figure 5A, lanes 3 and 5). Conversely, VIP1 antisense lines that did not develop *Agrobacterium*, resistance retained high levels of the sense VIP1 transcript (Figure 5A, lane 7) even though the antisense transcript was also expressed (Figure 5A, lane 8).

Quantification of these PCR products (Figure 5C) revealed that the sense VIP1 RNA, synthesized in the *Agrobacterium*-resistant antisense plants (bars 3 and 5), amounted to only 20% of that produced in the wild-type plants (bar 1), whereas the antisense RNA, undetectable in wild-type plants (bar 2), reached 25% of sense VIP1 RNA of the wild-type plants (bars 4 and 6). In *Agrobacterium*-sensitive antisense plants, the levels of the sense VIP1 RNA remained high (98% of the wild-type levels, bar 7) even in the presence of the antisense transcript (20% of sense VIP1 RNA of the wild-type plants, bar 8). In control experiments, analysis of actin-specific transcripts generated similar amounts of PCR products in all samples, indicating equal efficiencies of the RT-PCRs (Figure 5B). Thus, antisense expression of VIP1 cDNA in *Agrobacterium*-resistant transgenic tobacco substantially reduced transcription of the endogenous *VIP1* gene and, by implication, synthesis of the VIP1 protein (data not shown). Furthermore, that *Agrobacterium*-sensitive antisense lines retained high levels of the sense VIP1 transcript supported the correlation between the RT-PCR data and the antisense phenotype.

Next, we tested the ability of the VIP1 antisense plants to develop tumors following inoculation with wild-type, oncogenic *Agrobacterium*. Figure 6A shows that *Agrobacterium* elicited numerous and large tumors on leaf disks derived from the wild-type tobacco plants. Control transgenic plants transformed with an empty vector were equally susceptible to *Agrobacterium*-induced neoplastic growth (Figure 6C), indicating that the procedure used for generation of the transgenic plants did not render them resistant to the subsequent *Agrobacterium* infection. In contrast, two independent transgenic lines of VIP1 antisense plants exhibited a dramatic decrease in their susceptibility to *Agrobacterium* infection. Figure 6B shows that only very few and tiny tumors developed on leaf disks from one of these plants following inoculation with *Agrobacterium*.

Agrobacterium infectivity was then quantified by the number and weight of tumors induced on the inoculated leaf disks. Figure 6D shows the results of these measurements averaged for both antisense lines as compared with the wild-type and transgenic control plants. Wild-type tobacco plants and transgenic control lines supported

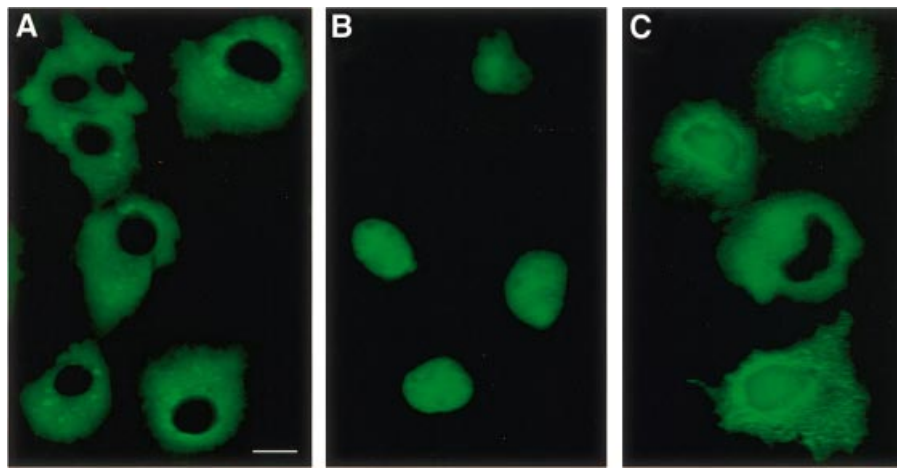


Fig. 4. VIP1-mediated nuclear import of VirE2 in mammalian cells. (A) COS-1 cells expressing GFP-VirE2. Dispersed fluorescence surrounding the signal-free, black cell nucleus represents the cytoplasmic localization of GFP-VirE2. (B) COS-1 cells expressing GFP-VIP1. The fluorescent signal is concentrated exclusively in the cell nucleus. (C) COS-1 cells co-expressing GFP-VirE2 and unlabeled VIP1. In most cells, part of the fluorescent signal enters the nucleus and part remains dispersed in the surrounding areas of the cytoplasm. Bar = 15 μ m.

formation of multiple tumors (4–8 per leaf disk), which expanded and fused into large neoplastic growths (200–300 mg). The VIP1 antisense plants, on the other hand, developed only a very low number (1.0–1.5 per leaf disk) of small tumors (20–40 mg). Thus, the tumor-inducing activity of *Agrobacterium* in VIP1 antisense plants was reduced to ~10% of that observed with the wild-type and transgenic control plants (Figure 6D). Progeny analysis demonstrated that this tumor-resistant phenotype of the VIP1 antisense plants co-segregated with the T-DNA inserts (data not shown).

All VIP1 antisense plants were indistinguishable from the wild-type plants in their morphology and seed viability (data not shown). Furthermore, shoot regeneration from uninfected VIP1 antisense leaf disks cultured on tobacco regeneration medium (Horsch *et al.*, 1985) was identical in its rate and efficiency to that of the wild-type plants (data not shown). Thus, VIP1 antisense expression most probably did not interfere with essential plant cellular functions.

Early stages of T-DNA gene expression are blocked in VIP1 antisense plants

Expression of genes contained on the *Agrobacterium* T-DNA takes place in two stages, early and late. Early gene expression, which reaches its maximum 2–4 days after infection (Janssen and Gardner, 1990; Nam *et al.*, 1999), is transient, occurring from the T-DNA molecules that have not yet integrated into the plant genome. In contrast, late gene expression, which occurs 10–14 days after infection (Janssen and Gardner, 1990), is stable, resulting from the integrated T-DNA. If VIP1 indeed participates in nuclear import of the T-DNA, VIP1 antisense plants are expected to display reduced levels of T-DNA gene expression already early in the infection process, i.e. before the T-DNA integration can take place.

To test this idea, we determined the efficiency of transient T-DNA gene expression by inoculating leaf disks derived from the wild-type and VIP1 antisense plants with *Agrobacterium* carrying on its T-DNA a *uidA* gene encoding a reporter enzyme β -glucuronidase (GUS).

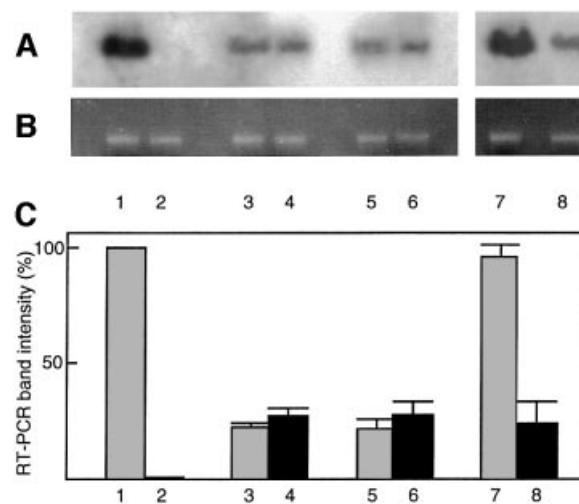


Fig. 5. Quantitative RT-PCR analysis of wild-type and VIP1 antisense plants. (A) Detection of sense and antisense VIP1 RNA. Lanes 1 and 2, RT-PCR of sense and antisense VIP1 RNA in wild-type plants; lanes 3 and 4, RT-PCR of sense and antisense VIP1 RNA in one line of *Agrobacterium*-resistant VIP1 antisense plants; lanes 5 and 6, RT-PCR of sense and antisense VIP1 RNA in another line of *Agrobacterium*-resistant VIP1 antisense plants; lanes 7 and 8, RT-PCR of sense and antisense VIP1 RNA in a line of *Agrobacterium*-sensitive VIP1 antisense plants. (B) Detection of sense actin RNA-specific product in the same samples shown in (A). (C) Quantification of sense and antisense VIP1 RNA. The amount of VIP1-specific RT-PCR products is expressed as a percentage of that obtained using sense VIP1-specific primers in wild-type plants. These data represent average values of three independent experiments with the indicated standard deviations.

Figure 7A shows multiple areas of GUS histochemical staining on leaf disks derived from the wild-type plants, indicating transient T-DNA expression within the *Agrobacterium*-infected cells; identical results were obtained using control transgenic plants (data not shown). VIP1 antisense plants, on the other hand, failed transiently to express GUS contained on the T-DNA (Figure 7B). Both wild-type and VIP1 antisense plants supported GUS expression resulting from biolistic delivery of the *uidA* gene (Figure 7C and D).

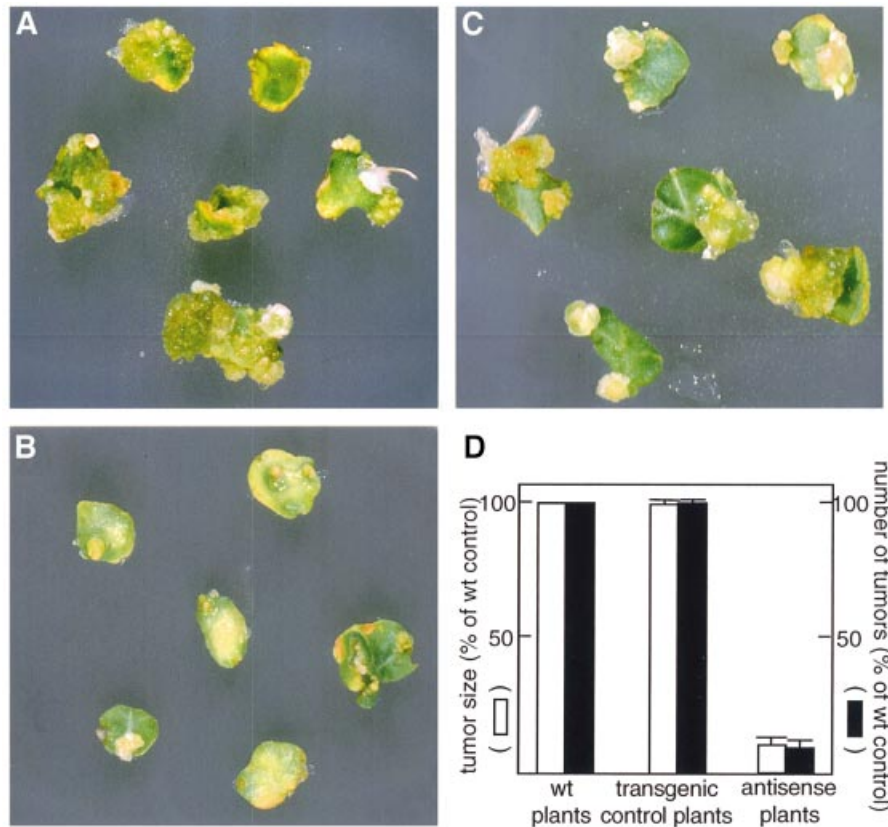


Fig. 6. Reduced tumor formation in *Agrobacterium*-infected VIP1 antisense plants. (A) Leaf disks from the wild-type tobacco plants. (B) Leaf disks from the VIP1 antisense transgenic plants. (C) Leaf disks from the control transgenic plants. (D) Summary of the number and sizes of *Agrobacterium*-induced tumors developed on leaf disks from two independent lines of VIP1 antisense plants and two lines of transgenic control plants relative to the wild-type (wt) plants.

Next, early expression of GUS in two independent VIP1 antisense transgenic lines was quantified using a sensitive fluorimetric assay. Because the *uidA* gene contained on the T-DNA lacked regulatory sequences required for its expression in bacterial cells (Janssen and Gardner, 1990), our measurements represented the GUS activity directed by the T-DNA after its transfer to the plant rather than its potentially leaky expression in *Agrobacterium*. Figure 7E shows that VIP1 antisense plants inoculated with *Agrobacterium* displayed ~20% transient GUS activity compared with the wild-type plants. No quantitative differences in GUS activity were detected when the *uidA* gene was introduced biolistically into the wild-type and VIP1 antisense plants (Figure 7E). Thus, VIP1 antisense plants specifically blocked early stages of the *Agrobacterium*-mediated *uidA* gene transfer but remained competent for efficiently expressing this gene when it was delivered by an *Agrobacterium*-independent technique.

Nuclear import of VirE2 is impaired in VIP1 antisense transgenic plants

To examine directly whether the reduced susceptibility of VIP1 antisense plants to *Agrobacterium*-mediated gene transfer was due to a decrease in VirE2 nuclear import, we compared the accumulation of VirE2 within the cell nuclei of the wild-type and VIP1 antisense tobacco tissues. Figure 8 shows that GUS–VirE2 expressed in the

mesophyll of wild-type tobacco leaves, following biolistic delivery of its encoding gene, accumulated in the cell nucleus (Figure 8A), co-localizing with the nucleus-specific stain, 4',6-diamidino-2-phenylindole (DAPI) (Figure 8B). However, GUS–VirE2 expressed in the leaf mesophyll cells of VIP1 antisense plants remained largely cytoplasmic (Figure 8C and D), supporting the notion of VIP1 involvement in VirE2 nuclear import. Importantly, nuclear import of GUS–VirD2 expressed in wild-type plants (Figure 8E and F) was identical to that in VIP1 antisense tissues (Figure 8G and H), indicating that VirE2 and VirD2 are imported into the host cell nucleus by different mechanisms and that antisense expression of VIP1 does not interfere non-specifically with the nuclear import reactions of the cell. As expected, free GUS expressed in wild-type (data not shown but see Table I, and Citovsky *et al.*, 1992, 1994) and VIP1 antisense plants (Figure 8I and J) remained cytoplasmic. Similarly to our observations in yeast and COS cells (see Figures 3 and 4B, respectively), VIP1 accumulated efficiently in the cell nucleus when expressed in wild-type tobacco plants (Figure 8K and L). Thus, the effect of VIP1 on nuclear transport of VirE2 most probably derives directly from the nuclear import capacity of VIP1 itself. Also, as expected, we detected no differences in nuclear import of GUS–VirE2 and GUS–VirD2 between the wild-type plants and control transgenic plants (data not shown), confirming that

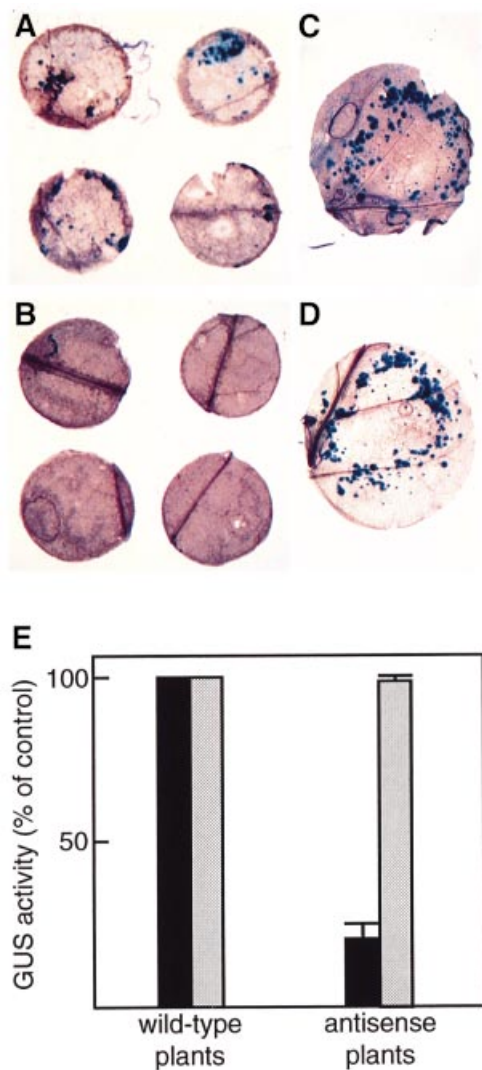


Fig. 7. Reduced transient expression of GUS activity contained within *Agrobacterium* T-DNA in VIP1 antisense plants. (A) Infected leaf disks from the wild-type tobacco plants. (B) Infected leaf disks from the VIP1 antisense transgenic plants. (C) Microbombarded leaf disk from the wild-type tobacco plants. (D) Microbombarded leaf disk from the VIP1 antisense transgenic plants. Note that microbombardment experiments (C and D) required larger leaf disks than that used in *Agrobacterium* inoculations (A and B). (E) Quantification of GUS activity. Black and white bars indicate transient GUS expression in *Agrobacterium*-infected and microbombarded tissues, respectively, derived from two independent lines of VIP1 antisense plants as compared with the wild-type control plants. GUS activity in control, wild-type plants was defined as 100%. All data represent average values of three independent experiments with the indicated standard deviations.

the procedure used for generation of the transgenic plants did not non-specifically affect their capacity for nuclear import.

We then analyzed the efficiency with which GUS–VirD2 and GUS–VirE2 were imported into the cell nucleus in the wild-type and VIP1 antisense plants. Table I demonstrates that, while no nuclear import of free GUS reporter was observed, all GUS–VirD2 accumulated in the nuclei of the expressing cells in both plants. In the wild-type plants, 100% of GUS–VIP1 and 97% of GUS–VirE2 was found within the nucleus, indicating

efficient nuclear import of this protein. In contrast, only 30% of GUS–VirE2 was imported into the nuclei of VIP1 antisense cells (Table I); note that the expression of GUS–VIP1 in these was difficult to determine, potentially due to its suppression by the antisense transgene. These observations further support the role of VIP1 in VirE2 nuclear import and also suggest that a smaller fraction of VirE2 (30%) may enter the plant cell nucleus by another, VIP1-independent pathway. Alternatively, residual amounts of VIP1 in the antisense plants may be responsible for this low level of import.

VIP1 forms a ternary complex with VirE2 and ssDNA

Our results suggest that VIP1 is involved in nuclear import of VirE2 and, by implication, the *Agrobacterium* T-complexes. Thus, VIP1 should be able to interact with VirE2 while the latter is bound to the ssDNA of the T-complex. We tested this assumption *in vitro*, using the agarose gel shift assay to monitor protein–ssDNA binding (Lohman *et al.*, 1986; Citovsky *et al.*, 1992). To approximate native T-complexes better, a relatively long (7.2 kb) M13mp18 ssDNA was used. Figure 9 shows that incubation of this ssDNA probe with VirE2 resulted in a gel shift due to formation of VirE2–ssDNA complexes (compare lanes 1 and 2). These results are consistent with the well-known ssDNA-binding activity of VirE2 (Gietl *et al.*, 1987; Christie *et al.*, 1988; Citovsky *et al.*, 1988, 1992; Das, 1988; Sen *et al.*, 1989). In contrast, incubation of the ssDNA probe with VIP1 produced no gel shift (Figure 9, lane 3), indicating that VIP1 does not bind ssDNA. Addition of VIP1 to the VirE2–ssDNA complexes decreased their electrophoretic mobility (Figure 9, lane 4) as compared with that observed in the absence of VIP1 (lane 2). This gel-shifted signal indeed represented protein–DNA complexes because it was not detected after treatment with proteinase K (Figure 9, lane 5). Thus, VIP1, which by itself was unable to associate with ssDNA, probably formed a ternary complex with VirE2 bound to ssDNA.

Discussion

T-DNA transport into the host cell nucleus is the central event in genetic transformation of plants by *Agrobacterium*. The bacterial protein VirE2 is one of the major players in this process. VirE2 cooperatively binds the transported DNA molecule, shapes it into a transferable form and, together with VirD2, mediates its nuclear uptake (reviewed by Tzfira and Citovsky, 2000; Tzfira *et al.*, 2000). Furthermore, VirE2 alone was shown actively to import ssDNA into the plant cell nucleus (Zupan *et al.*, 1996). Nuclear localization of the nopaline-type VirE2 is especially intriguing because, unlike nuclear import of VirD2, it occurs in plant but not in animal or yeast cells (Citovsky *et al.*, 1992, 1994; Guralnick *et al.*, 1996; Rhee *et al.*, 2000). On the other hand, octopine VirE2 has been shown to enter animal cell nuclei *in vitro* (Ziemienowicz *et al.*, 1999), suggesting that nopaline and octopine VirE2 proteins may differ in their ability to function in non-plant systems. Alternatively, nuclear import of VirE2 in a cell-free system may differ from that within living cells. Interestingly, however, octopine

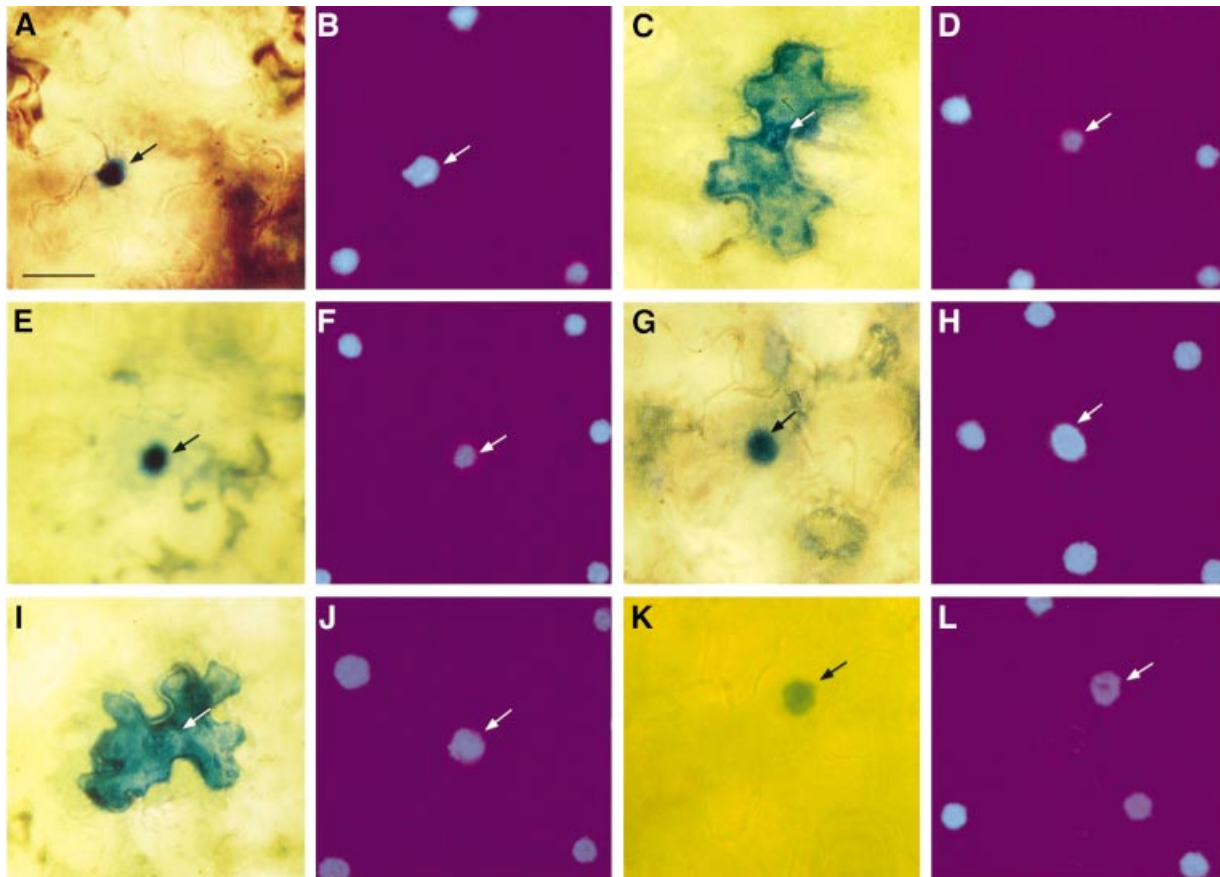


Fig. 8. Nuclear import of GUS–VirE2 and GUS–VirD2 in wild-type and VIP1 antisense plants. (A and B) GUS–VirE2 expressed in wild-type plants. (C and D) GUS–VirE2 expressed in VIP1 antisense plants. (E and F) GUS–VirD2 expressed in wild-type plants. (G and H) GUS–VirD2 expressed in VIP1 antisense plants. (I and J) Free GUS expressed in VIP1 antisense plants. (K and L) GUS–VIP1 expressed in wild-type plants. (A, C, E, G, I and K) GUS staining. (B, D, F, H, J and L) DAPI staining. Bar = 25 μ m.

VirE2 function in animal cells may still be impaired because it was unable to mediate nuclear import of ssDNA in this system (Ziemienowicz *et al.*, 1999).

The lack of nuclear import of VirE2 in living animal and yeast cells suggests involvement of plant-specific host cellular factors that interact with VirE2. Using the yeast two-hybrid system to probe protein–protein interactions (Fields and Song, 1989), we have isolated an *Arabidopsis* cDNA that encodes a VirE2-interacting protein. The deduced amino acid sequence of this protein, designated VIP1, contained a bZIP motif composed of a long basic domain followed by a leucine zipper, seven leucine repeats evenly separated from each other by six amino acid residues. Although the bZIP sequence is found in many transcription factors (van der Krol and Chua, 1991), the protein database search did not identify any yeast or animal homologs of VIP1. In contrast, bZIP proteins from several diverse plant species showed homology to VIP1. That VIP1 was more related to bZIP proteins of plant rather than animal or yeast origin and that plant bZIP proteins are known to localize to the cell nucleus (van der Krol and Chua, 1991) suggested (i) a role for VIP1 in plant-specific nuclear import of VirE2 and (ii) that bZIP motifs *per se* also found in non-plant proteins are not sufficient to mediate the VirE2 nuclear import.

Genetic and confocal microscopy experiments indeed demonstrated that VIP1 localized efficiently to the nucleus

Table I. Efficiency of nuclear localization of GUS–VirE2 and GUS–VirD2 in wild-type and VIP1 antisense plants

	GUS activity (% of maximal)	
	Nucleus	Cytoplasm
Wild-type plants		
GUS–VirE2	97 (2)	3 (2)
GUS–VirD2	100 (8)	0
GUS alone	0	100 (6)
GUS–VIP1	100 (2)	0
VIP1 antisense plants		
GUS–VirE2	30 (7)	70 (7)
GUS–VirD2	100 (4)	0
GUS alone	0	100 (5)

The data are derived from spectral analysis of photographic images of 20 independent GUS-positive cells; average values are given, and standard error values are indicated in parentheses. All other conditions were as described in Materials and methods and in Citovsky *et al.* (1992) and Howard *et al.* (1992).

in yeast and mammalian cells. Co-expression of VIP1 with VirE2 in these non-plant systems allowed VirE2 to be imported into the cell nucleus, suggesting that VIP1 may represent a cellular factor involved in the plant-specific nuclear uptake of VirE2. However, because the VIP1-

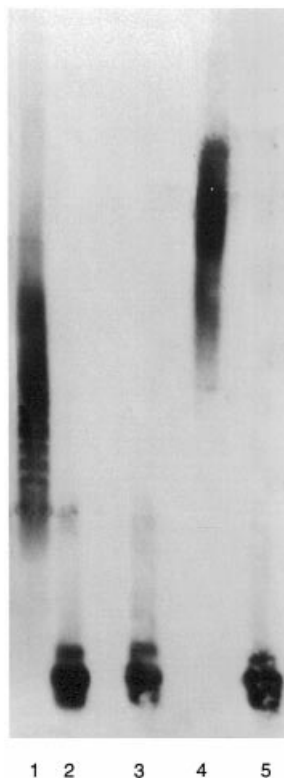


Fig. 9. *In vitro* formation of ternary complexes between VIP1, VirE2 and ssDNA. Gel shift assays were performed as described in Materials and methods. Lane 1, ssDNA incubated with VirE2; lane 2, ssDNA incubated alone; lane 3, ssDNA incubated with VIP1; lane 4, ssDNA incubated with VirE2 and VIP1; lane 5, ssDNA incubated with VirE2 and VIP1 and treated for 30 min at 37°C with 1 mg/ml of proteinase K.

mediated VirE2 nuclear import in mammalian cells was, although significant, still incomplete, other proteins may be involved in the VirE2 import pathway *in planta*, augmenting the VIP1 function. Facilitation of VirE2 nuclear import by VIP1 in yeast and mammalian cells also indicates that these two proteins specifically recognize and bind each other *in vivo*, within the cell cytoplasm.

If VIP1 is required for nuclear import of VirE2, which, in turn, is required for *Agrobacterium* tumorigenicity (Citovsky *et al.*, 1992; Gelvin, 1998), inactivation of VIP1 should lead to a decrease in *Agrobacterium*-induced tumor formation. This notion was tested using transgenic tobacco plants in which the expression of their *VIP1* gene was repressed by antisense expression of the *Arabidopsis* *VIP1* cDNA. The rationale for this heterologous antisense approach is 2-fold. First, it has been successful both in plant and animal systems; for example, antisense expression of an alfalfa gene suppressed its tobacco homolog (Schiene *et al.*, 2000) and a human gene was repressed by antisense expression of its mouse homolog (Oku *et al.*, 1998). This is because antisense expression of a transgene is well known to silence not only the same gene but also its closely related homologs (reviewed by Vaucheret *et al.*, 1998). Secondly, tobacco plants are much more suitable for standard assays of *Agrobacterium*-host interaction, e.g. tumorigenicity and transient and stable T-DNA expression, than *Arabidopsis* plants. Our results demon-

strated that one-half of all produced VIP1 antisense tobacco plants (five out of 10 lines) exhibited a considerably reduced production of the VIP1 RNA. That not all transgenic lines develop antisense suppression is common and has been observed before with other transgenes (see, for example, Stam *et al.*, 2000). Importantly, all antisense plants in which VIP1 expression was reduced developed a remarkable resistance to infection by *Agrobacterium*. *Agrobacterium* resistance of the VIP1 antisense plants was due to a blockage in early stages of T-DNA gene expression, which precede integration. Because these plants also failed to support efficient nuclear import of VirE2, we suggest that VIP1 activity during nuclear import of the invading T-complexes is a prerequisite for both transient T-DNA gene expression and tumor formation.

Specifically, we propose that VIP1 represents a cellular interactor for *Agrobacterium* VirE2. During *Agrobacterium* infection, VIP1 possibly recognizes VirE2 within the host cell cytoplasm. Because VIP1 itself is a nuclear protein, which is probably imported into the nucleus through the basic domain of its bZIP motif, binding of VIP1 to VirE2 may result in a 'piggyback' transport of VirE2 into the host cell nucleus. VirE2, in turn, is presumed to be bound cooperatively to the T-strand (Citovsky *et al.*, 1989; Sen *et al.*, 1989); thus, VIP1, most probably in concert with other cellular factors, may participate in the nuclear import of the entire *Agrobacterium* T-complex. This idea is consistent with our observations that VIP1 can bind to the VirE2-ssDNA complexes *in vitro*.

Interestingly, another protein component of the T-complex, VirD2, is transported into the plant cell nucleus by AtKAP α , a member of the *Arabidopsis* karyopherin α family (Ballas and Citovsky, 1997). That AtKAP α does not interact with VirE2 (Ballas and Citovsky, 1997) whereas VirD2 is not recognized by VIP1 suggests involvement of two distinct pathways in the T-complex nuclear import. Indeed, nuclear import of VirE2 but not of VirD2 was compromised in the VIP1 antisense plants. This dual pathway transport mechanism makes biological sense. It minimizes competition between a single molecule of VirD2 at the 5' end of the T-strand and the more abundant VirE2 that coats the rest of the ssDNA molecule. Thus, VirD2 would have a better chance to lead the T-complex into the nucleus and specify its import polarity, which is thought to be important for the subsequent integration event (Zambryski, 1992; Tinland and Hohn, 1995; Sheng and Citovsky, 1996; De Neve *et al.*, 1997).

In uninfected cells, similarly to many other plant bZIP proteins (van der Krol and Chua, 1991), VIP1 may be involved in transcription, associating with the chromosomal DNA either directly or through other components of transcription complexes. Thus, it is tempting to speculate that VIP1 and VirE2 may function in a multiprotein complex, which performs a dual function: it first facilitates nuclear targeting of VirE2 and then mediates intranuclear transport of VirE2 and its cognate T-strand to the site of integration. A similar dual role in nuclear and intranuclear transport has been suggested for the yeast Kap114p protein that functions to import the TATA-binding protein (TBP) into the cell nucleus and target it to the promoters of genes to be transcribed (Pemberton *et al.*, 1999). This model for VIP1-mediated intranuclear transport addresses the long-

standing question of how the invading T-complex finds its way to the host genome. Furthermore, because VIP1 probably interacts with the host cell chromatin during transcription, it may bring the T-complex to chromosomal regions where the host DNA is more exposed and, thus, more suitable for T-DNA integration.

Materials and methods

Plasmid construction

To use as bait in the yeast two-hybrid system, nopaline VirE2 ORF (Citovsky *et al.*, 1992) was amplified by PCR as a *Bam*HI fragment and fused with LexA by cloning into pBTM116 (TRP1+, Hollenberg *et al.*, 1995), producing pBTM116-VirE2.

For studies of nuclear import in yeast cells, VIP1, VirE2 (Citovsky *et al.*, 1992) and VirD2 ORFs (Ballas and Citovsky, 1997) were amplified by PCR and subcloned as *Bam*HI-*Sall*I, *Bam*HI-*Xho*I or *Bam*HI fragments, respectively, into the NIA plasmid pNIA (TRP1+; Rhee *et al.*, 2000), producing pNIA-VIP1, pNIA-VirE2 and pNIA-VirD2 constructs. VirD2 ORF was also inserted in the reverse orientation, resulting in the pNIA-VirD2 plasmid. For co-expression with VirE2, VIP1 ORF was subcloned as a PCR-amplified *Sall*I-*Bam*HI fragment into a galactose-inducible expression vector pSJ101 (UrA3+; Kironmai *et al.*, 1998), producing pSJ101-VIP1.

For expression in mammalian cells, VIP1 and VirE2 ORFs were PCR amplified and fused as *Sall*I-*Bam*HI or *Xho*I-*Bam*HI fragments, respectively, to the GFP reporter gene in pEGFP-C1 (Clontech), resulting in pEGFP-C1-VIP1 and pEGFP-C1-VirE2 constructs. For co-expression with GFP-VirE2, the VIP1 ORF was cloned as a *Sall*I-*Bam*HI fragment into a mammalian expression vector pCB6 that contains a cytomegalovirus promoter (obtained from Dr D. Brown, SUNY, Stony Brook), producing pCB6-VIP1.

Free GUS, GUS-VirE2 and GUS-VirD2 were expressed in plant tissues from pRTL2-GUS (Restrepo *et al.*, 1990), pRTL-GUSE2 (Citovsky *et al.*, 1992) and pGD plasmids (Howard *et al.*, 1992), respectively. Finally, for generation of transgenic VIP1 antisense plants, the VIP1 ORF was first inserted in reverse orientation as a PCR-amplified *Sall*I fragment into a plant expression vector, pCd, containing the 35S promoter of cauliflower mosaic virus, tobacco mosaic virus translational enhancer (Gallie *et al.*, 1989) and the nopaline synthase poly(A) signal. Then, the entire antisense expression cassette was subcloned as a *Bam*HI-*Xba*I fragment into the binary vector pBIN19, carrying a kanamycin selection marker, to produce pBIN19/VIP1-AS. All PCRs were performed using a high fidelity *Pfu* DNA polymerase (Promega) and their products were verified by dideoxynucleotide sequencing (Kraft *et al.*, 1988).

Yeast two-hybrid assay

Saccharomyces cerevisiae strain L40 [*MATa his3Δ200 trp1-901 leu2-3 112 ade2 lys2-801am URA3::(lexAop)8-lacZ LYS2::(lexAop)4-HIS3*; Hollenberg *et al.*, 1995] was grown in yeast extract/peptone/dextrose or the appropriate selective minimal medium using standard conditions (Kaiser *et al.*, 1994). Plasmids were introduced into yeast cells using a standard lithium acetate protocol (Kaiser *et al.*, 1994).

The *A.thaliana* (ecotype Colombia) cDNA library in pGAD424 (LEU3+, Clontech) (Ballas and Citovsky, 1997) was screened with pBTM116-VirE2 as bait as described (Hollenberg *et al.*, 1995; Ballas and Citovsky, 1997) and positive clones were selected on a histidine-deficient selective medium and confirmed by the β -galactosidase assay as described (Durfee *et al.*, 1993). False-positives were eliminated using human lamin C and topoisomerase I, known to function as non-specific activators in the two-hybrid system (Bartel *et al.*, 1993; Hollenberg *et al.*, 1995), as baits.

VIP1-VirE2 binding in vitro

Renatured blot overlay assay (Dorokhov *et al.*, 1999; Chen *et al.*, 2000) was used to examine interaction between VIP1 and VirE2 *in vitro*. Briefly, VirE2, VIP1, VirD2 and TMV MP were purified to near homogeneity (95–98% pure as determined by silver-stained SDS-polyacrylamide gels) following expression in *Escherichia coli* (Citovsky *et al.*, 1988, 1989, 1990; Chen *et al.*, 2000). VIP1 and VirD2 were then electrophoresed through a 12.5% SDS-polyacrylamide gel, electroblotted onto a PVDF membrane, depleted of SDS by guanidine hydrochloride extraction, renatured, blocked with 1% bovine serum albumin (BSA) and incubated with 10 μ g/ml of VirE2 as described (Chen *et al.*, 2000). Following

incubation, the membrane was washed and protein binding was detected using anti-VirE2 antibody and the ECL western blotting kit (Amersham). In control experiments, blotted VIP1 and VirD2 were incubated with purified VirD2 or TMV MP, and protein binding was detected using anti-VirD2 or anti-TMV MP antibodies, respectively.

Nuclear import in yeast and mammalian cells

For yeast nuclear import, L40 cells were transformed with pNIA-VIP1, pNIA-VirE2, pNIA-VirD2 or pNIA-VirD2 and grown in the presence or absence of histidine; histidine prototrophy indicated functional nuclear import (Rhee *et al.*, 2000). To examine the effect of VIP1 on nuclear import proteins expressed from pNIA constructs, they were co-transformed with pJS101-VIP1 into L40 cells and VIP1 expression was induced by growing cells in the presence of 10 mg/ml galactose.

For nuclear import in mammalian cells, pEGFP-C1-VIP1, pEGFP-C1-VirE2 or a mixture of pEGFP-C1-VirE2 and pCB6-VIP1 were introduced into 24-h-old COS-1 cells (2.5×10^4 cells/cm²) grown on 20 mm coverslips in 6-well tissue culture plates. Prior to transformation, cells were grown for 4 h at 37°C in 3 ml of fresh Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (BCS). The cells were then transformed with the expression constructs using FuGENE 6 (Roche) according to the manufacturer's instructions, and cultured for 24 h at 37°C to allow expression of the transfected genes. To visualize GFP expression, cells were washed briefly in phosphate-buffered saline (PBS), mounted on a fresh coverslip and observed under a Zeiss LSM 410 laser scanning confocal microscope equipped with a 485 nm excitation argon laser and a 527 nm GFP emission filter. For quantification of nuclear import, the signal intensity of intranuclear and cytoplasmic fluorescence was determined using the recorded images.

Generation of VIP1 antisense tobacco plants and assays for Agrobacterium tumorigenicity and transient T-DNA gene expression

The binary vector pBIN19/VIP1-AS was introduced into the disarmed *Agrobacterium* strain EHA105, which was then used to transform tobacco plants (*Nicotiana tabacum* cv. Turk) as described (Horsch *et al.*, 1985). Transgenic tobacco plants expressing VIP1 in the antisense orientation were selected on a kanamycin-containing medium and maintained for 1 month in sterile conditions on an MS basal medium (Murashige and Skoog, 1962) with no exogenous growth regulators. For transgenic controls, the plants were transformed with an empty pBIN19 binary vector and maintained under the same conditions as the antisense plants. Plants were then transferred to soil in a greenhouse, allowed to set seed, and the transgenic progeny were selected by germinating the seeds on MS agar in the presence of kanamycin.

For tumor assays, 9 mm disks were excised from mature leaves of 1-month-old wild-type plants and transgenic control and VIP1 antisense plants, submerged in an overnight culture of *Agrobacterium* strain C58 (OD₆₀₀ = 0.5), and incubated for 30 min at 25°C. The disks were then transferred to a hormone-free MS medium (Murashige and Skoog, 1962). After cultivation for 48 h at 25°C, the disks were washed three times in sterile distilled water, blotted dry and cultured on a hormone-free MS medium supplemented with 300 mg/l carbenicillin, to eliminate the remaining *Agrobacterium*. Four weeks later, the developed tumors were counted and their weight was determined.

For transient T-DNA gene expression, the leaf disks were co-cultivated for 48 h at 25°C on a hormone-free MS medium with *Agrobacterium* strain EHA105 (OD₆₀₀ = 0.5) harboring a GUS-expressing binary vector pKIWI105 (Janssen and Gardner, 1990). For histochemical detection of GUS activity, the leaf disks were stained with the chromogenic substrate X-Gluc as described (Nam *et al.*, 1999). For quantitative analysis, 20 leaf disks per experimental condition were combined, ground and assayed for GUS activity using the fluorescent substrate 4-methylumbelliferyl β -D-galactoside (MUG) as described (Nam *et al.*, 1999). In control experiments, GUS activity was determined in leaf disks microbombarded with pRTL2-GUS (Carrington *et al.*, 1991) followed by incubation for 24 h at 25°C to express the transfected DNA. All experiments were performed in triplicate and the resulting data represent average values with the indicated standard deviations.

Quantitative RT-PCR

Total RNA was extracted from 2.0 g of leaf tissue, treated with RQ1 RNase-free DNase and reverse-transcribed with M-MLV reverse transcriptase using strand-specific primers (i.e. forward or reverse to detect antisense or sense transcripts, respectively) derived from VIP1 and tobacco actin (DDBJ/EMBL/GenBank accession No. X63603; Thangavelu *et al.*, 1993) gene sequences. The resulting cDNAs were

PCR amplified as described (Kang *et al.*, 1995; Ni *et al.*, 1998), using a mixture of both forward and reverse primers. VIP1-specific RT-PCR products were then detected by Southern blot analysis using DIG-labeled full-length VIP1 cDNA as probe followed by autoradiography. The intensity of hybridized signals was quantified by scanning densitometry of the autoradiograms. Actin-specific RT-PCR products in the same reaction mixtures were detected by ethidium bromide staining of agarose gels. VIP1 forward and reverse primers, 5'-CGAACGGTGTGTTC-CTCCTAATCTCTT-3' and 5'-GCTCAGCAAGTCTATCACC-3', respectively, generated a 290 bp product, while actin forward and reverse primers, 5'-TCACTGAAGCACCTCTTAACC-3' and 5'-CAGCTCCA-TTCCAATCATTG-3', respectively, generated a 500 bp product. Note that the forward VIP1 primer was designed to span one of the introns predicted based on the genomic VIP1 sequence on *Arabidopsis* chromosome I (DDBJ/EMBL/GenBank accession No. AC009526).

Microbombardment

For expression of GUS-VirE2 and GUS-VirD2 fusion proteins, and GUS alone in plant tissues, 2 µg of DNA was adsorbed onto 50 µl of 1 µm gold particles according to the instructions of the manufacturer (Bio-Rad, CA) and microbombarded into the leaf mesophyll of greenhouse-grown wild-type and VIP1 antisense tobacco plants. Microbombardment was performed at a pressure of 150 p.s.i. using a portable Helios gene gun system (Model PDS-1000/He; Bio-Rad, CA). After incubation for 16 h at 25°C to allow expression of the transfected DNA, the leaf disks were stained for GUS activity for 3 h (Nam *et al.*, 1999) and observed under a Zeiss Axiophot microscope. DAPI staining was used to verify the location of the cell nucleus. Each experiment was repeated at least six times. The intensity of indigo dye formed during the GUS assay in the cell cytoplasm and nucleus was quantified by photodensitometry of the images recorded on a 35 mm film exactly as described (Citovsky *et al.*, 1992; Howard *et al.*, 1992). Nuclear localization of GUS-VirD2 was defined as 100% activity, and GUS alone was defined as 0% activity. Average values and standard deviations were calculated based on 20 independent measurements.

Gel shift assay for protein-ssDNA binding

Purified VirE2 (3 µg) and/or VIP1 (10 µg) proteins were incubated for 30 min at 4°C in 30 µl of buffer L [10 mM Tris-HCl pH 8.0, 200 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride (PMSF)] with 0.25 µg of M13mp18 ssDNA (New England Biolabs). For formation of VIP1-VirE2-ssDNA complexes, VirE2 was first allowed to bind to ssDNA for 10 min at 4°C and then VIP1 was added, and the incubation continued for another 20 min. After incubation, samples were electrophoresed on a 0.3% agarose gel as described (Lohman *et al.*, 1986; Citovsky *et al.*, 1992) and analyzed by Southern blotting (Ausubel *et al.*, 1987), using an M13mp18 ssDNA labeled with DIG according to the manufacturer's instructions (Amersham).

DDBJ/EMBL/GenBank accession number

The accession number for the sequence reported in this paper is AF225983.

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