The VirD2 pilot protein of *Agrobacterium*-transferred DNA interacts with the TATA box-binding protein and a nuclear protein kinase in plants

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The bacterial virulence protein VirD2 plays an important role in nuclear import and chromosomal integration of *Agrobacterium*-transferred DNA in fungal, plant, animal, and human cells. Here we show that in nuclei of alfalfa cells, VirD2 interacts with and is phosphorylated by CAK2Ms, a conserved plant ortholog of cyclin-dependent kinase-activating kinases. CAK2Ms binds to and phosphorylates the C-terminal regulatory domain of RNA polymerase II largest subunit, which can recruit the TATA box-binding protein. VirD2 is found in tight association with the TATA box-binding protein *in vivo*. These results indicate that recognition of VirD2 is mediated by widely conserved nuclear factors in eukaryotes.

transferred DNA integration | transcription-coupled repair | transcription factor IIH

Transferred DNA (T-DNA) is a segment of *Agrobacterium* Ti (tumor-inducing) and Ri (root-inducing) plasmids, which is flanked by 25-bp imperfect direct border repeats. The T-DNA is transferred as a single-stranded intermediate (T-strand) from agrobacteria into plant cells, where it is integrated into the nuclear genome (1, 2). Genes carried by the T-DNA are expressed properly only in plants, and code for enzymes and regulatory factors modifying plant hormonal balance, cell differentiation, transcription, and metabolism (3, 4). The T-DNA genes are not required for DNA transfer and integration. Hence, any DNA sequence inserted between T-DNA borders can be transferred from agrobacteria into plants, as well as into fungal, animal, and human cells, when bacterial virulence genes are present in cis or trans (1, 5–7).

Expression of virulence (*vir*) genes leads to the assembly of a border-processing complex carrying VirD1 and VirD2 subunits. After binding to T-DNA borders, VirD2 introduces single-strand-specific nicks between nucleotides 3 and 4 of 25-bp border repeats and forms a covalent phosphodiester bond through its tyrosine 29 residue with the 5′ end of nicked T-strand (8–9). The VirD2–T-strand complex is probably released by strand-replacement DNA synthesis and targeted by VirD4 to T-pili formed by the VirB proteins (10–11). T-pili are type IV transport channels, which transfer the VirD2–T-strand complex and bacterial virulence proteins into the host cells (12–13). During transport, presumably in the host cell, the T-strand assembles with, and is covered along its entire length by, the VirE2 ssDNA-binding protein (14–15). VirE2 carries bipartite nuclear localization sequences (NLSs), which are not recognized in yeast and animal cells, but function properly when VirE2 is expressed in dicotyledonous plants (16–17). A single amino acid exchange in the NLS, however, enables VirE2 to transfer ssDNA into *Drosophila* and *Xenopus* nuclei (6). In plants VirE2 interacts with a nuclear bZIP protein, VIP1, which is proposed to facilitate "piggyback" transport of VirE2-covered T-strand through the nuclear pores (18).

In addition to VirE2, the VirD2 pilot protein also affects the nuclear import of the T-strand (15, 19–20). VirD2 carries a C-terminal bipartite NLS, which interacts with AtKAPα, an α-importin nuclear import receptor in *Arabidopsis* (21–22). Upstream of the NLS, VirD2 binds several plant cyclophilins. Cyclophilin-binding drugs are reported to abolish *Agrobacterium* transformation, suggesting that cyclophilins may either protect or ensure proper folding of VirD2 (23). After nuclear import, interaction of VirD2 with unknown host factors is thought to target the T-strand to specific chromosomal integration sites. Comparative sequence analysis of insert junctions and target sites suggests that the T-DNA is preferentially integrated into promoters and AT-rich sequences of transcribed chromatin domains by illegitimate recombination (24–27). VirD2 may facilitate joining the 5′ end of T-strand with free 3′ ends of nicks or gaps in the host DNA. Precise ligation of VirD2-protected 5′ end of T-strand is inhibited by an R129G amino acid exchange in a histidine triad-like motif of N-terminal VirD2 relaxase-domain (28–30). Recombination events at the 3′ end of the T-strand probably involve nonhomologous end joining (NHEJ). Mutations inactivating NHEJ proteins inhibit T-DNA integration in yeast (31). T-DNA insertions trapped in preformed double-strand breaks show frequent deletions at the 5′ end of T-strand (i.e., T-DNA right border junction, 32). This finding suggests that T-DNA integration can also occur by NHEJ in the absence of functional VirD2. Nonetheless, dramatic reduction of transformation frequencies and formation of aberrant T-DNA insertions in plants transformed with *virD2* mutant agrobacteria indicate that VirD2 is important for interaction with host factors mediating T-DNA integration (19–20).

Here we show that VirD2 is tightly associated with the TATA box-binding protein (TBP) in transformed *Arabidopsis* cells, which correlates with frequent integration of the T-DNA into transcribed chromatin domains. In nuclei of alfalfa cells, VirD2 is phosphorylated *in vivo* by CAK2Ms, a member of the conserved cyclin-dependent kinase-activating kinase family (33–36). CAK2Ms isolated by immunoprecipitation phosphorylates cyclin-dependent kinase (CDK2) and the C-terminal domain (CTD) of RNA polymerase II largest subunit, which serves as a TBP-binding platform. Remarkable functional conservation of TBP and CDK-activating protein kinase (CAK2) orthologs in eukaryotes suggests that these nuclear VirD2-binding factors provide a link between T-DNA integration and transcription-coupled repair (37–38).

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Abbreviations: T-DNA, transferred DNA; T-strand, single-stranded intermediate; NLS, nuclear localization sequence; CDK, cyclin-dependent kinase; CAK, CDK-activating protein kinase; TBP, TATA box-binding protein; CTD, C-terminal domain; HA, hemagglutinin; MBP, maltose-binding protein; TFIIH, transcription factor IIH.

Data deposition: The sequence reported in this paper for the alfalfa CAK2Ms protein kinase has been deposited in the GenBank database (accession no. AF302013).

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Materials and Methods

Construction of Bacterial and Plant Expression Vectors. The *VirD2* gene from pETVirD2 (39) was cloned as *Bam*HI fragment in pGEX-3X (Amersham Pharmacia) to yield pGEXVirD2. An *Eco*RI fragment of pGEXVirD2, encoding the C terminus of VirD2, was moved into pGEX-5X1 (40) to obtain pGSTVirD2ΔNT. Deletion of this fragment from pGEXVirD2 yielded pGST-VirD2ΔCT encoding a GST fusion with the N-terminal VirD2 domain of 266 aa. In pBS-HA an oligonucleotide carrying an *Nde*I site and encoding a hemagglutinin (HA) epitope (MAYPYDVPDYASL) was inserted into filled-in *Apa*I–*Hin*dIII sites of Bluescript SKII (Stratagene). The VirD2-coding region from pGEXVirD2 was cloned into *Nde*I–*Bam*HI sites of pBS-HA to obtain pBS-HA-VirD2. An *Nco*I–*Bam*HI fragment of pBS-HA-VirD2 was cloned in pET28a (Novagen) and pRT100 (41) to express HA-VirD2, with an N-terminal HA-tag in *Escherichia coli* and plant protoplasts, respectively. *VirD2* was moved from pGEXVirD2 as *Bam*HI fragment into pMENCHU (42), and then the *Not*I plant expression cassette was inserted into *Sma*I–*Sac*I sites of pPCV812 (42) by blunt-end ligation to express HA-VirD2 in *Arabidopsis*. An *Eco*RV–*Acc*I fragment of cDNA clone C43 (43), encoding the CTD of *Arabidopsis* RNA polymerase II largest subunit, was cloned by blunt-end ligation into *Eco*RI–*Pst*I sites of pMALc2MS (Biolabs, Beverly, MA) to express an N-terminal fusion of CTD with the maltose-binding protein (MBP). An *Eco*RV cDNA fragment encoding CAK2Ms was obtained by PCR amplification and cloned into a filled-in *Bam*HI site of pPE1000 (44) to express HA-CAK2Ms in plant protoplasts.

Purification of Proteins Expressed in *E. coli*. *E. coli* cultures were grown at 28°C to an OD_{600} of 0.5–0.8, induced by 1 mM isopropyl β-D-thiogalactoside (IPTG) for 4 h, harvested by centrifugation (10,000 × *g* for 30 min), resuspended at 4°C in lysis buffers specified below, and disrupted by French press to prepare cleared extracts by centrifugation (30,000 × *g* for 30 min). GST-fusion with VirD2, VirD2ΔNT, VirD2ΔCT, and human CDK2 (45) were affinity purified on glutathione-Sepharose as described (40), and were further purified on HiTrap-Q columns (Amersham Pharmacia). *E. coli* cells harboring pET28a-HA-VirD2 were disrupted in lysis buffer (50 mM sodium phosphate/10 mM Tris·HCl, pH 8.0/250 mM NaCl/0.25% Nonidet P-40/2 mM EDTA/10 mM 2-mercaptoethanol (2-ME)/1 mM PMSF/10 mg/liter aprotinin/1 mg/liter leupeptin and antipain). HA-VirD2 was precipitated from the cleared extract by using 20–40% ammonium sulfate saturation, dialyzed in buffer H (25 mM Tris·HCl, pH 7.8/100 mM NaCl/0.025% Tween 20/2 mM EDTA/10 mM 2-ME/5 mM $MgCl_2$/0.2 mM PMSF/1 mg/liter aprotinin), and purified on a HiTrap heparin column (Amersham Pharmacia) fitted to a fast-performance liquid chromatography system by using a linear NaCl (0.1–1 M) gradient. After dialysis in buffer Q, HA-VirD2 was purified to apparent homogeneity on HiTrap-Q by using a linear NaCl gradient (0.05–1 M). *E. coli* BL21 cells carrying pMALc2MS-CTD were disrupted in lysis buffer (20 mM Tris·HCl, pH 7.5/2 mM EDTA/1 mM DTT/10% glycerol/0.05% Tween 20/100 mM NaCl/1 mM PMSF/0.1 mM benzamide/10 mg/liter aprotinin). MBP-CTD was purified on amylose-agarose (Biolabs) as described (45) and was dialyzed in buffer D (20 mM Tris·HCl, pH 7.5/1 mM EDTA/1 mM DTT/10% glycerol/0.05% Tween 20/50 mM NaCl/1 mM PMSF/0.1 mg/liter aprotinin and leupeptin). *E. coli* BL21(DE3)pLysS cells carrying pET14b-TBP1 (provided by N.-H. Chua, The Rockefeller University, New York) were disrupted in lysis buffer (20 mM Tris·HCl, pH 7.8/500 mM NaCl/5 mM 2-ME/10% glycerol/1 mM PMSF/2 mg/liter each of antipain, leupeptin, and pepstatin) to isolate His_6-TBP by Ni^{2+}-nitrilotriacetic acid agarose (Qiagen) chromatography, as recommended by the manufacturer. Subsequently, His_6-TBP was purified to apparent homogeneity by size fractionation on Sephacryl S200 in buffer S (25 mM sodium phosphate, pH 7.8/200 mM NaCl/0.2 mM EDTA/1 mM KCl/8% glycerol).

Preparation and Fractionation of Plant Cell Extracts. Cultured *Arabidopsis* and alfalfa cells harvested by filtration were extracted by grinding in buffer EB (25 mM Tris·HCl, pH 7.6/15 mM $MgCl_2$/15 mM EGTA/75 mM NaCl/60 mM β-glycerolphosphate/1 mM DTT/0.1% Nonidet P-40/0.1 mM Na_3VO_4/20 mM NaF/1 mM PMSF/10 mg/liter aprotinin and leupeptin/5 mg/liter chymostatin and pepstatin) to prepare whole-cell extracts by centrifugation (45,000 × *g* for 30 min at 4°C). Cytoplasmic and nuclear protein fractions were isolated as described (46). Purified nuclei were extracted with EB buffer containing 275 mM NaCl, cleared by centrifugation as above, and stored at −70°C. Nuclear proteins were size fractionated by linear glycerol-gradient (10–35%) centrifugation (115,000 × *g* for 15 h at 4°C) in buffer G (25 mM Tris·HCl, pH 7.8/15 mM $MgCl_2$/200 mM NaCl/1 mM DTT/0.1% Nonidet P-40/20 mM glycerolphosphate/10 mg/liter each of aprotinin, leupeptin, and antipain) by using thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), and ovalbumin (44 kDa) molecular mass standards.

Protein Kinase and *in Vitro* Protein Interaction Assays. Protein extracts and glycerol-gradient fractions were incubated at 26°C for 20 min with 5 μg of GST-VirD2, or MBP-CTD, or GST-CDK2 in 40 μl of kinase buffer (25 mM Tris·HCl, pH 7.8/15 mM $MgCl_2$/1 mM DTT) containing 10 μM (5 μCi; 1 Ci = 37 GBq) [γ-^{32}P]ATP, then 35 μl of 35% (vol/vol) glutathione-Sepharose or amylose-agarose was added to bind the phosphorylated substrates for 30 min. After washing with TBST (50 mM Tris·HCl, pH 8.0/150 mM NaCl/0.1% Tween 20), the bound proteins were eluted by boiling the resins in Laemmli buffer (40), and fractionated on SDS/10% PAGE gels, which were stained with Coomassie brilliant blue, dried, and subjected to autoradiography. In pull-down kinase assays, the matrices were loaded with GST-VirD2 or MBP-CTD and control GST or MPB proteins, respectively, washed with binding buffer (50 mM Tris·HCl, pH 7.8/150 mM NaCl/5 mM EDTA/10% glycerol/0.1% Nonidet P-40), and blocked for 2 h at 4°C in binding buffer containing 0.2% gelatin. The substrate-affinity resins were incubated with glycerol-gradient fractions for 2 h at 4°C, washed with binding buffer, and suspended in kinase buffer to perform phosphorylation assays as described above.

Yeast Two-Hybrid Assays and Expression of Proteins in Plant Cells. Protein interactions in the yeast two-hybrid system were assayed as described (47). PEG-mediated transformation of alfalfa protoplasts with pPE1000-HA-CAK2Ms and pRT100-HA-VirD2 sense and antisense DNA constructs was performed as described (48), by culturing the transformed cells in MS medium (49) containing 0.4 M sucrose and 1 mg/liter 2,4-D. Samples containing 5 × 10^5 protoplasts were either disrupted in Laemmli buffer for immunoblot analyses or lysed in EB buffer for pull-down kinase assays. For *in vivo* labeling of VirD2, 6 × 10^6 protoplasts were cultured in phosphate-free MS medium with 1 mCi of [^{32}P]orthophosphate (Amersham Pharmacia) for 5 h, washed with MS medium, and homogenized in lysis buffer (25 mM Tris·HCl, pH 7.8/150 mM NaCl/0.5% Nonidet P-40/2 mM EDTA/50 mM NaF/0.2 mM Na_3VO_4/10 mM $Na_4P_2O_7$/25 mM β-glycerolphosphate/0.5 mM PMSF/10 mg/liter each of aprotinin, leupeptin, pepstatin, and chymostatin) to prepare a cleared lysate by centrifugation (14,000 × *g* for 30 min at 4°C) for immunoprecipitation with a monoclonal anti-HA IgG (42). *Agrobacterium*-mediated transformation of *Arabidopsis* cells was performed as described (42).

Abs. A polyclonal rabbit anti-VirD2 Ab was blot-affinity-purified as described (50). His_6-TBP was immobilized on CNBr-activated Sepharose to purify rabbit anti-TBP IgG from a polyclonal serum (51). An N-terminal cysteine-tagged (C-GLKPPNRPT) peptide of CAK2Ms was coupled to maleinimide-conjugated keyhole limpet hemocyanin for rabbit immunization. The anti-CAK2Ms and anti-R2Os (45) IgGs were isolated by peptide affinity purification (51). Detection of HA-epitope was performed with a monoclonal anti-HA IgG (clone 16B12, Babco, Richmond, CA).

Results

VirD2 Interacts with Multiple Plant Cyclophilins and α-Importin Nuclear Receptors. VirD2 was reported to interact with an *Arabidopsis* α-importin, AtKAPα (At3g06720) (22) and three cyclophilins (ROC1, ROC4 and AtCYP1/ROC5/CypA) (23) *in vitro* and in the two-hybrid system. To search for VirD2-binding proteins, we performed yeast two-hybrid screens by using VirD2 with an N-terminal Gal4 DNA-binding domain as bait, and a pACT2 prey cDNA library prepared from cultured *Arabidopsis* cells (47). Characterization of 50 confirmed VirD2-interacting partners identified 12 ROC1, 12 ROC2, 9 ROC3, and 8 ROC5 full-length cDNAs (52), indicating that two additional cytoplasmic cyclophilins can recognize VirD2 in yeast. The remaining nine cDNAs encoded three from the seven members of *Arabidopsis* α-importin family (At1g02690, At1g09270, and At4g02150), which differed from AtKAPα, indicating that VirD2 can interact with at least four different *Arabidopsis* nuclear import receptors (data not shown). As recurrent saturating two-hybrid screens failed to identify any other *Arabidopsis* protein, we chose an alternative biochemical approach to search for additional VirD2-interacting factors.

VirD2 Is a Phosphoprotein *in Planta* and Is Phosphorylated by a Nuclear Protein Kinase *in Vitro*. Inspection of pTiC58 VirD2 sequence with the NETPHOS 2.0 program (53) suggested several potential phosphorylation sites, including S/T-P motives for proline-directed protein kinases. To test whether VirD2 is phosphorylated by plant protein kinases *in vitro*, a purified GST-VirD2 fusion protein was used as substrate, along with GST as control, in kinase assays performed with whole-cell extract, and purified nuclear and cytosolic protein fractions isolated from cultured alfalfa cells. VirD2-kinase activity was detected in the whole-cell extract and nuclear protein fraction, whereas only a trace level of activity was observed in the cytosolic fraction (Fig. 1*A*). The control GST substrate was not labeled in these assays indicating a specific phosphorylation of VirD2-domain in the GST-VirD2 fusion protein.

Based on earlier detection of a potential casein kinase II consensus sequence (21), it was speculated that a plant protein kinase could phosphorylate the C-terminal VirD2 NLS and thereby facilitate nuclear import of VirD2 (1). To map the VirD2 phosphorylation sites recognized by a kinase in the nuclear fraction, the phosphorylated GST-VirD2 substrate was subjected to CNBr fragmentation. After size fractionation on SDS/PAGE and autoradiography, two phosphopeptides of 13 and 23 kDa were detected, the size of which was in good agreement with the predicted size of peptides located between amino acid positions 2–109 (12.2 kDa) and 248–447 (22 kDa) of VirD2 (Fig. 1*B*). This phosphopeptide pattern suggested that at least two regions of VirD2 were phosphorylated, one located close to the N terminus in the relaxase domain, and another within the C-terminal domain carrying the NLS.

To examine whether VirD2 is phosphorylated in plant cells *in vivo*, HA-VirD2 labeled with an N-terminal HA-epitope was expressed transiently in alfalfa protoplasts by using the promoter of cauliflower mosaic virus 35S RNA in pRT100 (41). As control, the protoplasts were transformed with pRT100 carrying the HA-VirD2 coding domain in antisense orientation. Within 12–36 h after transformation with the sense gene construct, high-level expression of HA-VirD2 was detected by immunoblotting with anti-HA IgG, whereas no HA-VirD2 signal was observed in cells transformed with the control antisense construct (Fig. 1*C*). In parallel transformation experiments, the transformed protoplasts were cultured for 5 h with [^{32}P]orthophosphate, and then the HA-VirD2 protein was isolated by immunoprecipitation with anti-HA IgG and analyzed by SDS/PAGE and autoradiography. An immunoprecipitated phosphoprotein showing the same molecular mass as HA-VirD2 purified from *E. coli* was detected in protoplasts expressing HA-VirD2, but not in cells transformed with the control antisense vector (Fig. 1*D*), demonstrating that VirD2 was indeed phosphorylated in plant cells.

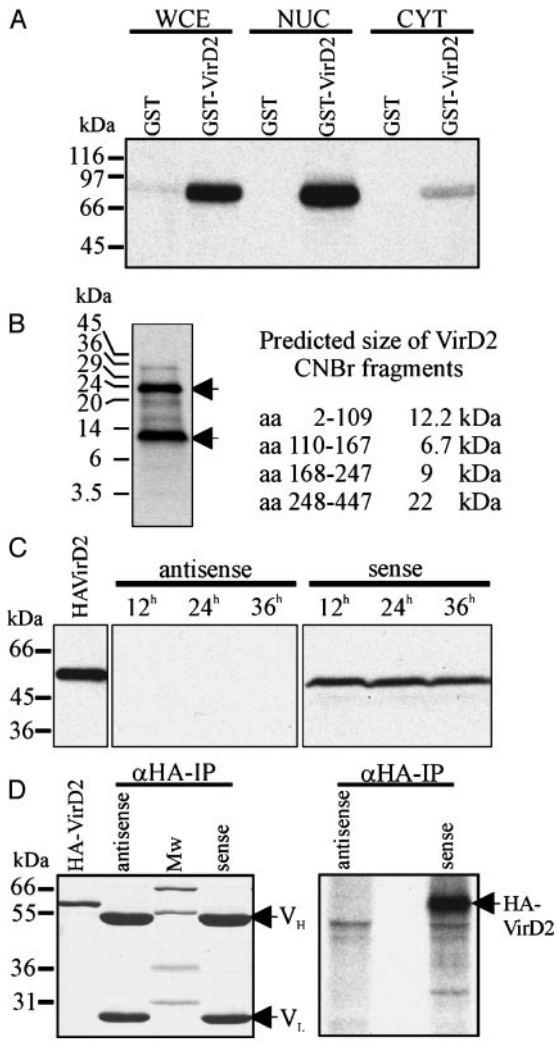

Fig. 1. VirD2 is phosphorylated by and interacts with a nuclear protein kinase. (*A*) *In vitro* protein kinase assays with alfalfa whole-cell extract (WCE) and purified nuclear (NUC) and cytoplasmic (CYT) protein fractions by using GST and GST-VirD2 substrates. (*B*) Cyanbromide fragmentation of GST-VirD2 after phosphorylation with alfalfa nuclear extract shows ^{32}P labeling of peptides located between VirD2 amino acid positions 2–109 (12.2 kDa) and 248–447 (22 kDa). (*C*) Expression of HA-VirD2 detected by Western blotting with anti-HA IgG in protoplasts 12, 24, and 36 h after transformation with pRT100-HA-VirD2 sense DNA. Controls to the left show HA-VirD2 purified from *E. coli* and a lack of HA-VirD2 expression in cells transformed with an antisense construct. (*D*) Immunoprecipitation of ^{32}P-labeled HA-VirD2 from alfalfa protoplasts. After Coomassie blue staining (*Left*), the gel was subjected to autoradiography (*Right*). [^{32}P]HA-VirD2 shows similar mobility as HA-VirD2 purified from *E. coli*. V_H and V_L are the heavy and light chains of IgG.

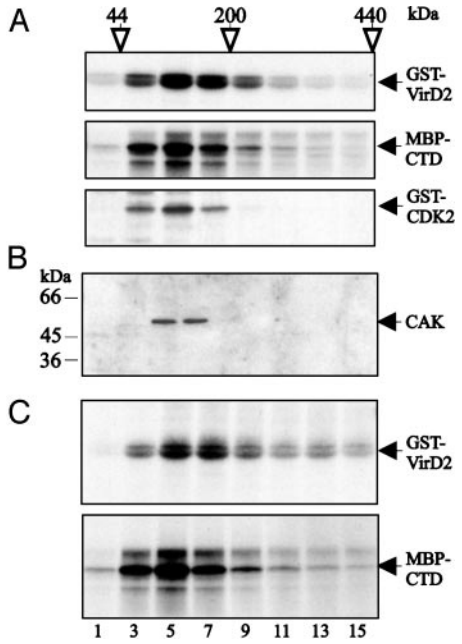

Fig. 2. Nuclear VirD2-kinase fractions purified by glycerol-gradient centrifugation phosphorylate RNA polymerase II CTD and CDK2 and are immunoprecipitated by anti-R2Os IgG. (*A*) Phosphorylation assays with glycerol-gradient fractions of nuclear VirD2-kinase using GST-VirD2, MBP-CTD, and GST-CDK2 substrates *in vitro*. (*B*) Western blotting of glycerol-gradient fractions with anti-R2Os IgG. (*C*) From the glycerol-gradient fractions, anti-R2Os IgG immunoprecipitates a protein kinase that phosphorylates GST-VirD2 and GST-CTD *in vitro*. Positions of glycerol-gradient and SDS/PAGE size markers (kDa) are shown above *A* and to the left of *B*.

The VirD2-Kinase Cofractionates with CDK2- and RNA Polymerase II CTD-Kinase Activities. For initial biochemical analysis of the VirD2-kinase, alfalfa nuclear proteins were size fractionated by 10–35% glycerol-gradient centrifugation. The VirD2-kinase activity migrated with a peak size of 120 kDa (Fig. 2*A*). To characterize the kinase, the gradient fractions were analyzed for their ability to phosphorylate a set of standard protein kinase substrates (data not shown). These assays revealed that the VirD2-kinase fractions phosphorylated the MBP-CTD and GST-CDK2 substrates, respectively, which carried the CTD of *Arabidopsis* RNA polymerase II largest subunit in fusion with an MBP-tag (43), and the human cyclin-dependent protein kinase CDK2 fused to an N-terminal GST-tag (GST-CDK2; ref. 45 and Fig. 2*A*). The CTD- and CDK2-kinase activities cosedimented precisely with the VirD2-kinase, which failed to phosphorylate other standard substrates and the control MBP and GST proteins.

Identification of the VirD2-Kinase CAK2Ms. Phosphorylation of RNA polymerase II CTD and CDK2 indicated that the substrate specificity of VirD2-kinase is very similar to that of conserved CAKs, including yeast Mcs6/Mop1, rice R2Os, and mammalian MO15/CDK7 (33–36). Immunoblotting of glycerol-gradient fractions with an Ab raised against the rice R2Os kinase (35) detected a protein of 48 kDa, suggesting that the VirD2-kinase from alfalfa nuclear extracts cofractionated with a protein immunologically related to the R2Os CAK-kinase (Fig. 2*B*). The VirD2-kinase fractions were immunoprecipitated with the anti-R2Os kinase Ab, and the immunocomplexes were assayed for kinase activity by using GST-VirD2 and MBP-CTD. The immunoprecipitated kinase complexes showed specific VirD2 and CTD phosphorylation activities, suggesting that the catalytic subunit of VirD2-kinase is a R2Os kinase-related protein (Fig. 2*C*). As observed before, the anti-R2Os IgG-precipitated VirD2-kinase failed to phosphorylate other protein kinase substrates, including histone H1 and casein, which are recognized by plant cyclin-dependent CDKs and casein kinases, respectively.

To confirm that the VirD2-kinase contains a R2Os kinase-related catalytic subunit, the anti-R2Os Ab was exploited to screen a *Medicago sativa* λZAPII cDNA expression library (54). From several clones obtained, the longest cDNA (1,670 bp) carried a coding region of 1,239 bp for a protein of 412 aa (AF302013; 46.5 kDa), which showed a high sequence identity with the cyclin-H dependent *Arabidopsis* CAK2At and rice R2Os protein kinases (Fig. 3*A*). To confirm that the identified *CAK2Ms* cDNA indeed encoded a VirD2-kinase, we cloned the coding region in pPE1000 (44) and expressed HA-CAK2Ms, carrying an N-terminal HA-

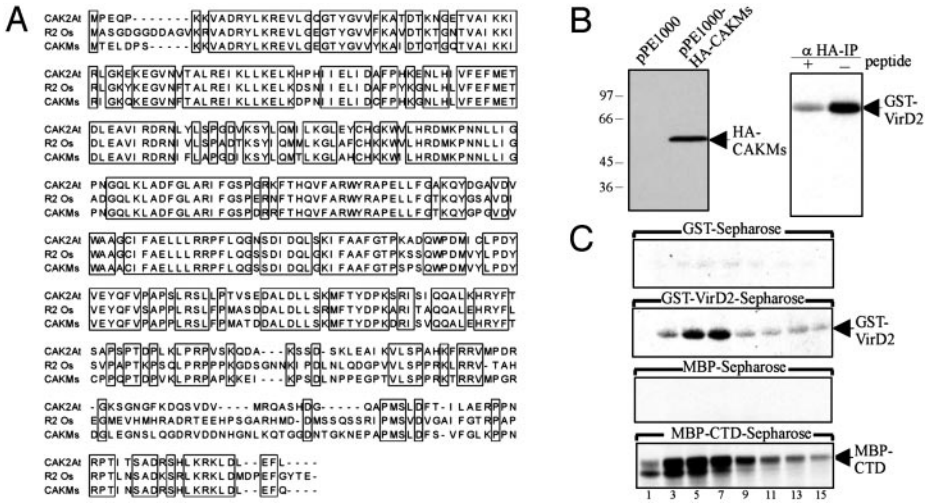

Fig. 3. CAK2Ms, a close homolog of *Arabidopsis* CAK2At and rice R2Os protein kinases, interacts with and phosphorylates VirD2 and RNA polymerase II CTD. (*A*) Alignment of CAK2Ms (AAK97227), R2Os (CAA41172), and CAK2At (BAB62843) kinase sequences. Frames mark regions with identical amino acids. (*B*) (*Left*) Detection of HA-CAK2Ms expression in alfalfa protoplasts by immunoblotting with anti-HA IgG after transformation with pPE1000-HA-CAK2Ms DNA. Cells transformed with the empty vector pPE1000 show no signal. (*Right*) Immunoprecipitation of HA-CAK2Ms from alfalfa protein extracts in the presence (+) or absence (−) of competitor HA-peptide, followed by kinase assays with GST-VirD2. (*C*) VirD2-kinase from the glycerol-gradient fractions (Fig. 2) is selectively retained on GST-VirD2 (*Left*) and MBP-CTD (*Right*) affinity resins and phosphorylates these substrate proteins in kinase pull-down assays.

epitope in alfalfa protoplasts. Transient expression of HA-CAK2Ms was observed 12–48 h after transformation by immunoblotting with anti-HA IgG. The Ab detected no crossreacting protein in control cells, which were transformed with the empty pPE1000 vector (Fig. 3*B Left*). The monoclonal anti-HA Ab immunoprecipitated the VirD2-kinase activity from protein extracts of HA-CAK2Ms expressing protoplasts. Binding of the VirD2-kinase to anti-HA IgG was almost completely abolished by preincubation of the Ab with an excess of HA-epitope peptide (Fig. 3*B Right*). These results demonstrated that in the transformed cells HA-CAK2Ms assembled into an active kinase complex, which was able to phosphorylate VirD2.

CAK2Ms Interacts with VirD2 and RNA Polymerase II CTD. Conserved members of the CAK-kinase family, such as MO15/CDK7, occur in a trimeric complex with cyclin H and MAT1, phosphorylate the RNA polymerase II CTD, and show association with the basal transcription factor TFIIH (33–36). In addition, human CDK7 and yeast Kin28 interact with the histidine-triad (HIT) proteins Hint/PKCI-1 and Hnt1, which are characterized by a conserved His-Xaa-His-Xaa-His motif, and are involved in the regulation and cellular targeting of CAKs (29–30). Surprisingly, we found that a HIT-motif is present in pTiC58 VirD2 (HPHLH, positions 131–135), and is conserved in the functionally essential N-terminal relaxase-domain in all known VirD2 proteins (28). This observation prompted us to investigate whether CAK2Ms can directly interact with VirD2 in pull-down kinase assays. Glutathione-Sepharose affinity matrices carrying purified GST and GST-VirD2 proteins were incubated with VirD2-kinase glycerol-gradient fractions and, after removing the unbound proteins, the beads were assayed for retained kinase activity by using free GST-VirD2 substrate. The VirD2-kinase was extracted by GST-VirD2, but not by control GST beads, from all glycerol-gradient fractions, which were previously observed to phosphorylate VirD2 (Fig. 3*C*). Binding of the VirD2-kinase to RNA polymerase II CTD was similarly tested by using immobilized MBP and MBP-CTD proteins. The VirD2-kinase interacted with and phosphorylated the CTD *in vitro*, resulting in a supershift in the SDS/PAGE mobility of MBP-CTD (Fig. 3*C*). These results showed that in addition to VirD2, CAK2Ms could physically recruit and phosphorylate the RNA polymerase II CTD, as other members of the conserved CAK-kinase family (55).

VirD2 Interacts with the TATA-Binding Protein *in Vitro* and in *Agrobacterium*-Transformed *Arabidopsis* Cells. VirD2 is thought to target the T-complex to free 3′ DNA ends of nicks and gaps in chromatin domains, where the dsDNA is melted (25). Recent demonstration (37–38) that the TBP can bind to lesions in the DNA to initiate transcription-coupled repair has raised the question whether TBP can recruit the VirD2 protein. First, we have analyzed the interaction of VirD2 with *Arabidopsis* TBP *in vitro*. Equimolar amounts of purified GST-VirD2 and His_6-TBP were coincubated, and the preformed protein complex was bound to glutathione-Sepharose. As control, the binding of TBP and VirD2 to RNA polymerase II CTD was similarly assayed by using MBP-CTD and pull-down with amylose agarose. The analysis of protein interactions by SDS/PAGE revealed that GST-VirD2 interacted with His_6-TBP, but not with MBP-CTD. By contrast, His_6-TBP bound to nonphosphorylated MBP-CTD, as described for human and yeast TBPs (ref. 56; Fig. 4*A*). The observed interaction between GST-VirD2 and His_6-TBP proved to be specific for the VirD2 portion of GST-VirD2 fusion protein, because control glutathione and GST beads retained only trace amounts of His_6-TBP. *In vitro* binding of VirD2 to His_6-TBP was resistant up to 500 mM NaCl (Fig. 4*B Upper*). The TBP-binding region was mapped to the highly conserved N-terminal VirD2 relaxase-domain harboring the HIT-motif. An N-terminally truncated form of VirD2, lacking the first 266 aa in GST-VirD2ΔNT, failed to interact with His_6-TBP. By contrast, a GST fusion protein carrying a C-terminal truncated, NLS-free form

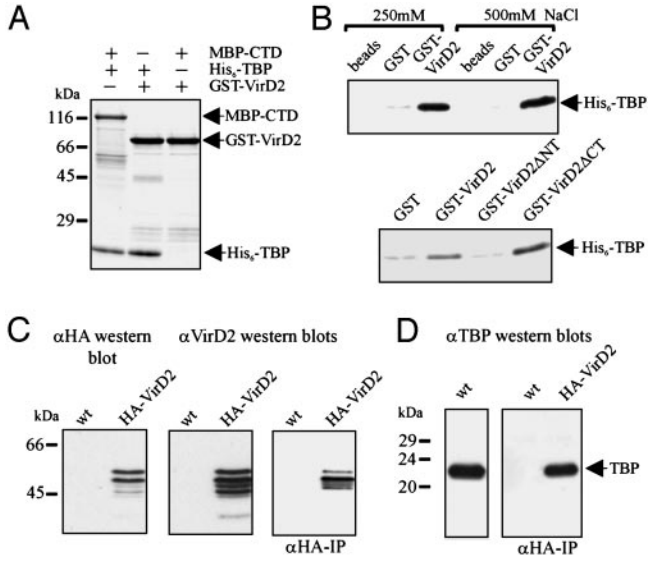

Fig. 4. Molecular interactions among VirD2, TBP, and RNA polymerase II CTD. (*A*) SDS/PAGE analysis of *in vitro* protein interactions shows that MBP-CTD binds TBP, but not VirD2, whereas VirD2 recruits TBP. (*B*) (*Upper*) His_6-TBP forms a salt-resistant complex with GST-VirD2 but does not bind specifically to control glutathione-Sepharose and GST-loaded beads. (*Lower*) The N-terminal VirD2 domain is necessary for TBP binding. GST-VirD2ΔNT, carrying an N-terminal VirD2 deletion of 266 aa, and the control GST protein show only trace levels of TBP binding. GST-VirD2 and GST-VirD2ΔCT, carrying a C-terminal VirD2 deletion, show strong interaction with TBP. (*C*) Transient expression of HA-VirD2 by *Agrobacterium* transformation in *Arabidopsis* cells. Multiple forms of HA-VirD2 protein are observed by immunoblotting with anti-HA and anti-VirD2 IgGs, as well as by anti-HA immunoprecipitation (αHA-IP), followed by Western blotting with anti-VirD2 IgG. (*D*) (*Left*) Detection of TBP in wild-type cells by Western blotting with anti-TBP IgG. (*Right*) Detection of TPB after immunoprecipitation of protein extracts from wild-type cells (wt) and HA-VirD2-expressing *Agrobacterium*-transformed *Arabidopsis* cells with anti-TBP Ab.

of VirD2 showed salt-resistant interaction with His_6-TBP as the full-length VirD2 protein (Fig. 4*B Lower*).

To demonstrate that VirD2 occurs in a stable complex with TBP *in vivo*, VirD2 was labeled with an intron-tagged HA-epitope in pMENCHU, and expressed in *Arabidopsis* cells by *Agrobacterium* transformation (42). The presence of an intron in the HA-epitope coding sequence prevented the expression of HA-VirD2 protein in *Agrobacterium* (data not shown). Thus, HA-VirD2 could be specifically detected in plant cell extracts by immunoblotting with anti-HA and anti-VirD2 Abs, as well as by immunoprecipitation with anti-HA IgG, followed by detection with anti-VirD2 Ab (Fig. 4*C*). Whereas VirD2 expressed by DNA transformation (i.e., in the absence of the T-strand, see Fig. 1*C*) appeared as a single protein band on the immunoblots, HA-VirD2 was detected in *Agrobacterium*-transformed cells as a series of bands, suggesting either degradation or posttranslational modification of VirD2. Immunoprecipitation of nuclear protein extracts from transformed and control wild-type cells with anti-HA IgG pulled down TBP only from HA-VirD2 expressing *Agrobacterium*-transformed *Arabidopsis* cells, demonstrating that VirD2 was associated with TBP *in vivo* (Fig. 4*D*).

Discussion

Analysis of the crystal structure of *Arabidopsis* TBP demonstrated that the TBP is one of the most conserved nuclear proteins in eukaryotic cells (57). TBP does not only play a defined role in the regulation of transcription, but is also implicated in the control of transcription-coupled repair (TCR), which ensures preferential and effective removal of DNA lesions

from transcribed genes (58). TCR depends on RNA polymerase II that becomes stalled at DNA lesions, to which TBP is selectively recruited (37–38). Subsequent recruitment of the basal transcription factor complex TFIIH is essential and specific for TCR, which is abolished by inactivation of TFIIH subunits (59).

In analogy to TBP, the TFIIH-associated CAK-kinase subunits show a remarkable conservation in eukaryotes. Plant members of this kinase family, such as *Arabidopsis* CAK2At and rice R2Os, can suppress the *cak/civ1* mutation of a yeast CAK-activating kinase and interact with cyclin H, as their animal ortholog CDK7/MO15, a subunit of TFIIH (60). Our data show that VirD2 interacts with and is phosphorylated both *in vitro* and *in vivo* by the nuclear CAK2Ms kinase in alfalfa, a close homolog of CAK2At and rice R2Os. CAK2Ms can also bind to and phosphorylate the C-terminal regulatory domain of RNA polymerase II, suggesting a functional similarity to TFIIH-associated CAKs. Nonetheless, further resolution of the subunit structure of purified VirD2-kinase is necessary to learn whether CAK2Ms is indeed a subunit of a plant TFIIH complex, which recognizes the conserved HIT-motif of VirD2. Further analysis of VirD2-CAK2Ms interaction can also answer the questions how CAK2Ms-mediated phosphorylation modulates VirD2 activity *in vivo*, and whether CAK2Ms would correspond to a kinase, which was predicted to regulate nuclear import of VirD2 (1).

Our data indicate that TBP is tightly associated with VirD2 in *Agrobacterium*-transformed cells, yet it is still unknown whether VirD2 would recruit CAK2Ms to TBP. We have also shown that the nonphosphorylated CTD of RNA polymerase II can bind both TBP and CAK2Ms. This raises the question whether TBP or CAK2Ms can target VirD2 to the CTD. To understand the functional consequences and regulation of VirD2 interactions with TBP and CAK2Ms, it will also be important to determine whether VirD2 can target CAK2Ms to DNA-bound TBP, and whether phosphorylation of VirD2 by CAK2Ms would regulate this interaction and affect covalent linkage of VirD2 with the 5′ end of integrating T-strand. Further studies of nuclear VirD2-binding factors identified in this article may thus help to better understand the mechanisms controlling T-DNA integration in *Agrobacterium*-transformed eukaryotic cells.

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