# The Plant VirE2 Interacting Protein 1. A Molecular Link between the Agrobacterium T-Complex and the Host Cell Chromatin?[1]

**Abraham Loyter, Joseph Rosenbluh, Nehama Zakai, Jianxiong Li, Stanislav V. Kozlovsky, Tzvi Tzfira, and Vitaly Citovsky***

Department of Biological Chemistry, Alexander Silberman Institute of Life Sciences, Hebrew University of Jerusalem, 91904 Jerusalem, Israel (A.L., J.R., N.Z.); and Department of Biochemistry and Cell Biology, State University of New York, Stony Brook, New York 11794–5215 (J.L., S.V.K., T.T., V.C.)

The microbe *Agrobacterium tumefaciens* is harmful to plants and useful to scientists for one and the same reason: It transfers DNA into plant genomes. Found in soil worldwide, Agrobacterium causes disease in plants by transferring its own DNA into plant cells. But in the laboratory, the ability to move foreign genes into plants has made the microbe a standard tool for investigating plant genetics and modifying crops. During genetic transformation, a single stranded copy (T-strand) of the bacterial transferred DNA (T-DNA) and several virulence (Vir) proteins are exported from Agrobacterium into the plant cell cytoplasm, within which a mature transport (T) complex is assembled that contains a T-strand molecule covalently attached at its 5′-end to a single molecule of the VirD2 protein and packaged by multiple molecules of VirE2 into a telephone cord-like coiled structure. This complex is then imported into the plant cell nucleus with the help of both VirD2 and VirE2. Once inside the nucleus, the T-complex is targeted to the plant chromatin, uncoated of its protein components, and integrated into the host DNA, which must also be exposed for integration (for review, see Zupan et al., 2000; Tzfira and Citovsky, 2002; Gelvin, 2003). The molecular pathways by which most of these diverse steps of the transformation process occur have been identified; for example, DNA and proteins are exported from Agrobacterium via the type IV secretion system (for review, see Christie, 2004), the T-complex is imported into the host cell nucleus by the karyopherin $\alpha$-dependent pathway (for review, see Tzfira and Citovsky, 2002) and is likely uncoated by targeted proteolysis via the Skp1/Cdc53-cullin/F-box pathway (Tzfira et al., 2004). But how T-complex is targeted to the host chromatin remains completely unknown. Here, we offer a hypothesis that the Arabidopsis (*Arabidopsis thaliana*) VirE2 Interacting Protein 1 (AtVIP1), which associates with the T-complexes by binding to their VirE2 component (Tzfira et al., 2001), acts as a molecular link between the T-complex and the histone constituents of the host chromatin. This model is based on our observations that AtVIP1 interacts with histones in vitro and in planta.

## ATVIP1 BINDS CORE HISTONES IN VITRO

To examine AtVIP1-histone interactions in vitro, we first used a quantitative ELISA-based protein interaction assay, in which histone is immobilized on a plastic surface, incubated with AtVIP1 tagged with biotinylated bovine serum albumin (bBSA) in the presence of excess of free BSA to quench nonspecific interactions, and the degree of binding is quantified using peroxidase-conjugated avidin (Citovsky et al., 2004). Because animal histones are easily available and are homologous, especially H3 and H4, to plant histones, and plant and animal chromatin have similar nucleosomal structures (for review, see Goodrich and Tweedie, 2002), we utilized purified Xenopus histones H2A, H2B, H3, and H4 (Luger et al., 1997) in our initial experiments. Figure 1A shows that AtVIP1 bound H2A in a dose-dependent and saturable fashion; the binding was strong, with a low apparent dissociation constant ($K_d$ value) of 18 nM. These characteristics are typical for specific protein-protein interactions (e.g. Hu and Jans, 1999; Sondermann et al., 2000).

Because histones are sticky proteins (Muhlhausser et al., 2001), it was important to further address the specificity of the AtVIP1-H2A interaction. One of the standard criteria for binding specificity is inhibition by a specific unlabeled competitor and lack of inhibition by a nonspecific competitor (e.g. Sondermann et al., 2000). Here, we used unlabeled AtVIP1 as specific competitor, whereas BSA chemically coupled to the SV40 large T-antigen nuclear localization signal (BSA-

[1] This work was supported by the U.S.-Israel Binational Science Foundation (grant to A.L. and V.C). The work in our labs is also supported by the National Institutes of Health, National Science Foundation, U.S. Department of Agriculture (grants to V.C.), the U.S.-Israel Binational Agricultural Research and Development Fund (grants to V.C., T.T., and A.L.), and the Human Frontiers Science Program (grant to T.T.).

* Corresponding author; e-mail vitaly.citovsky@stonybrook.edu; fax 631–632–8575.

www.plantphysiol.org/cgi/doi/10.1104/pp.105.062547.

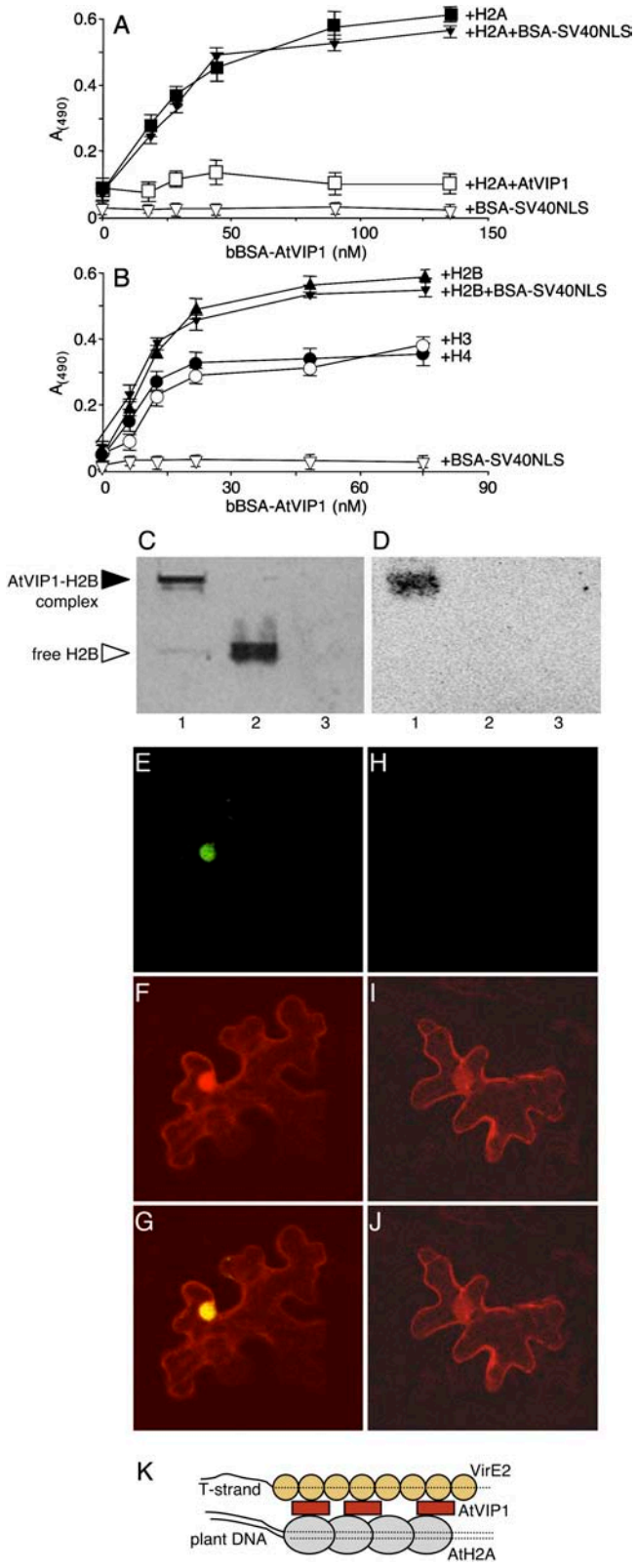


**Figure 1.** A, Immobilized *Xenopus* H2A coincubated with the indicated amounts of bBSA-AtVIP1 (black squares) or bBSA-AtVIP1 and 200 molar excess of either unlabeled specific competitor, AtVIP1, (white squares) or unlabeled nonspecific competitor, BSA-SV40NLS conjugate (black inverted triangles). B, Immobilized Xenopus H2B (black triangles), H3 (white circles), or H4 (black circles) coincubated with the indicated amounts of bBSA-AtVIP1. Immobilized H2B was also coincubated with the indicated amounts of bBSA-AtVIP1 in the presence of 200 molar excess of either unlabeled AtVIP1 (white triangles) or unlabeled BSA-SV40NLS (black inverted triangles). For negative control, immobilized BSA-SV40NLS was coincubated with the indicated amounts of bBSA-AtVIP1 (white inverted triangles). SDS based on at least three independent experiments are indicated for each experimental condition. C and D, Detection of Xenopus H2B-AtVIP1 binding by native PAGE. C, Detection of biotinylated H2B (b-H2B); D, detection of AtVIP1. Lane 1, b-H2B+AtVIP1; lane 2, b-H2B alone; lane 3, AtVIP1 alone. Black and white arrowheads indicate the H2B-AtVIP1 complex and free H2B, respectively. Note that, in a native gel, free AtVIP1 moved in the direction opposite of H2B and run off, making its detection impossible. E to G, nYFP-AtVIP1, cYFP-AtH2A, and free DsRed2. H to J, nYFP-AtVIP1, unfused cYFP, and free DsRed2. D and G show YFP signal, E and H show DsRed2 signal, and F and I show merged signals. All images are single confocal sections. K, A diagram suggesting how AtVIP2 may juxtapose and the T-complex packaged by VirE2 to the plant DNA packaged by histones (e.g. AtH2A). Note that while biological evidence indicates that AtVIP1 can form ternary complexes with VirE2 and VirF, a bacterial F-box protein likely involved in proteasomal uncoating of the T-complex (Tzfira et al., 2004), the ability of AtVIP1 to bind both VirE2 and host histones remains to be demonstrated. Xenopus histones and AtVIP1 were expressed in *Escherichia coli* and purified (Luger et al., 1997, 1999; Citovsky et al., 2004), tagged with bBSA, and the ELISA-based binding assays were performed exactly as described (Citovsky et al., 2004). Apparent dissociation constants ($K_d$ values) were calculated as concentrations of AtVIP1 that yielded half-maximal binding as described (Hu and Jans, 1999). For binding on an 8% native polyacrylamide gel, proteins resolved by electrophoresis from the positive to negative pole were stained with Coomassie Brilliant Blue, and the relative mobility of H2B directly tagged with biotin and AtVIP1 was assayed by western-blot analyses. For BiFC, YFP was dissected into two parts: the N-terminal part (nYFP) that terminated at amino acid residue 174, and the C-terminal part (cYFP) that began with a Met residue preceding the residue 175 of YFP. For pSAT6-nYFP-C1 and pSTA6-cYFP-C1, PCR-amplified nYFP and cYFP were ligated into sites *Nco*I-*Bam*HI of pSAT6-EYFP-C1 (accession no. AY818380; Tzfira et al., 2005), replacing enhanced YFP (EYFP). For cYFP fusion, AtH2A-1 (accession no. AF204968.1) was cloned into the *Sal*I-*Bam*HI sites of pSTA6-cYFP, and for nYFP fusion, AtVIP1 was cloned into the *Sal*I-*Bam*HI sites of pSTA6-nYFP-C1. The tested pairs of constructs were cobombarded into tobacco leaves together with pGDR that expresses free DsRed2 (Goodin et al., 2002) followed by confocal microscopy as described (Tzfira et al., 2004; Lacroix et al., 2005). The transformation efficiency was estimated by the number of DsRed2 expressing cells; each experiment examined 50 to 100 DsRed2-expressing cells and was repeated ≥3 times, with 30% to 40% of the transformed cells exhibiting BiFC.

SV40NLS) was used as nonspecific competitor; BSA-SV40NLS was chosen because it is unrelated to AtVIP1 on the one hand, but, like AtVIP1, it is a soluble protein carrying an active basic-type NLS. Figure 1A shows that the presence of unlabeled AtVIP1 significantly (by about 80%) inhibited binding. In contrast, the presence of BSA-SV40NLS had no effect on the AtVIP1-H2A interaction across the entire range of the AtVIP1 concentrations used in the binding assay. Furthermore, BSA-SV40NLS also did not bind AtVIP1 when used as an immobilized ligand rather than as competitor (Fig. 1A).

Next, we examined AtVIP1 interaction with other core histones. Figure 1B shows that AtVIP1 also bound H2B, H3, and H4. While the overall binding saturation levels were higher with H2B than with H3 and H4, all of them possessed high binding affinities to AtVIP1, with $K_d$ values of approximately 10 nM (Fig. 1B). Similarly to the AtVIP1-H2A interaction, AtVIP1 binding to H2B, H3, and H4 was inhibited by unlabeled AtVIP1, but not by BSA-SV40NLS (Fig. 1B, and data not shown).

We then used native PAGE and western-blot analysis as an independent in vitro assay for binding of AtVIP1 to H2B; in this approach, the samples were analyzed for the presence of H2B and AtVIP1. Coincubation of AtVIP1 and H2B resulted in formation of protein complexes (Fig. 1C, lane 1, upper band, and Fig. 1D, lane 1) with electrophoretic mobility much lower than that of free H2B (Fig. 1C, lane 2). Binding of H2B to AtVIP1 was incomplete as indicated by the presence of residual amounts of unbound H2B (Fig. 1C, lane 1, lower band). As expected, no retarded AtVIP1-H2B complexes were observed with H2B incubated alone (Fig. 1, C and D, lanes 2). Free AtVIP1 electrophoresed on a native gel moved in the direction opposite of H2B and, thus, run off the gel, preventing its detection when not in complex with H2B (Fig. 1, C and D, lanes 3). Detection of H2B (Fig. 1C, lane 1) and AtVIP1 (Fig. 1D, lane 1) in the same samples confirmed that the protein complexes with reduced electrophoretic mobility indeed contained both H2B and AtVIP1. Similar results were obtained with H2A, H3, and H4 histones (data not shown). Collectively, our data suggest that the association of AtVIP1 with histones observed in our in vitro binding assays represent specific protein interactions.

## ATVIP1 BINDS H2A IN PLANTA

We confirmed the AtVIP1-H2A interaction in planta, using the bimolecular fluorescence complementation (BiFC) assay; in this approach, a molecule of yellow spectral variant of GFP (YFP) is separated into two parts, N-terminal (nYFP) and C-terminal (cYFP), neither of which fluoresces when expressed alone, but the fluorescence is restored when nYFP and cYFP are brought together as fusions with interacting proteins (Hu et al., 2002; Bracha-Drori et al., 2004; Tzfira et al., 2004; Walter et al., 2004; Lacroix et al., 2005). The interaction between AtVIP1 and Arabidopsis AtH2A was examined in tobacco (*Nicotiana tabacum*) leaf cells, and the location of the cell nucleus was determined using coexpression of free DsRed2, a small protein that partitions between the cytoplasm and the nucleus, conveniently visualizing and identifying both of these cellular compartments (Goodin et al., 2002). Figure 1 shows that nYFP-tagged AtVIP1 interacted with cYFP-tagged AtH2A, producing a strong nuclear signal of the reconstructed YFP (Fig.1E), colocalizing with the nuclear DsRed2 in the same cell (Fig. 1F); indeed, the merged image of the reconstructed YFP and DsRed2 showed overlapping signal (Fig. 1G) within the cell nucleus, confirming the predominantly nuclear location of the interacting AtVIP1 and AtH2A molecules. In negative control experiments, no YFP signal was detected following coexpression of nYFP-AtVIP1 with unfused cYFP (Fig. 1, H–J) or cYFP-AtH2A with unfused cYFP (data not shown).

## DISCUSSION

We propose that AtVIP1 attaches to plant chromosomes via histones, and that this attachment results in active and efficient chromatin targeting of the invading Agrobacterium T-complex (Fig. 1K). This notion helps explain the important, yet insufficiently understood, role of AtH2A in T-DNA integration (Mysore et al., 2000; Yi et al., 2002) as well as the unique ability of Agrobacterium to promote stable genetic transformation of cells with the efficiency substantially higher than that which can be obtained even when massive amounts of foreign DNA are introduced into plant cells by other methods, for example, by electroporation or microbombardment. Because AtVIP1 is also involved in recruiting the targeted proteolysis machinery of the host cell (Tzfira et al., 2004), it is tempting to hypothesize that the AtVIP1-histone association may trigger a coordinated and localized proteolytic uncoating of the T-strand and the juxtaposed host DNA to allow integration. Future experiments will test these ideas and explore potential correlations between chromatin targeting of the T-complex and histone modifications that determine the chromatin state, which may underlie the instances of high T-DNA insertion frequency within the regulatory regions of genes (Szabados et al., 2002; Alonso et al., 2003) and low frequency in silent chromatin regions (Alonso et al., 2003).

## RECENT DEVELOPMENTS

Correlation between AtVIP1 binding to AtH2A and plant susceptibility to Agrobacterium tumorigenesis was reported by Li et al. (2005).

## ACKNOWLEDGMENT

We thank Drs. K. Luger and S. Gelvin for their generous gifts of Xenopus and Arabidopsis histones.

Received March 8, 2005; revised March 17, 2005; accepted March 17, 2005; published July 11, 2005.

## LITERATURE CITED

**Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, et al** (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science **301:** 653–657

**Bracha-Drori K, Shichrur K, Katz A, Oliva M, Angelovici R, Yalovsky S,**

**Ohad N** (2004) Detection of protein-protein interactions in plants using bimolecular fluorescence complementation. Plant J **40:** 419–427

**Christie PJ** (2004) Type IV secretion: the *Agrobacterium* VirB/D4 and related conjugation systems. Biochim Biophys Acta **1694:** 219–234

**Citovsky V, Kapelnikov A, Oliel S, Zakai N, Rojas MR, Gilbertson RL, Tzfira T, Loyter A** (2004) Protein interactions involved in nuclear import of the *Agrobacterium* VirE2 protein *in vivo* and *in vitro.* J Biol Chem **279:** 29528–29533

**Gelvin SB** (2003) *Agrobacterium*-mediated plant transformation: the biology behind the ''gene-jockeying'' tool. Microbiol Mol Biol Rev **67:** 16–37

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

**Goodrich J, Tweedie S** (2002) Remembrance of things past: chromatin remodeling in plant development. Annu Rev Cell Dev Biol **18:** 707–746

**Hu CD, Chinenov Y, Kerppola TK** (2002) Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. Mol Cell **9:** 789–798

**Hu W, Jans DA** (1999) Efficiency of importin alpha/beta-mediated nuclear localization sequence recognition and nuclear import. J Biol Chem **274:** 15820–15827

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

**Li J, Krichevsky A, Vaidya M, Tzfira T, Citovsky V** (2005) Uncoupling of the functions of the Arabidopsis VIP1 protein in transient and stable plant genetic transformation by Agrobacterium. Proc Natl Acad Sci USA **102**: 5733–5738

**Luger K, Rechsteiner TJ, Flaus AJ, Waye MM, Richmond TJ** (1997) Characterization of nucleosome core particles containing histone proteins made in bacteria. J Mol Biol **272:** 301–311

**Luger K, Rechsteiner TJ, Richmond TJ** (1999) Expression and purification of recombinant histones and nucleosome reconstitution. Methods Mol Biol **119:** 1–16

**Muhlhausser P, Muller EC, Otto A, Kutay U** (2001) Multiple pathways contribute to nuclear import of core histones. EMBO Rep **2:** 690–696

**Mysore KS, Nam J, Gelvin SB** (2000) An Arabidopsis histone H2A mutant is deficient in Agrobacterium T-DNA integration. Proc Natl Acad Sci USA **97:** 948–953

**Sondermann H, Becker T, Mayhew M, Wieland F, Hartl FU** (2000) Characterization of a receptor for heat shock protein 70 on macrophages and monocytes. Biol Chem **381:** 1165–1174

**Szabados L, Kovács I, Oberschall A, Ábrahám E, Kerekes I, Zsigmond L, Nagy R, Alvarado M, Krasovskaja I, Gál M, et al** (2002) Distribution of 1000 sequenced T-DNA tags in the Arabidopsis genome. Plant J **32:** 233–242

**Tzfira T, Citovsky V** (2002) Partners-in-infection: host proteins involved in the transformation of plant cells by Agrobacterium. Trends Cell Biol **12:** 121–129

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

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

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

**Walter M, Chaban C, Schütze K, Batistic O, Weckermann K, Näke C, Blazevic D, Grefen C, Schumacher K, Oecking C, et al** (2004) Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. Plant J **40:** 428–438

**Yi H, Mysore KS, Gelvin SB** (2002) Expression of the Arabidopsis histone *H2A-1* gene correlates with susceptibility to Agrobacterium transformation. Plant J **32:** 285–298

**Zupan J, Muth TR, Draper O, Zambryski PC** (2000) The transfer of DNA from *Agrobacterium tumefaciens* into plants: a feast of fundamental insights. Plant J **23:** 11–28