***Agrobacterium* VirD2-Binding Protein Is Involved in Tumorigenesis and Redundantly Encoded in Conjugative Transfer Gene Clusters**

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Agrobacterium tumefaciens can transfer oncogenic T-DNA into plant cells; T-DNA transfer is mechanistically similar to a conjugation process. VirD2 is the pilot protein that guides the transfer, because it is covalently associated with single-stranded T-DNA to form the transfer substrate T-complex. We used the VirD2 protein as an affinity ligand to isolate VirD2-binding proteins (VBPs). By pull-down assays and peptide-mass-fingerprint matching, we identified an *A. tumefaciens* protein designated VBP1 that could bind VirD2 directly. Genome-wide sequence analysis showed that *A. tumefaciens* has two additional genes encoding proteins highly similar to VBP1, designated *vbp2* and *vbp3*. Like VBP1, both VBP2 and VBP3 also could bind VirD2; all three VBPs contain a putative nucleotidyltransferase motif. Mutational analysis of *vbp* demonstrated that the three *vbp* genes could functionally complement each other. Consequently, only inactivation of all three *vbp* genes highly attenuated the bacterial ability to cause tumors on plants. Although *vbp1* is harbored on the megaplasmid pAtC58, *vbp2* and *vbp3* reside on the linear chromosome. The *vbp* genes are clustered with conjugative transfer genes, suggesting linkage between the conjugation and virulence factor. The three VBPs appear to contain C-terminal positively charged residues, often present in the transfer substrate proteins of type IV secretion systems. Inactivation of the three *vbp* genes did not affect the T-strand production. Our data indicate that VBP is a newly identified virulence factor that may affect the transfer process subsequent to T-DNA production.

Additional keywords: VirB; *vir* genes.

Agrobacterium tumefaciens is a gram-negative soilborne bacterium. It causes crown gall tumor disease in dicotyledonous plants by transferring a segment of DNA (T-DNA) from its Ti-plasmid to plant cells (Gelvin 2000; Zhu et al. 2000; Zupan et al. 2000). The T-DNA is delineated by 25-bp direct repeat sequences, which are called T-borders. Regardless of the sequence or origin, any DNA fragment placed between the two 25-bp direct repeats can be transferred into the plant cells and integrated into the host genome. In addition to its natural plant hosts, *A. tumefaciens* has been shown to transfer DNA to yeast (Bundock et al. 1995; Piers et al. 1996), fungal (Groot et al. 1998), and mammalian cells (Kunik et al. 2001) under laboratory conditions.

The T-DNA transfer process is triggered by plant wound signal compounds, such as some phenolic compounds and sugars. These signal compounds induce the expression of virulence (*vir*) genes; the Vir proteins encoded by the *vir* genes are essential for the processing, transfer, and eventual integration of the T-DNA into plant chromosome. The expression of the genes encoded on the T-DNA leads to crown gall tumor formation (Gelvin 2000, 2003; McCullen and Binns 2006; Tzfira and Citovsky 2006; Tzfira et al. 2000; Zupan et al. 2000).

VirD2 plays a crucial role in T-DNA transfer. In the presence of VirD1, VirD2 nicks T-DNA at the 25-bp direct repeat sequences of double-stranded (ds) Ti plasmid to produce the T-strand (Filichkin and Gelvin 1993; Scheffele et al. 1995; Yanofsky et al. 1986). Purified VirD2 alone can cleave a single-stranded (ss) T-border sequence in vitro (Jasper et al. 1994; Pansegrau et al. 1993). The VirD2-catalyzed nicking is site- and strand-specific (Jayaswal et al. 1987; Yanofsky et al. 1986). After the cleavage, VirD2 remains covalently associated with the 5' end of the T-strand. Mutational analysis of VirD2 indicated that tyrosine 29 is required for the covalent association of VirD2 with the T-strand (Pansegrau et al. 1993; Vogel and Das 1992). VirD2 contains two distinct functional nuclear localization signals (NLSs). The monopartite NLS is located in the N-terminal region of the protein; the bipartite NLS resides in the C-terminal region. VirD2 also is postulated to direct the movement of the T-strand in a 5'–3' directional manner and target it to the plant cell nucleus (Howard et al. 1992; Tinland et al. 1992).

The 11 VirB proteins and VirD4 are involved in the biogenesis of the T-complex transfer apparatus, which is a member of the type IV secretion system (T4SS) family (Backert and Meyer 2006; Christie 2004; Christie et al. 2005). T4SS represents a growing class of evolutionarily conserved transporters that transfer DNA and proteins into a wide variety of organisms, including bacterial and eukaryotic cells (Backert and Meyer 2006; Christie et al. 2005). The VirB proteins are homologous to the transfer proteins essential for the conjugative transfer of bacterial plasmids and those essential for the secretion of the *Bordetella pertussis* toxin protein (Backert and Meyer 2006; Christie 2004; Christie et al. 2005; Lessl et al. 1992). VirB10 and VirB11 have homologs in the Dot virulence system of *Legionella pneumophila*, which exports protein substrates (Christie et al. 2005; Vogel et al. 1998). The *virB*-encoded transport apparatus, the Tra systems of the broad host range plasmids, and the Dot virulence system of *L. pneumophila* also can promote the conjugative transfer of a common substrate, the non-self-transmissible IncQ plasmid RSF1010 (Backert and Meyer 2006; Beijersbergen et al. 1992; Christie et al. 2005; Haase et al. 1995; Vogel et al. 1998).

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In order to identify any additional protein or proteins that may be involved in the T-DNA transfer, we chose the VirD2 protein as a “bait” to “fish out” bacterial proteins that can interact with VirD2. We identified an *A. tumefaciens* protein designated VirD2-binding protein (VBP)1 that could bind VirD2. Two genes encoding proteins highly similar to VBP1 also are found on the linear chromosome of *A. tumefaciens* and designated *vbp2* and *vbp3*. To understand the role of these *vbp* genes in *A.*

tumefaciens, we mutated three *vbp* genes; a triple mutant of the three *vbp* genes exhibited attenuated bacterial ability to cause tumors on plants. The VBP did not affect the T-strand production. These suggest that VBP is an additional virulence factor important for the transfer process subsequent to T-DNA production.

RESULTS

Identification of VBP1.

To identify additional bacterial proteins that may be involved in T-DNA processing and transfer, we fused VirD2 to maltose-binding protein (MBP); the MBP-VirD2 fusion protein was used as an affinity ligand to pull down VBPs from crude extracts of *A. tumefaciens* cells induced by acetosyringone (AS). One unique protein was present in the samples pulled down by MBP-VirD2 (Fig. 1, arrowed in lane 4) and not in the controls. We extracted the surrounding proteins of the pull-down samples from the gels and identified them using matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). We found that only the unique protein gave an apparent match with an *A. tumefaciens* protein: a hypothetical protein Atu5117 (accession no. AAL45807) in the University of Washington (UW) version of *A. tumefaciens* genome (Wood et al. 2001) or AAK90491 in the Cereon version (Goodner et al. 2001). All other identified proteins matched with MBP, VirD2 or VirJ, indicating that they are part of the ligands (data not shown).

This unique protein then was characterized as a putative VBP; a MALDI-TOF mass spectrometer was used to obtain the peptide mass map (Fig. 2). MS-Fit search of the peptide mass map indicated that the top two search hits had significantly higher molecular weight search (MOWSE) scores than other hits. Both of them indicated a molecular weight consistent with the unique protein. The corresponding proteins are encoded by an identical DNA sequence in the two genome versions (Goodner et al. 2001; Wood et al. 2001). The 12 main peptide masses (Fig. 2) matched with the calculated tryptic peptide masses from the top search hit (Fig. 3). Therefore, the MS-Fit results indicated an unambiguous identification of an *A. tumefaciens* protein that may bind to VirD2. Subsequently, we sequenced five peptides of tryptic digestion by quadrupole (Q)-TOF tandem mass spectrometry (MS/MS) to further con-

Fig. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of proteins pulled down by maltose-binding protein (MBP)-VirD2. *Escherichia coli* cells expressing MBP, MBP-VirJ, and MBP-VirD2 were extracted. The extracts were loaded onto amylose resin columns; the unbound proteins were washed away. As negative controls, MBP (lane 1), MBP-VirJ (lane 2), and MBP-VirD2 (lane 3) were eluted with maltose directly. For the pull-down assay, amylose resins bound with MBP-VirD2 (lane 4), MBP-VirJ (lane 5), and MBP (lane 6) were further incubated with the protein extract of acetosyringone-induced *Agrobacterium tumefaciens* cells. After washing, the bound proteins were eluted with maltose. Proteins were resolved by 12% SDS-PAGE gel and stained with Coomassie brilliant blue. The unique band protein is indicated by an arrow (lane 4).

Fig. 2. MALDI time-of-flight (TOF) peptide mass map of a putative VirD2-binding protein. The unique protein from the MBP-VirD2 pull-down sample was excised from gel and in-gel digested by trypsin. The resulting peptides were analyzed using a MALDI-TOF mass spectrometer to obtain the mass map. Peptide peaks that corresponded to the calculated tryptic peptide masses of an *Agrobacterium tumefaciens* protein are indicated by triangles.

firm the identification. The sequences of these five peptides matched 100% with the *A. tumefaciens* protein (data not shown). A peptide fragment matched exactly with the N-terminus of the top search hit, indicating that the start codon predicted by the UW genome version (Wood et al. 2001) is correct. We designated this protein VBP1.

VBP homologs.

When we conducted the BLAST search, we found that the *A. tumefaciens* genome encodes two additional proteins with similar amino acid sequences, Atu4860 (accession no. AAL45654) and Atu4856 (accession no. AAL45650), in the UW version of *A. tumefaciens* genome (Wood et al. 2001); we designated them VBP2 and VBP3, respectively. The genes encoding them also can be found in the Cereon version of the *A. tumefaciens* genome (Goodner et al. 2001). In the UW version of the *A. tumefaciens* genome, the VBP1, VBP2, and VBP3 proteins are predicted to contain 313, 311, and 308 amino acids, respectively (Fig. 3). The three VBPs are highly similar to each other. VBP1 exhibits 72% identity and 80% similarity with VBP2; VBP1 shares 56% identity and 71% similarity with VBP3 (Fig. 3).

Each of the three VBPs contains a putative nucleotidyltransferase motif in their N-terminal region and a putative higher eukaryotes and prokaryotes nucleotide-binding (HEPN) domain in their C-terminal region (Fig. 3). A very diverse spectrum of proteins may contain a nucleotidyltransferase motif; the substrates of nucleotidyltransferases are extremely diverse (Aravind and Koonin 1999). It remains elusive whether VBPs possess any nucleotidyltransferase activity or how nucleotidyltransferase activity might contribute to the VBP function in tumorigenesis.

Binding between VirD2 and each of the VBPs.

During the initial experiments, the pulled-down proteins were analyzed by using Coomassie blue-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then MALDI-TOF MS. To efficiently detect more interactions, we analyzed the pulled-down proteins by Western blot using VBP antibodies. We used several control fusion proteins to determine whether the binding between VirD2 and VBP1 is specific. One protein cross-reacting with the VBP1 antibody was pulled down by MBP-VirD2 (Fig. 4A, lane 6) and not by amylose resin (lane 2), MBP (lane 3) or MBP-VirJ (lane 4). This protein was not pulled down by MBP-C-VirD2 (lane 5), which is a truncated version of MBP-VirD2 and has only the C-terminal half of VirD2 fused onto VirD2. It appeared that the N-terminal half of VirD2 is important for the interaction. The protein interacting with VirD2 should be identified as VBP1, because MALDI-TOF MS unambiguously identified only VBP1 and not other VBPs in the pull-down samples from the wild-type strain (Figs. 2 and 3). The data suggest that VBP1 could bind to VirD2 specifically.

To confirm the specific interaction between VirD2 and VBP1, we reversed the binding partners and used histidine (His)-VBP1 as the affinity ligand to pull down the native VirD2 protein from AS-induced *A. tumefaciens* cells. His-KatA fusion protein was used as a negative control, because KatA is a catalase (Xu et al. 2001) and, thus, was not expected to bind VirD2. We used a denaturing buffer to dissolve both His-VBP1 and His-KatA, because most of the His-VBP1 fusion protein overexpressed in *Escherichia coli* was insoluble (data not shown). The denatured His-VBP1 and His-KatA were allowed to bind to TALON metal affinity resin; then, the denaturant urea and salt were removed. Sub-

Fig. 3. Sequences comparison of VirD2-binding proteins (VBPs) and coverage map of the matched peptides. The amino acid sequences of VBP1, VBP2, and VBP3 are aligned and compared. Identical amino acids are shaded in black; similar amino acids are shaded in gray. The numbers in the right indicate the amino acid positions. The sequences of three VBP are from the University of Washington version of the genome data (Wood et al. 2001). The matched peptide fragments of VBP1 are indicated by solid lines above the VBP1 sequence. The molecular weights of the corresponding peptides are given above the lines. A pair of peptides having the same molecular weight (845 D) is marked with *; another pair (2038 D) is marked with ‡.

sequently, the denatured His-VBP1 and His-KatA were allowed to refold, before they were used to pull down VirD2. Only His-VBP1 could pull down VirD2 (Fig. 4B, lane 2). Therefore, the binding between VBP1 and VirD2 is a specific interaction.

Because VBP2 and VBP3 are highly similar to VBP1, we wanted to determine whether VBP2 and VBP3 have the same function as VBP1. We used His-VBP2 and His-VBP3 to conduct the pull-down assays to test whether VBP2 and VBP3 also could pull down VirD2. Both VBP2 and VBP3 could pull down VirD2 from the protein extract of AS-induced *A. tumefaciens* cells (Fig. 4C). This showed that both VBP2 and VBP3 also can bind to VirD2, just like VBP1.

Direct interaction between VBP1 and VirD2.

To determine whether VirD2 interacts directly with the VBPs, we used purified MBP-VirD2 fusion protein to conduct the pull-down assay with purified His-VBP1. Both MBP-VirD2 and His-VBP1 were expressed and purified from *E. coli*, which does not contain any *vbp* homolog gene and was not introduced with any *A. tumefaciens* gene other than *virD2* or *vbp*. Thus, no potential co-factor or co-factors from *A. tumefaciens* might contribute to the binding between VirD2 and VBP1 in the pull-down assay. In addition, the proteins were denatured and then purified; and the renatured proteins were used for the binding; any potential co-factor or co-factors from *E. coli* could have been removed. Indeed, no *E. coli* protein was specifically pulled down by VirD2 or VBP1 in the assays, based on our MALDI-TOF identification. The purified MBP-VirD2 could still bind to the purified His-VBP1 (Fig. 4D, lane 1). This suggests that VirD2 interacts with VBP directly.

A redundantly encoded role of *vbp* in tumorigenesis.

To test the effect of *vbp* mutation on bacterial ability to cause tumors, we generated *vbp* mutants in *A. tumefaciens* strains A348 and GMI9017. Because *vbp1* is harbored on the plasmid pAtC58, the effect of *vbp1* may be removed by using *A. tumefaciens* strain GMI9017, which lacks the *vbp1*-containing plasmid pAtC58 (Rosenberg and Huguet 1984). Therefore, we focused on generating mutations at *vbp2* and *vbp3* and obtained different *vbp* mutant strains: AMV2, GMV12, AMV23, and GMV123 (Table 1). We inoculated the *vbp* mutants and their parent strains onto the leaves of *Kalanchoe* plants. Only GMV123 that contained no functional *vbp* gene was highly attenuated in the ability to cause tumors on plants (Fig. 5A). The other three *vbp* mutant strains, AMV2, GMV12, and AMV23, that contained one or two *vbp* genes did not show any apparent difference in tumor formation when compared with their parent strains, A348 and GMI9017. This suggests that all three *vbp* genes, *vbp1*, *vbp2*, and *vbp3*, could functionally complement each other.

To confirm this, we constructed three plasmids, pCBV1, pCBV2, and pCBV3, which carry the full-length *vbp1*, *vbp2*, and *vbp3*, respectively, as well as their flanking sequences. We introduced each of these three plasmids into GMV123 (lacking any functional *vbp*) to test whether introduction of any single *vbp* gene could restore the tumorigenicity. Introduction of any single *vbp* gene indeed fully restored the ability of the *vbp* triple mutant to cause tumors (Fig. 5B).

To further compare the virulence of GMV123 and its parent strain, GMI9017, we infected *Kalanchoe* plants with different cell concentrations. GMV123 induced much less and smaller tumors on plants than GMI9017 at a high concentration of 1×10^9 cells/ml (Fig. 5C). At concentrations of 1×10^7 or 1×10^8

Fig. 4. Binding between VirD2 and each of the VirD2-binding proteins (VBPs). **A** through **C**, Proteins indicated on the right were detected by Western blot with rabbit His-VBP1 or His-VirD2 antibody. **A**, The pull-down assay with maltose-binding protein (MBP) fusion proteins. Protein extract (lane 1) of acetosyringone-induced *Agrobacterium tumefaciens* cells was pulled down by using amylose resin (lane 2), MBP (lane 3), MBP-VirJ (lane 4), MBP-C-VirD2 (lane 5), or MBP-VirD2 (lane 6). **B**, Binding between His-VBP1 and VirD2. His-VBP1 (lane 2) and His-KatA (lane 4) were used for the pull-down assay. Purified His-VBP1 (lane 1) and His-KatA (lane 3) were used as the negative controls; protein extract (lane 5) of *A. tumefaciens* cells was used as a control. **C**, Pulling down of VirD2 by VBP1, VBP2, or VBP3. His-KatA (lane 2), His-VBP1 (lane 3), His-VBP2 (lane 4), and His-VBP3 (lane 5) were used for the pull-down assay. Protein extract (lane 1) of *A. tumefaciens* cells was used as a control. **D**, Binding between VBP1 and VirD2, both purified from *Escherichia coli*. After purification, MBP-VirD2 (lanes 1 and 2) and MBP (lanes 3 and 4) were used to pull down purified His-VBP1 (lanes 1 and 3) and purified His-KatA (lanes 2 and 4), respectively. The pulled-down proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and detected by Coomassie brilliant blue staining.

cells/ml, GMV123 did not induce any tumors on plants. We wondered if the *vbp* mutation could affect the growth rate of *A. tumefaciens* cells. Therefore, we compared the growth curves of GMV123 and GMI9017. No significant difference in growth rate was observed between GMV123 and GMI9017 in both rich and minimal media (MG/L and induction broth [IB; Cangelosi et al. 1991]) (data not shown). Therefore, *vbp* is important for the bacterial virulence to cause tumors on plants; the three *vbp* genes can functionally complement each other; and the *vbp* function in tumorigenesis is redundantly encoded in *A. tumefaciens*.

Clustering of *vbp* with conjugative transfer genes.

We determined the genomic locations of VBP-encoding genes based on the genome data. Gene *vbp1* is located on the plasmid pAtC58; both *vbp2* and *vbp3* are located on the linear chromosome and very close to each other (Fig. 6). Interestingly, *vbp1* is located only 3 kb away from the conjugative transfer genes (Fig. 6), which are part of the gene clusters for the conjugation of a megaplasmid pAtC58 (Chen et al. 2002). The

vbp2 and *vbp3* genes are located in the same cluster of genes that are highly similar to conjugative transfer genes (Leloup et al. 2002) (Fig. 6). A *vbp* homolog found in the *A. radiobacter* K84 genome (Kim et al. 2006) also is clustered with the conjugative transfer genes of plasmid pAgK84 (Fig. 6).

VBP is not required for T-strand production.

The *vbp* mutation highly attenuated the bacterial ability to cause tumors on plants; therefore, we wanted to know how VBP is involved in the tumorigenesis process. To analyze the effect of VBP on T-strand production, we developed a polymerase chain reaction (PCR)-based method to detect T-strand. Total DNA isolated from AS-induced *A. tumefaciens* cells was used as the template to amplify the *ipt* gene fragment of the T-strand. Restriction enzyme *Bcl*II was used to remove the contaminating ds-Ti-plasmid template, because it can digest dsDNA but not ssDNA like the T-strand. T-strand was detected in both GMI9017 (*vbp*⁺) and GMV123 (*vbp*⁻) cells (Fig. 7, lanes 4 and 6). As expected, no T-strand was detected in the GMV123d2 cells (lane 5), in which *virD2* was inactivated and

Fig. 5. Effect of *vbp* mutation on tumorigenesis. Different *Agrobacterium tumefaciens* strains were grown in MG/L medium (Cangelosi et al. 1991) at 28°C for 1 day. The cell concentration was adjusted to 1×10^9 , 1×10^8 , or 1×10^7 cells/ml. Then, 2 μ l of cell suspension was inoculated onto each wound area on the leaves of *Kalanchoe* plants. The tumors were photographed 35 to 42 days after inoculation. **A**, Comparison of *A. tumefaciens* strains A348, GMI9017, AMV2, GMV12, AMV23, and GMV123 to cause tumors. The leaf inoculated with 1×10^8 cells/ml is presented as the representative. **B**, Complementation of GMV123 (lacking all the three VirD2-binding proteins) by any *vbp* gene harbored on a plasmid. The leaf inoculated with 1×10^8 cells/ml is presented as the representative. **C**, The effect of the cell concentrations on the tumorigenesis of GMV123 and GMI9017. The leaves inoculated with different cell concentrations are presented.

T-strand could not be produced (Table 1). When the total DNA was not digested by *Bcl*I, more PCR product was generated, presumably because both the ds-Ti-plasmid and ss-T-strand could be used as the templates (lanes 1 to 3). Further, less PCR product was amplified from the *virD2* mutant GMV123d2 in the absence of *Bcl*I. The data suggest that our PCR-based method could indicate the abundance of the T-strand in the bacterial cells, although the method is not quantitative. The abundance of the PCR product generated from GMV123 was similar to that from GMI9017 (lanes 4 and 6), indicating that the abundance of T-strand is normal in the *vbp* triple mutant and VBP is not required for the T-strand production.

VBPs contain clusters of positively charged residues at C-termini.

Because the *vbp* triple mutant produces a normal level of T-strand in the bacterial cells, the VBP function must be important for the next step of tumorigenesis: the T-DNA transfer. Analysis of the VBP amino acid sequences showed that the three VBPs appear to contain clusters of positively charged residues at their C-termini (Fig. 8), which have been found to

be an important common feature shared among T4SS substrates (Christie 2004; Vergunst et al. 2005). The positive-charge clusters are believed to be the transport signals of T4SS substrates and to mediate the interactions between the secretion substrate and VirD4, a component of the *A. tumefaciens* T4SS apparatus (Christie 2004; Hohlfeld et al. 2006; Vergunst et al. 2005). This suggests that the VBPs might be able to interact with the T4SS apparatus.

DISCUSSION

VirD2 plays a crucial role in the *Agrobacterium*-mediated T-DNA transfer into plant cells; it participates in the entire transfer process, from the processing to the integration of T-DNA into host plant genome (Gelvin 2000, 2003; McCullen and Binns 2006). Using an MBP-VirD2 fusion protein as the affinity ligand to perform the pull-down assay, we identified a VBP, designated VBP1. Besides *vbp1*, *A. tumefaciens* has two additional genes, *vbp2* and *vbp3*, encoding similar proteins. To determine the biological function of *vbp* genes, we generated mutations at *vbp*. The *vbp* triple mutant GMV123 that had no

Table 1. Bacterial strains and plasmids used in this study

Bacterial strain and plasmid	Relevant characteristics ^a	Source or reference
Strains		
<i>Escherichia coli</i>		
DH5α	<i>EndA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 (argF-lacZYA)</i> U169 φ80d _{lacZ} , for DNA cloning	Bethesda Research Laboratories
BL21(DE3)	<i>F⁻ ompT hsdS_B(r_B⁻m_B⁻) gal dcm (DE3)</i> , for protein expression	Invitrogen
<i>Agrobacterium tumefaciens</i>		
C58	Wild-type, nopaline-type pTiC58 plasmid	Laboratory collection
A348	Wild-type, A136 (pTiA6NC) (octopine-type)	Knauf and Nester 1982
GMI9017	C58 cured of pAtC58; lacking <i>vbp1</i> ; Sm ^r , Sp ^r , Rf ^r	Rosenberg and Huguet 1984
GMV12	Derivative of GMI9017 in which <i>vbp2</i> was deleted	This study
GMV123	Derivative of GMI9017 in which <i>vbp2</i> and <i>vbp3</i> were mutated; Cb ^r	This study
GMV123d2	Derivative of GMI9017 in which <i>vbp2</i> , <i>vbp3</i> and <i>virD2</i> were mutated; Cb ^r	This study
AMV2	Derivative of A348 in which <i>vbp2</i> was deleted	This study
AMV23	Derivative of A348 in which <i>vbp2</i> and <i>vbp3</i> were mutated; Cb ^r	This study
Plasmids		
pUC19	Cloning vector, <i>ColE1 oriV bla</i> ; Ap ^r	U.S. Biochemical
pEX18Tc	Counterselectable plasmid carrying <i>sacB</i> marker, <i>oriT</i> ; Tc ^r	Hoang et al. 1998
pCB301	A mini binary vector; Km ^r	Xiang et al. 1999
pQM118	Derivative of pEX18Tc in which Tc ^r was replaced by <i>nptIII</i> from pCB301; Km ^r	This study
pMAL-cRI	Expression vector encoding the full-length maltose-binding protein (MBP); Ap ^r	New England Biolabs
pSQ166	pMAL-cRI containing a <i>malE::virD2</i> fusion encoding MBP-VirD2 with MBP fused onto VirD2 at the <i>Pst</i> I site of <i>virD2</i> ; Ap ^r	This study
pSQ190	pMAL-cRI containing a <i>malE::virJ</i> fusion encoding MBP-VirJ with MBP fused onto VirJ at the <i>Pst</i> I site of <i>virJ</i> ; Ap ^r	This study
pSQ203	pMAL-cRI containing a <i>malE::virD2</i> fusion encoding MBP-C-VirD2 with MBP fused onto VirD2 C-terminal domain at the <i>Bam</i> HI site of the <i>virD2</i> gene; Ap ^r	This study
pXQ15	pRSET-A carrying a 2170 bp <i>Xho</i> I- <i>Kpn</i> I fragment encoding the full-length KatA ORF fused in-frame with (His) ₆ ; Ap ^r	Xu et al. 2001
pEXKm1	pQM118 carrying at <i>Hind</i> III a 2,183-bp fragment downstream of <i>vbp2</i> ; Km ^r	This study
pEXKm2	pEXKm1 carrying at <i>Bam</i> HI a 1,395-bp fragment upstream of <i>vbp2</i> ; Km ^r	
pEXKd2	pQM118 carrying at <i>Bam</i> HI a 1,211-bp fragment containing the upstream and downstream sequences of <i>virD2</i> ; Km ^r	
pUCV3	pUC19 carrying at <i>Bam</i> HI a 596-bp fragment encoding N-terminal half of <i>vbp3</i> ORF without the start code; Ap ^r	This study
pRV1	pRSET-A carrying at <i>Xho</i> I a 947-bp fragment encoding the full-length <i>vbp1</i> ORF fused in-frame with (His) ₆ ; Ap ^r	This study
pRV2	pRSET-B carrying at <i>Bam</i> HI a 952-bp fragment encoding the full-length <i>vbp2</i> ORF fused in-frame with (His) ₆ ; Ap ^r	This study
pRV3	pRSET-B carrying at <i>Bam</i> HI a 936-bp fragment encoding the full-length <i>vbp3</i> ORF fused in-frame with (His) ₆ ; Ap ^r	This study
pCBV1	pCB301 carrying at <i>Hind</i> III a 2,054-bp fragment containing the 941-bp full-length <i>vbp1</i> ORF as well as its 597-bp upstream and 516-bp downstream sequences; Km ^r	This study
pCBV2	pCB301 carrying at <i>Hind</i> III a 2,031-bp fragment containing the 935-bp full-length <i>vbp2</i> ORF as well as its 602-bp upstream and 494-bp downstream sequences; Km ^r	This study
pCBV3	pCB301 carrying at <i>Hind</i> III a 2,027-bp fragment containing the 926-bp full-length <i>vbp3</i> ORF as well as its 597-bp upstream and 504-bp downstream sequences; Km ^r	This study

^a Ap^r, Cb^r, Gm^r, Km^r, Rf^r, Sm^r, Sp^r, and Tc^r = Resistant to ampicillin, carbenicillin, gentamicin, kanamycin, rifampicin, streptomycin, spectinomycin, and tetracycline, respectively; ORF = open reading frame.

functional *vbp* gene exhibited attenuated virulence. This strongly indicates that VBP is an additional virulence factor in *Agrobacterium* tumorigenesis.

Computer analysis revealed that all three VBPs have a putative nucleotidyltransferase motif and a putative HEPN domain. Nucleotidyltransferases are classified into more than 10 distinct superfamilies (Aravind and Koonin 1999). The member proteins catalyze a similar chemical reaction: the coupling of nucleoside triphosphates to a free hydroxyl group via elimination of pyrophosphate (Aravind and Koonin 1999). Nucleotidyltransferases are involved in diverse biological functions that range from DNA repair, telomere maintenance, and immunoglobulin gene rearrangement to regulation of biosynthetic pathways and signal transduction (Aravind and Koonin 1999). For instance, protein nucleotidyltransferases, such as protein uridylyl transferases (GlnD) and protein adenylyl transferases (GlnE), could catalyze the nucleotidylation of specific proteins to regulate the functions of the modified proteins (Aravind and Koonin 1999). In view of the diverse spectrum of possible nucleotidyltransferase activities and substrates, it remains elusive whether any nucleotidyltransferase activity might be involved in the T-DNA transfer.

Interestingly, VBP and its homologs might be part of a DNA transfer and replication (Dtr) system (Fig. 6). Gene *vbp1* is located adjacent to conjugative transfer genes for conjugation of a plasmid pAtC58 (Chen et al. 2002). The *vbp2* and *vbp3* genes are located in the same cluster of genes that are related to conjugation (Leloup et al 2002). A *vbp* homolog in *A. radiobacter* K84 also is clustered with the conjugative transfer genes responsible for the conjugation of plasmid pAgK84 (Kim et al. 2006). Recently, we found that VBP was required for efficient transfer of a conjugative plasmid (M. Guo, unpublished data). These suggest linkage between the conjugative function and virulence factor in *A. tumefaciens* tumorigenesis.

This is consistent with the accumulating evidence indicating that the processing and transfer of T-DNA from *A. tumefaciens* to plant cells is similar to the interbacterial conjugation of a

variety of plasmids (Lessl and Lanka 1994; Lessl et al. 1992). In both processes, transferred DNA is cleaved at specific *cis*-acting sites (conjugation at *oriT* and T-DNA transfer at the T-borders) and transferred in a single-stranded form. Like the conjugative relaxase, the protein VirD2 that catalyzes the T-DNA scission remains bound to the 5' end of the T-strand molecule (Lessl and Lanka 1994; Pansegrau et al. 1993).

Plasmid conjugation can be conceptually divided into two function systems: the Dtr system and the mating pair formation (Mpf) system. In the Dtr system, relaxase and auxiliary proteins, which can be host or plasmid encoded, assemble at *oriT* to form a nucleoprotein complex called the relaxosome. There are extensive sequence similarities between *A. tumefaciens* *virB*-encoded T4SS apparatus and the Mpf system encoded on plasmids (Christie 2004; Lessl et al. 1992). Conjugative intermediate of plasmid RSF1010 can inhibit *A. tumefaciens* virulence by competing with the VirD2-T-strand or VirE2 for a common export site (Cascales et al. 2005; Stahl et al 1998).

Fig. 7. Detection of the T-strand by polymerase chain reaction. Total DNA was isolated from acetosyringone-induced *Agrobacterium tumefaciens* GM19017 (lanes 1 and 4), GMV123d2 (lanes 2 and 5), and GMV123 (lanes 3 and 6) cells. The same concentration of the total DNA was directly used as the template (lanes 1 through 3) or digested by *BclI* and then used (lanes 4 through 6) to amplify a DNA fragment within the T-strand.

Fig. 6. Gene organizations in the regions of *vbp* homologs in *Agrobacterium* spp. The *vbp* homologs are shown in black arrows. Genes with significant homology similarity to conjugative transfer genes are shown in filled box arrows of similar patterns. Genes with no significant homology similarity are shown in empty box arrows. The numbers indicate the nucleotide positions in the genomic sequences indicated by the genome Refseq numbers in parenthesis.

A. tumefaciens virB-encoded T4SS has the capacity to mobilize other relaxosomes (Beijersbergen et al. 1992; Christie et al. 2005). There is extensive evidence for functional relatedness between T-DNA transfer and conjugation (Beijersbergen et al. 1992; Cascales et al. 2005; Lessl and Lanka 1994; Lessl et al. 1992; Stahl et al. 1998).

A protein called the coupling protein (CP) is needed to couple the Dtr to the Mpf in conjugation. The CP determines the recognition between relaxosome and Mpf (Hamilton et al. 2000; Liosa et al. 2003); several CP-relaxase interactions have been demonstrated in vitro. For instances, TraG_{RP4} CP interacts with TraI_{RP4} relaxase/helicase (Schröder et al. 2002) and with Mob relaxase of the mobilizable plasmid pBHR1 (Szipirer et al. 2000). The TrwB_{R388} CP interacts with TrwC_{R388} relaxase/helicase; the TraD_F CP interacts with TraM_F, a Dtr protein that participates in the processing by binding at sites near *oriT* of the F plasmid (Disque-Kochem and Dreiseikelmann 1997).

VirD4 is highly similar to TraG of RP4 and functions as a CP that may link the T-DNA processing system and the mating channel (Backert and Meyer 2006; Christie et al. 2005; Hamilton et al. 2000). However, there is no evidence indicating that the VirD4 CP strongly interacts with the VirD2 relaxase, although the transfer DNA immunoprecipitation showed an in vivo weak interaction between T-DNA and VirD4 (Cascales and Christie 2004). In addition, VirD2 can be transferred to plant cells in the absence of T-DNA (Vergunst et al. 2005). It remains unknown how T-complex is recruited to the transfer apparatus.

As a newly identified virulence factor, VBP is not required for the T-strand production (Fig. 7), suggesting that VBP plays a role in the transfer process subsequent to T-DNA production. The VBPs can bind to VirD2 and they appear to have a common feature shared among T4SS substrates (Fig. 8), suggesting that VBP also may interact with the transfer apparatus, because T4SS substrates pass through the transfer apparatus and the common feature may be involved in the interaction with the

apparatus. Because VirD2 is the pilot protein guiding T-DNA transfer, VBP might be involved in the interaction between VirD2 and the transfer apparatus and, thus, is involved in the T-DNA transfer process.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and bacterial cultures.

Bacterial strains and plasmids used in this study are described in Table 1. The sequences of the primers used in this study are described in Table 2. *E. coli* strains were grown at 37°C in Luria-Bertani liquid or agar medium (Sambrook et al. 1989). *A. tumefaciens* strains were grown at 28°C in MG/L or IB medium (Cangelosi et al. 1991).

DNA manipulations.

DNA manipulations followed standard molecular protocols (Sambrook et al. 1989). *E. coli* DH5α was used routinely as the host for cloning experiments. *E. coli* BL21(DE3) was used for protein overproduction. DNA was transferred into *E. coli* cells by heat shock (Sambrook et al. 1989). Plasmids were introduced into *A. tumefaciens* by electroporation (Cangelosi et al. 1991). Total DNA of *A. tumefaciens* was prepared according to Charles and Nester (1993).

Preparation of recombinant proteins.

The plasmids containing genes that encode MBP and MBP fusion proteins MBP-VirD2, MBP-VirJ, and MBP-C-VirD2 (Table 1) were introduced into *E. coli*. The bacterial cells were grown to approximately 5×10^8 cells/ml; isopropyl β-D-thiogalactoside (IPTG) was added to the cultures at a final concentration of 0.3 mM to induce expression of the fusion proteins. The bacterial cultures were allowed to grow for additional 2 to 3 h. All the subsequent operations were performed at 4°C. The cells of 500 ml of culture were harvested by centrifugation and resuspended in 25 ml of lysis buffer (150 mM NaCl, 10 mM

Known VirB/D4 T4SS substrates

VirE2 ----KLPSDAAQLLAEPSDRYSRAFVRPEPALPPISDSRRTYSRPRSQSVNSF
 VirE3 ----NSEQQALLNELLSVPLPGPLPKADHERPRVLESSRSRERSMSGGLSL
 VirF ----GGPNAVGLTTNESFEKNINEVYNRTRAELDASSRDRSRSGLSR
 VirD2 ----ATDSLATAHLQRRGVLSKRPREDDDGEPSERKRERDERSKDGRGGNRR
 MobA-RSF1010 ----ALARPDATDNRGRLDLAALGGPMKNDRTLQAIGRQLKAMGCER

Relaxases known to bind with coupling proteins

TraI-F ----LREPQRVREAVREIARENLLQERLQQMERDMVRDLQKEKTLGGD
 TraI-RP4 ----LLRKENDEILVLPVDKATVQRMKRLAIGDPVTVTPRGSLKTTRGRSR

VBP proteins

VBP1 ----AYVKARYSSQYKISQEALDWIGERATLLLSLVGEVCRDHISALERDAGREIAEQGAAEE
 VBP2 ----AYVKARYSKHFEISEALGWLGERTAHLLELVKVVCDEHIEKVDCGVRMGGSSESSQ
 VBP3 ----AYVKARYSKRFEVSEEAALTWLQERTAELHKLVETLCREHIEKLEHAAGQAANSSD

Fig. 8. Three VirD2-binding proteins (VBPs) contain clusters of positively charged amino acid residues at their C termini. Positively charged residues Arg (R), Lys (K), and His (H) were bolded and underlined. This positive charge bias of VBPs is an important common feature found in type IV secretion system (T4SS) substrates, including VirB/D4 T4SS substrates and relaxases that interact with coupling proteins.

EDTA, 10 mM EGTA, 20 mM Tris, 2 mM dithiothreitol [DTT], 2 mM phenylmethylsulfonyl fluoride [PMSF], 0.25% Tween-20, leupeptins at 10 mg liter⁻¹, and antipain at 5 mg liter⁻¹, pH 7.4). The cell suspension was sonicated to near clarity and centrifuged at 17,000 × g for 20 min. Aliquots of the supernatant were stored at -20°C. The fusion proteins were purified as described by Pan and associates (1995) with minor modifications. Briefly, 2 ml of amylose resin (New England Biolabs, Beverly, MA, U.S.A.) was washed five times with column buffer (30 mM NaCl, 2 mM EDTA, 2 mM EGTA, 20 mM Tris, 1 mM DTT, 2.5 mM MgCl₂, 1 mM PMSF, leupeptins at 2 mg liter⁻¹, and antipain at 1 mg liter⁻¹, pH 7.4). Then, 15 ml of *E. coli* lysate, which had been diluted five times from 3 ml of supernatant with 12 ml of column buffer, was added to the amylose resin and incubated for 45 min with rocking. The protein-bound amylose resin was loaded to a column. The column was washed with 15 ml of column buffer; the bound proteins were eluted with the column buffer containing 10 mM maltose. The purity of the eluted fusion proteins were examined by 10% SDS-PAGE. The purified fusion proteins were concentrated by Centra-prep and freeze dried.

Pull-down assay for identifying VBPs.

To prepare *A. tumefaciens* protein extracts, A348 was grown at 28°C in MG/L medium for 16 to 18 h. The bacterial cells were collected by centrifugation, washed once with water, and then resuspended to 4 × 10⁸ cells/ml in the IB medium containing 200 μM AS. The cells were incubated at 28°C for another 16 to 18 h with shaking. The cells were harvested from 5 liters of cell cultures, washed once with water, and resuspended in 15 ml of *A. tumefaciens* lysis buffer A (30 mM NaCl, 10 mM MgCl₂, 5 mM EDTA, 5 mM EGTA, 20 mM Tris, 2 mM DTT, 2 mM PMSF, 1% Triton X-100, leupeptins at 10 mg

liter⁻¹, and antipain 5 mg liter⁻¹, pH 7.4). The cells were disrupted by French Press (cell pressure: 35K psi). The protein extract was collected by centrifugation at 12,000 × g for 15 min at 4°C and diluted with an equal volume of lysis buffer A (but without Triton X-100). The aliquots of the extract were stored at -20°C until the pull-down assay.

To conduct the pull-down assay, *E. coli* protein extracts containing MBP, MBP-VirJ, MBP-C-VirD2, and MBP-VirD2 were prepared as described above and allowed to incubate with pre-equilibrated amylose resins (50% vol/vol) for 45 min at 4°C with rocking. Care was taken to ensure that an equal amount of each of the proteins remained bound to the resin. The protein-bound amylose resins were washed with the column buffer. Then, an equal amount of *A. tumefaciens* protein extract was added to each of the protein-bound amylose resins and incubated for 60 min at 4°C with rocking. The resulting amylose resins were loaded to columns and washed with 6 to 10 column volumes of the column buffer. The bound protein complexes were eluted with the column buffer containing 10 mM maltose and concentrated by Centra-prep. The concentrated pull-down protein samples were freeze dried and stored at -20°C.

In-gel digestion, MALDI-TOF analysis, and tandem MS sequencing.

All the pull-down protein samples were adjusted to the same protein concentration and separated by 10 or 12% SDS-PAGE gel. Proteins were excised carefully from Coomassie blue-stained gels; the gels were cut into small pieces and then destained with several washes of 50 mM ammonium bicarbonate in 50% aqueous acetonitrile. The gel pieces were dehydrated with 100% acetonitrile and dried in Savant Speed-vac. The dried gel pieces were rehydrated with a solution containing 10 mM

Table 2. Primers used in this study

Primers	Sequence	Purpose
Pvbp1-1	5'-ccgctcgagcatgaacatcgctcg-3'	To amplify <i>vbp1</i>
Pvbp1-2	5'-ccgctcgagcccgctattcttcag-3'	To amplify <i>vbp1</i>
Pvbp1-3	5'-cgctgacgaactacacgcgcgcg-3'	To sequence <i>vbp1</i> in the expression vector
Pt7	5'-aatacgactcactatagg-3'	To sequence the genes in pRSET vector
Pt7r	5'-ctagtattgctcagcg-3'	To sequence the genes in pRSET vector
Pdow3	5'-tcaaagcttgatcatatcccgacag-3'	To amplify the downstream sequence of <i>vbp2</i>
Pdow4	5'-cccaagcttcagcgcgcgacacag-3'	To amplify the downstream sequence of <i>vbp2</i>
Pup10	5'-acagatgccctctgcccagcc-3'	To amplify the upstream sequence of <i>vbp2</i>
Pup11	5'-gtgggatccatgaactttatagcttc-3'	To amplify the upstream sequence of <i>vbp2</i>
Pscreen1	5'-ctcgagagagagacgcacg-3'	To screen the <i>vbp2</i> -deletion mutants
Pscreen2	5'-cagcgatcctcgaactctc-3'	To screen the <i>vbp2</i> -deletion mutants
Pvbp3-1	5'-atgggatccagcctgaacatttgc-3'	To amplify a fragment of <i>vbp3</i>
Pvbp3-3	5'-gcgggatccactgctgatgg-3'	To amplify a fragment of <i>vbp3</i>
Ppuc1	5'-cggagcctatgaaaaagcc-3'	To screen <i>vbp3</i> -disrupting mutants
Pvbps1	5'-cctctggaatggcccgtagag-3'	To screen <i>vbp3</i> -disrupting mutants
Pvbps3	5'-gaggttgtagcagcaggg-3'	To screen <i>vbp3</i> -disrupting mutants
Pd2du	5'-ctaggatcccatctgccagacga-3'	To amplify the upstream of <i>virD2</i> and screen <i>virD2</i> -deletion mutant
Pd2dul	5'-ctgaattctggatgactccttcg-tcagtgctacaagcgctttcag-3'	Over-lapping primer; to amplify the upstream of <i>virD2</i>
Pd2dd	5'-actggatcctctctatctctatttc-3'	To amplify the downstream of <i>virD2</i> and to screen for <i>virD2</i> -deletion mutant
Pd2ddl	5'-ctgaaagacgcctgtgactcga-gcaaggagatcatcagaattcag-3'	Over-lapping primer; to amplify the downstream of <i>virD2</i>
Pvbp2p1	5'-gatggatccctccctgatcatatc-3'	To amplify <i>vbp2</i>
Pvbp2p2	5'-attggatcctgctttgacacctcg-3'	To amplify <i>vbp2</i>
Pvbp3p1	5'-catggatccagcctgaacatttg-3'	To amplify <i>vbp3</i>
Pvbp3p2	5'-tgcggatcctggaagctcaatcg-3'	To amplify <i>vbp3</i>
nptIIIF	5'-gaagatctctcgagttggcagcatcacc-3'	To amplify <i>nptIII</i>
nptIIIR	5'-gaagatctactaaacaattcatccag-3'	To amplify <i>nptIII</i>
Pex18F	5'-gaagatctgtgtaatactatactctc-3'	To amplify the backbone of pEX18Tc
Pex18R	5'-gaagatctgtcagaccaagtgttactcat-3'	To amplify the backbone of pEX18Tc
Pvbp1C1	5'-gaggaagcttaagatcctcgccg-3'	To amplify <i>vbp1</i> with its flanking sequence
Pvbp1C2	5'-gcggaagcttgatgcttgtagc-3'	To amplify <i>vbp1</i> with its flanking sequence
Pvbp2C1	5'-gatcaagcttccaataattgcggcc-3'	To amplify <i>vbp2</i> with its flanking sequence
Pvbp2C2	5'-gtctcaagcttgcccgatagtg-3'	To amplify <i>vbp2</i> with its flanking sequence
Pvbp3C1	5'-ccaaaagcttaaacgcgctgcaag-3'	To amplify <i>vbp3</i> with its flanking sequence
Pvbp3C2	5'-cgagaagcttgaaccgggtcatg-3'	To amplify <i>vbp3</i> with its flanking sequence

DTT and 100 mM ammonium bicarbonate and incubated at 57°C for 60 min to reduce the disulfide bonds. The reduced sulfhydryl group was alkylated by 55 mM iodoacetamide in 100 mM ammonium bicarbonate at room temperature for 60 min. After alkylation, the gel pieces were washed with 100 mM ammonium bicarbonate solution, dehydrated with 100% acetonitrile at least six times, and then dried in Savant Speed-vac again. Sequencing grade modified trypsin (12.5 mg liter⁻¹ in 50 mM ammonium bicarbonate, pH 8.0) was added to the dried gel pieces and incubated overnight at 37°C. The resulting trypsinized peptides were extracted with 20 mM ammonium bicarbonate solution, 5% formic acid in 50% aqueous acetonitrile, and 100% acetonitrile, respectively. The extracted peptides were combined, lyophilized, and then resuspended in 0.1% trifluoroacetic acid (TFA). An aliquot of the resuspended peptides was spotted onto the stainless steel MALDI sample plate and overlaid with an equal volume of matrix solution (α -cyano-4-hydroxycinnamic acid at 20 g liter⁻¹ in 0.1% TFA, and 50% aqueous acetonitrile). The sample/matrix mixture was allowed to air dry. An Applied Biosystems Voyager-DE STR MALDI mass spectrometer was used to acquire the MALDI-TOF spectra. The instrument was operated in the positive reflector delayed extraction mode. Peptide mass spectra were searched against theoretically derived spectra from proteins in the nonredundant databases of National Center for Biotechnology Information (NCBI) to match peptide mass fingerprints.

Partial amino acid sequence of a single peptide was obtained by Q-TOF hybrid MS/MS. For Q-TOF MS/MS analysis, the trypsinized peptide samples were concentrated and desalted by the Zip-Tip method. Based on the mass spectra acquired by MALDI-TOF, dominant peptides were selected for MS/MS analysis. After peptides were selected, the Q-TOF mass spectrometer was automatically switched from MS to MS/MS mode and the Q-TOF MS/MS spectra were collected. The amino acid sequences were determined by searching the Q-TOF MS/MS spectra against the NCBI nonredundant protein databases.

Pull-down assay for detecting the binding between VBP and VirD2.

The full-length *vbp1*, *vbp2*, and *vbp3* coding sequences were amplified from *A. tumefaciens* C58 genomic DNA by PCR and were subcloned into the vector pRSET-A or pRSET-B to make in-frame fusions to the His tag. The resulting plasmids pRV1, pRV2, and pRV3 were introduced to *E. coli* strain BL21(DE3) to overexpress His-VBP1, His-VBP2, and His-VBP3, respectively. The *E. coli* cells harboring the plasmids were grown and induced by IPTG; they were collected and resuspended in lysis buffer 2 (300 mM NaCl, 2 mM PMSF, and 50 mM sodium phosphate, pH 7.4). The cells were disrupted by French Press (cell pressure: 35K psi). The pellet was collected by centrifugation at 17,000 $\times$ g for 15 min at 4°C, resuspended in denaturing column buffer (8 M urea, 300 mM NaCl, 2 mM PMSF, and 50 mM sodium phosphate, pH 7.4), and then incubated at room temperature for 1 h with shaking. The suspension was centrifuged; the supernatant was collected and then incubated with the pre-equilibrated TALON metal affinity resin (Clontech, Palo Alto, CA, U.S.A.) at room temperature for 1 h. After His-VBP was bound to TALON metal affinity resin, the resin was loaded to a column and washed with 10 to 15 resin volumes of the denaturing column buffer. His-VBP was eluted with the denaturing column buffer plus 150 mM imidazol. The purity of His-VBP was assessed by SDS-PAGE. The purified His-VBPs were injected into rabbits to generate His-VBP antibodies.

To detect the binding between VBP and VirD2, the denaturant urea and salt were removed from the protein-bound resin.

His-VBP-bound resin was washed with buffers having NaCl gradient from 300 to 0 mM and urea gradient from 8 to 0 mM. Eventually, the resin was washed with incubating buffer (2.5 mM MgCl₂, 50 mM NaCl, 2 mM PMSF, leupeptines at 20 mg liter⁻¹, and 30 mM Tris-HCl, pH 7.4). To refold the denatured His-VBP protein, the His-VBP-bound resin was incubated with the incubating buffer at 4°C for 60 min with rocking, and then washed three times with the incubating buffer. VirD2-containing *A. tumefaciens* extract was prepared as described earlier for the pull-down assay for identifying VBPs, except that *A. tumefaciens* lysis buffer A was replaced by the incubating buffer. His-KatA (Xu et al. 2001) was used as a negative control. The resin-bound VirD2 was eluted with the incubating buffer containing 150 mM imidazol and analyzed by Western blot.

SDS-PAGE and Western analysis.

SDS-PAGE analysis was performed according to Laemmli (1970). Gels either were stained with Coomassie brilliant blue or processed for Western blot analysis. For Western analysis, proteins were transferred electrophoretically to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, U.S.A.) and detected with the ECL Western blotting analysis system (Amersham Life Science, Buckinghamshire, U.K.). Blocking, washing, and exposure were conducted according to the recommendations by the manufacturer. Polyclonal antibodies against His-VirD2 and His-VBP were generated in rabbits and used for the detection.

Generation of *A. tumefaciens* mutants.

To delete the *vbp2* gene, the *sacB*-based gene replacement strategy (Hoang et al. 1998) was employed. To obtain a *sacB*-based suicide vector suitable for *A. tumefaciens* gene replacement, we replaced the tetracycline-resistant (Tc^r) gene of the plasmid pEX18Tc (Hoang et al. 1998) with a kanamycin-resistant (Km^r) gene. Primers Pex18F and Pex18R were used to amplify a 5-kb fragment without the Tc^r gene from pEX18Tc. A 1.04-kb Km^r cassette fragment was amplified by primers nptIIIF and nptIIIR from plasmid pCB301 (Xiang et al. 1999). These two fragments were ligated to generate the Km^r plasmid pQM118. The *vbp2* gene replacement vector was constructed as follows. First, a 2.183-kb fragment downstream of *vbp2* was amplified by primers Pdown3 and Pdown4 and inserted into pQM118 at *Hind*III to generate plasmid pEXKm1. Then, primers Pup10 and Pup11 were used to amplify a 1.395-kb fragment upstream of *vbp2*. The upstream fragment was inserted into pEXKm1 at *Bam*HI to produce pEXKm2. The plasmid pEXKm2 was introduced to *A. tumefaciens* cells by electroporation. The cells were plated on kanamycin-containing MG/L agar plates to select for single cross-over transformants. The Km^r colonies were purified once by streaking on fresh kanamycin-containing MG/L plates, and then were plated on MG/L agar plates containing 5% sucrose to select for the double cross-over colonies. The *vbp2* deletion mutants were identified among the Km-sensitive, sucrose-resistant colonies by PCR. The PCR products were sequenced to confirm that the *vbp2* gene was deleted as expected. The *vbp2*-deletion mutants derived from A348 and GMI9017 were designated AMV2 and GMV12, respectively.

To generate a mutation at *vbp3*, single cross-over strategy was adopted to disrupt the *vbp3* gene (Miller and Mekalanos 1988) because construction of a *vbp3* deletion was unsuccessful. A 591-bp *vbp3* fragment was amplified from the nucleotide positions 4 to 595 of the *vbp3*-coding sequence (which is 936 bp) with primers Pvbp3-1 and Pvbp3-3. The fragment was ligated into pUC19 at *Bam*HI to form the ampicillin-resistant plasmid pUCV3. The plasmid then was electroporated into

A. tumefaciens cells to generate *vbp3* insertional mutants due to homologous recombination of the plasmid into the genome. The *vbp3* insertional mutants were selected on MG/L agar plates containing carbenicillin and screened by PCR. The PCR products were sequenced to confirm that the plasmid was inserted at the expected site. The *vbp3* insertional mutants derived from AMV2 and GMV12 were designated AMV23 and GMV123, respectively.

To generate a *virD2* mutant in the *vbp* triple mutant background, the *sacB*-based gene replacement strategy (Hoang et al. 1998) was employed as described for the generation of the *vbp2* deletion mutant. Primers Pd2du and Pd2dl were used to amplify a 522-bp fragment upstream of *virD2*. Primers Pd2dd and Pd2ddl were used to amplify a 689-bp fragment downstream of *virD2*. Because primers Pd2dl and Pd2ddl are overlapping primers, the DNA fragments of the two PCR reactions were mixed together as the template and then were used to conduct the second round of PCR by using primers Pd2du and Pd2dd. This generated a 1.211-kb fragment, which contains the upstream and downstream sequences of *virD2* but not the 689-bp *virD2* coding sequence. This 1.211-kb fragment was inserted into pQM118 at *Bam*HI to generate pEXKd2. The plasmid pEXKd2 was introduced to GMV123 by electroporation. The *virD2* mutant GMV123d2 then was obtained as described earlier for generating the *vbp2* deletion mutants.

Virulence assays.

A. tumefaciens cells were grown in MG/L liquid medium overnight at 28°C supplemented with appropriate antibiotics. The bacterial cells were collected by centrifugation and resuspended in fresh MG/L medium. The cell concentrations were adjusted to 1×10^7 , 1×10^8 , and 1×10^9 cells/ml. The leaves of *Kalanchoe* plants were wounded with a hypodermic needle. Bacterial cell suspension (2 μ l) was inoculated onto each wound area. Each strain was inoculated in three different cell concentrations. Each inoculation was repeated at least two times on different leaves. The tumors were photographed 30 to 40 days after inoculation.

Detection of T-strand by PCR.

Total DNA was isolated from AS-induced *A. tumefaciens* cells and adjusted to the same concentration based on the absorption at 260 nm. Two primers, which anneal to the *ipt* gene within the T-DNA of C58, were used to amplify the T-strand. To prevent the ds-Ti-plasmid from acting as the template for this PCR reaction, the total DNA was digested by restriction endonuclease *Bcl*II because the *ipt* gene fragment contains a *Bcl*II site between the two primer sequences. The PCR products were analyzed by electrophoresis.

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