Overexpression of the Victoriocin Gene in *Helminthosporium (Cochliobolus) victoriae* Enhances the Antifungal Activity of Culture Filtrates

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ABSTRACT

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We have previously reported the isolation and characterization of the broad-spectrum antifungal protein, victoriocin, from culture filtrates of a virus-infected isolate of the plant-pathogenic fungus *Helminthosporium* (teleomorph: *Cochliobolus*) *victoriae*. We predicted that the 10-kDa mature victoriocin is derived in vivo from a preprotoxin precursor that is processed by a signal peptidase and kexin-like endopeptidase. We also presented evidence that the victoriocin precursor is encoded by a host gene, designated the *victoriocin* (*vin*) gene. In the present study, an *H. victoriae* genomic DNA library was constructed in the cosmid vector pMLF-2, and a cosmid clone carrying the *vin* gene and flanking sequences was isolated and used to generate constructs for transformation of virus-free and virus-infected *H. victoriae* isolates with the *vin* gene. Culture filtrates of the virus-free *vin* transformants exhibited high levels of antifungal activity compared with that revealed by the nontransformed virus-free wild-type strain, which exhibited little or no antifungal activity.

Moreover, transformation of the wild-type virus-infected *H. victoriae* strain with the *vin* gene resulted in still higher production of victoriocin and higher antifungal activity in the culture filtrates of the *vin* transformants compared with the virus-infected wild-type strain. As previously predicted, the presence in the *vin* transformants of the preprovictoriocin and its post-translationally generated products, the provictoriocin and the mature victoriocin, was clearly demonstrated. Processing of the victoriocin preprotoxin requires eukaryotic host factors because no processing occurred in an in vitro translation system or in bacteria. It is of interest that some of the virus-free isolates transformed with the *vin* gene exhibited some features of the virus-induced disease phenotype, including moderate stunting and sectoring. Present data suggests that victoriocin may play an indirect role in disease development. Taken together, these results indicate that victoriocin is the primary protein responsible for the antifungal activity in culture filtrates of virus-infected *H. victoriae* isolates and that virus infection upregulates the expression of victoriocin. Overproduction of victoriocin may give the slower-growing virus-infected fungal strains some competitive advantage by inhibiting the growth of other fungi.

Two isometric double-stranded (ds)RNA viruses, the totivirus *Helminthosporium victoriae* virus 190S (HvV190S) and the chrysovirus *Helminthosporium victoriae* virus 145S (HvV145S), are associated with the transmissible disease of *H. victoriae* (teleomorph: *Cochliobolus victoriae*), the causal agent of Victoria blight of oat (5,8). Diseased *H. victoriae* isolates have reduced growth, excessive sectoring, aerial mycelial collapse, and generalized lysis (5–8,11). Culture filtrates of virus-infected but not virus-free isolates exhibit broad-spectrum antifungal activity (2). De Sá et al. (2) described the isolation and characterization of a broad-spectrum antifungal protein of ~10 kDa, designated victoriocin, which was secreted by virus-infected isolates of *H. victoriae*. Victoriocin, like several other antifungal proteins, including the killer toxins of yeasts and smuts, was predicted, based on its structure, to be expressed in vivo as a preprotoxin precursor consisting of a hydrophobic N-terminal secretion signal, followed by a pro-region and terminating in a classical Kex2p endopeptidase cleavage site that generates the N terminus of the mature victoriocin. The endogenous gene encoding the victoriocin precursor (preprovictoriocin), designated the *victoriocin* (*vin*)

gene, was isolated and characterized (2, this study). Because culture filtrates from virus-free fungal isolates exhibit little or no antifungal activity, it was concluded that virus infection induces overexpression of the *vin* gene. This study was undertaken to determine whether overexpression of the *vin* gene in a virus-free fungal isolate would enhance the antifungal activity to higher levels comparable with those of virus-infected isolates and to document the occurrence in vivo of the predicted victoriocin precursors.

MATERIALS AND METHODS

Fungal and bacterial strains. The *H. victoriae* virus-free and virus-infected strains, B-2ss (American Type Culture Collection [ATCC] 42020) and A-9 (ATCC 42018), respectively, were previously described (2,7). *Penicillium chrysogenum* (ATCC 9480) was used in the antifungal activity “well assays”, as previously described (2).

Escherichia coli strain DH5 α was used as the host strain for cloning of polymerase chain reaction (PCR)-amplified fragments in pGEM-T-Easy (Promega Corp., Madison, WI) and for cloning and propagation of the cosmid vector pMLF2 (1), the bacterial protein expression vector pET-22b(+) (Novagen-EMD, San Diego, CA) and of the fungal expression vector pCB1004 (15). *E. coli* strain BL21(DE3)plysS (Novagen-EMD) was used for expression of recombinant proteins from pET-22b(+).

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Constructs for victoriocin expression in *E. coli*. A cDNA clone containing the entire coding region of the victoriocin precursor (20 kDa) was recovered from a cDNA library of *H. victoriae* strain A9 (2,14) and used as a template for PCR amplification with DyNAzyme DNA polymerase (Finnzymes Inc., Woburn, MA) to generate constructs for expression of victoriocin in the bacterial expression vector pET-22b(+). To generate the construct pET-victoriocin, forward and reverse primers (5'-GCGCTGGCCATGGATATGAACCTTTGTCTATTC-3' and 5'-ATATAATTGCGGCCGCTCAGCAGCCACAGCCAC-3', respectively) were designed with restriction enzyme sites (underlined) for insertion of the coding region of the victoriocin precursor within the *NcoI* and *NotI* restriction sites of pET-22b(+), thus allowing for the addition of an N-terminal *pelB* leader sequence but not for a histidine tag at the C terminus of the bacterially expressed protein. The PCR product was gel purified, digested with *NcoI* and *NotI*, and ligated into a similarly digested vector.

The plasmid pET-victoriocin and the vector pET-22b(+) were transformed into *E. coli* strain BL21(DE3)pLysS and the bacterial cultures were grown in Luria-Bertani (LB) broth amended with chloramphenicol (50 µg/ml) and ampicillin (50 µg/ml) to a density of absorbance at 600 nm = 0.4 and the cultures were induced with 1.0 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). Induced and noninduced bacterial cultures were grown at 37°C, and samples were collected before induction and 3 h postinduction. Cells were collected by centrifugation at 10,000 × g for 10 min, frozen overnight, and resuspended in Bug Buster reagent with added Benzonase (Novagen-EMD) for extraction of proteins from soluble and insoluble fractions according to manufacturer's instructions.

Aliquots of total cell extracts, soluble and insoluble fractions, and culture media were analyzed by electrophoresis on 15% sodium dodecyl sulfate (SDS) polyacrylamide gels followed by staining in Coomassie brilliant blue or subjected to western blot analysis using the victoriocin-specific antiserum AS-P10 (2).

In vitro transcription and translation of cloned *vin* cDNA. The TNT T7 Quick coupled transcription and translation system (Promega Corp.) was used according to the manufacturer's instructions to assess the expression of cloned *vin* cDNA. The recombinant plasmid pET-victoriocin and the vector pET-22b(+) (≈1 µg each) were added to the transcription and translation reaction mix in the presence of [³⁵S]methionine. A plasmid encoding luciferase, which was included in the kit, was used as a positive control reaction and a reaction mix without added DNA was used as a negative control. The translation products were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on 15% polyacrylamide gels, stained in Coomassie brilliant blue (R-250), and dried. The dried gel was then exposed to a phosphor-imager screen and the images were visualized with a Phosphor-Imager 445 SI system and analyzed with the ImageQuant 4.1 software program system (Amersham Biosciences, Piscataway, NJ).

Construction of *H. victoriae* genomic DNA library. An ordered genomic DNA library of *H. victoriae* strain A-9 was constructed in the cosmid vector pMLF2 (1) essentially as previously described (1,4,12). Strain A-9 was grown in Czapek-Dox (CZ) broth minimal medium in 1-liter flasks under agitation for 72 h at 23°C. The mycelium was collected by filtering through Miracloth (Calbiochem, San Diego, CA), freeze dried, and then pulverized in liquid nitrogen. DNA was extracted from mycelium using lysis buffer (50 mM Tris-HCl, pH 8.0, containing 50 mM EDTA, pH 8.0, 0.5 M NaCl, and 1% SDS) that was heated to 65°C. The slurry was then mixed with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and the DNA was precipitated with isopropanol, spooled onto a glass rod, washed with 70% ethanol, air dried, and resuspended in Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0, containing 0.1 mM EDTA). High

molecular weight DNA (>200 Kbp) was partially digested with *MboI* and then size fractionated on a continuous sucrose gradient (10 to 40%) by centrifugation at 28,000 × g for 20 h at 20°C. Gradient fractions were collected and aliquots were analyzed by CHEF electrophoresis (CHEF-DR1; Bio-Rad Laboratories, Hercules, CA) to identify those containing genomic DNA fragments of 30 to 45 kbp. DNA was precipitated from the relevant fractions and ligated to *Bam*HI-digested pMLF2. The ligation reaction was packaged using Giga Pack III Gold Packaging Extract (Stratagene, La Jolla, CA), and *E. coli* DH5α cells were transfected with the packaged ligation reaction. Colonies were transferred to 384-well plates (Nalge Nunc International Corp., Rochester, NY) including 70 µl of LB containing 10% freezer medium (16). The plates were then incubated overnight at 37°C and a 384-pin replicator (Nalge Nunc International Corp., Rochester, NY) was used to array colonies onto replicate Pall Biodyne B membranes (Nalge Nunc International Corp.) placed on LB-ampicillin (100 µg/ml) plates. The colonies were grown at 37°C for 12 h and then lysed to release DNA onto the membranes following a previously described protocol (4). The membranes were then hybridized overnight at 42°C with a denatured [α-³²P]dCTP-labeled *vin* probe (2). The hybridized membranes were washed in 2× SSPE (0.3 M NaCl; 20 mM NaPO₄; 2 mM EDTA, pH 7.7) at 42°C for 10 min, 2× SSPE at 50°C for 15 min, followed by a wash in 0.1× SSPE containing 0.1% SDS for 15 min at 50°C. The membranes were exposed to photographic films that were matched to the 384-well plates and positive clones were further selected by Southern hybridization analysis of purified DNAs (2).

Constructs for victoriocin overexpression in *H. victoriae*. A cosmid clone containing the *vin* gene (pMLF-L5B), as verified by Southern blot analysis, was used to generate constructs in the fungal transformation vector pCB1004 for overexpression of the *vin* gene in *H. victoriae*. The pMLF-L5B cosmid clone was digested with *Bam*HI and *Not*I and the digestion products were separated by electrophoresis on 0.7% agarose followed by Southern blot analysis to identify a DNA fragment containing the *vin* gene. A positively identified fragment was gel purified and ligated into the pCB1004 transformation vector that was previously digested with *Bam*HI and *Not*I to generate the plasmid construct pCB-6. The construct pCB-6 was digested with *Sna*BI and then with *Not*I to generate a *Sna*BI-*Not*I fragment that was subcloned into pCB1004 predigested with *Not*I and *Eco*RV to create the construct pCB-SnaBI/*Not*I (Fig. 1). Deletion derivatives of pCB-6 were generated by separate restriction enzyme digestions with *Cla*I, *Sma*I, and *Eco*RI and religation of pCB-6, and the resulting constructs were designated pCB-ClaI, pCB-SmaI, and pCB-EcoRI (Fig. 1). All constructs were sequenced using M13 forward and reverse universal primers and two gene-specific primers, GSP1-F (5'-CTGGTGCTGGAGGAAGTGG-3') and GSP5-R (5'-TAGACAGAAGCTTCATTTTGG-3'), to verify the presence of the *vin* gene. Two additional primers were designed to identify sequences 5' and 3' to the *vin* gene: 5'VIN-F (5'-TTCTTCCATTGATTGCTATCTTCG-3') and VIN3'-R (5'-CTTCTTACACGGCTCCTTTATTC-3').

Transformation of *H. victoriae* with *vin* constructs. Protoplasts from the virus-free *H. victoriae* strain B-2ss were prepared from mycelium according to the method described by Yoder (17), except that Glucanex (Sigma-Aldrich, St. Louis) at 10 mg/ml was used in place of Novozyme 234 in the enzyme osmoticum solution. In all, ≈5 × 10⁷ protoplasts were transformed with the four constructs pCB-ClaI, pCB-SnaBI/*Not*I, pCB-SmaI, and pCB-EcoRI as well as with the transformation vector pCB1004 alone, as previously described (18). The transformants were recovered in regeneration medium (10 ml of 0.1% yeast extract, 0.1% casein hydrolyzate, 1 M sucrose, and 1.75% agar) containing hygromycin B (50 µg/ml). To ensure nuclear homogeneity, transformed isolates were purified by two rounds of single

conidia isolations and grown on potato dextrose agar medium containing 0.5% (wt/vol) yeast extract and hygromycin B (50 µg/ml) for 1 to 2 weeks. The virus-infected *H. victoriae* strain A-9 was also transformed with the pCB-ClaI construct as described above. Expression of victoriocin was examined by northern and western blot analysis, and antifungal activity of culture filtrates was verified by the well assay (2).

Northern and western blot analysis. Total RNA and total protein were extracted from mycelium of *H. victoriae* grown in CZ medium and subjected to northern and western blot analyses, respectively. Total RNA was prepared by pulverizing 1 g of mycelium in liquid nitrogen and adding to the powdered mycelium 10 ml of RNA extraction buffer (0.2 M LiCl; 0.2 M Tris-HCl, pH 8.0; 0.02 M EDTA; and 2% SDS); then, Tris-equilibrated phenol heated to 80°C was added at a ratio of 1:1. The resulting slurry was mixed for 5 min, then centrifuged (15,300 × *g*, 10 min), and 4 M LiCl was added to the upper phase. Isopropanol and 3 M sodium acetate were used in the final RNA precipitation step. The RNA was glyoxal denatured and fractionated by electrophoresis on 0.8% agarose gels in 10 mM phosphate buffer, pH 6.3, as previously described (12). The RNA was transferred to Hybond X-L membranes by semi-dry transfer using the PowerPak 200 (Bio-Rad Laboratories) transfer apparatus and were UV cross-linked. The membranes were hybridized overnight at 42°C with the denatured *vin* probe in hybridization solution (50% formamide; 5× Denhardt's solution; 5× SSC; 0.5% [wt/vol] SDS; and boiled, sheared salmon sperm DNA at 100 µg/ml). The membranes were washed using high-stringency conditions (2× SSC and 0.1% SDS at room temperature for 10 min, 1× SSC and 0.1%

SDS at 65°C for 15 min, and 0.1× SSC and 0.1% SDS at 65°C for 10 min) and the membranes were then air dried, exposed to a phosphorimager screen, and visualized with the PhosphorImager 445 SI system.

For analysis of protein expression, proteins were extracted from fungal mycelium with a modification of the procedure of Oshero and May (10). Lyophilized mycelium (10 mg) was ground in liquid nitrogen and 0.5 ml of 10% trichloroacetic acid was added to the powdered mycelium; the resultant slurry was mixed by vortexing and incubated at room temperature for 5 min. The slurry was centrifuged (12,000 × *g* for 5 min) and the pellet was washed three times with a solution of 90% acetone with 20 mM HCl and air dried. The pellet was resuspended in 0.2 ml of sample buffer (1% SDS, 9 M urea, 25 mM Tris-HCl, 1 mM EDTA, and 0.7 M β-mercaptoethanol) and samples were loaded onto 15% SDS-polyacrylamide gels for electrophoresis and western blot analysis. Proteins resolved by electrophoresis were transferred to polyvinylidene difluoride membranes for western blotting and probed with the victoriocin-specific antiserum AS-P10 (2).

Sequencing and sequence analysis. Sequencing of constructs for protein expression, fungal transformation, and cosmid DNA was performed by automated DNA dideoxy-termination sequencing using the Big Dye terminator sequencing kit (Applied Biosystems Inc., Foster City, CA) on an ABI600 automated sequencer using M13 forward and reverse primers and gene-specific primers. The sequences were assembled and analyzed using programs in the University of Wisconsin GCG software package (3) and DNA Strider (9).

Fig. 1. Schematic representation of the *vin* constructs used for transformation of the virus-free *Helminthosporium victoriae* strain B-2ss. All constructs contain the coding region of the preprevictoriocin but differ in the length of the upstream sequences presumed to contain promoter elements.

RESULTS

Expression of victoriocin precursor in *E. coli*. We generated the construct pET-victoriocin in the bacterial vector pET-22b(+) for expression of the victoriocin precursor in *E. coli* strain BL21 (DE3)pLysS, and analyzed the products synthesized by western blotting using the victoriocin-specific antiserum AS-P10 (2). A protein band of ≈ 25 kDa was detected in the soluble fraction of cultures of BL21-(DE3)pLysS transformed with the pET-victoriocin plasmid and grown at 37°C for 3 h following induction with 1 mM IPTG (Fig. 2A, lane 1). No such band was detected in the soluble fraction of the noninduced cultures transformed with the pET-victoriocin (Fig. 2A, lane 2). Furthermore, the band corresponding to the victoriocin precursor was also absent in the soluble fraction of induced or noninduced cultures of BL21(DE3)pLysS transformed with the control pET-22b(+) (Fig. 2A, lanes 3 and 4, respectively).

In vitro transcription and translation of cloned *vin* cDNA. TNT coupled in vitro transcription and translation of the construct pET-victoriocin produced a protein band of ≈ 25 kDa (Fig. 2B, lanes 1 and 2), which was absent as a translation product when pET-22b(+) DNA or the luciferase DNA was added, or in reactions with no added DNA (Fig. 2B, lanes 3 and 4, respectively). The anomalous behavior of the victoriocin precursor (with calculated molecular mass of ≈ 20 kDa) on SDS-PAGE is probably due to its cysteine-rich nature and high stability (2).

Construction of *H. victoriae* genomic library and *vin* fungal transformation vectors. The *H. victoriae* genomic DNA library had a titer of 3.2×10^5 CFU/ μ g of ligation mixture and an average insert size of 40 kbp. In total, 8,448 clones were screened, and one cosmid clone (pMLF-L5B) was positive for the *vin* gene as indicated by Southern hybridization analysis. The pMLF-L5B cosmid clone was used to generate four constructs in the fungal transformation vector pCB1004 (see Materials and Methods). The construct pCB-Clal was the largest of the four constructs and was completely sequenced. Sequence analysis indicated that the pCB-Clal plasmid has an insert of 2,205 bp of *H. victoriae* genomic

DNA sequences that included the *vin* open reading frame (ORF), sequences of 1,067 bp upstream of the ATG initiation codon, and sequences of 587 bp downstream of the TGA terminator codon (Fig. 1). As deduced from the sequence of the pCB-Clal construct and verified by sequencing, all three of the other constructs contain the *vin* ORF and 587 bp of sequence downstream of the *vin* ORF. The lengths of the sequences upstream of the *vin* ORF, which presumably contain putative promoter sequences, were 1,067, 803, 296, and 73 bp in clones pCB-Clal, pCB-SnaBI/NotI, pCB-SmaI, and pCB-EcoRI, respectively (Fig. 1). The nucleotide sequence pCB-Clal clone has been deposited in GenBank under accession number EF587239.

Analysis of *vin* transformants. Expression of the *vin* gene and processing of the preprovictoriocin were analyzed in the virus-free *H. victoriae* isolate B-2ss transformed with pCB1004 alone (vector) as well as with four *vin* constructs. Western blot analysis, using an antiserum specific to victoriocin (AS-P10), showed that transformants with constructs pCB-Clal, pCB-SmaI, and pCB-SnaBI/NotI (transformant SnaB1) accumulated higher levels of preprovictoriocin (ppV), provictoriocin (pV) and victoriocin (V) than transformants with construct pCB-EcoRI or with the empty vector (Fig. 3A). Northern hybridization results, using a *vin*

Fig. 2. Bacterial expression and in vitro coupled transcription and translation of the victoriocin precursor. **A**, Western blot analysis of expression products of preprovictoriocin in induced and noninduced cultures of *Escherichia coli* strain BL21(DE3)pLysS transformed with the pET-victoriocin plasmid (lanes 1 and 2, respectively). Lanes 3 and 4, induced and noninduced cultures of BL21(DE3)pLysS transformed with the control plasmid pET-22b(+). **B**, Analysis of in vitro translation products of victoriocin precursor coding sequences by sodium dodecyl sulfate polyacrylamide gel electrophoresis on an 8% polyacrylamide gel and autoradiography. Recombinant plasmid pET-victoriocin of cloned *vin* cDNA was translated in vitro using the TNT T7 coupled transcription and translation system in the presence of [35 S]methionine (lanes 1 and 2). In vitro translation products of the control pET-22b(+) and luciferase coding sequences are shown in lanes 3 and 4, respectively. Position of the victoriocin precursor is indicated by an arrowhead to the left of the panels and the migration of standard proteins with known molecular masses is shown to the right.

Fig. 3. Analysis of the *vin* transformants. **A**, Western blot analysis of proteins extracted from a virus-free *Helminthosporium victoriae* isolate transformed with the *vin* constructs pCB-SnaBI/NotI (lanes SnaB1 and SnaB2), pCB-EcoRI (lane EcoR), pCB-Clal (lane Cla), pCB-SmaI (lane Sma), and empty vector (pCB1004; lane vector). ppV, preprovictoriocin; pV, provictoriocin; and V, mature victoriocin. See Figure 1 for diagrams of the *vin* constructs. **B**, Northern hybridization analysis of RNA extracts from the same transformants used for Western blotting analysis, including transformants pCB-SnaBI/NotI (lanes SnaB1 and SnaB2), pCB-EcoRI (lane EcoR), pCB-Clal (lane Cla), and empty vector (pCB1004; lane vector) (a low level of the *vin* transcript could be observed after extended exposure).

probe, were consistent with the victorin western results (Fig. 3B). Thus, the length of the sequence upstream of the *vin* ORF in the various constructs appears to correlate with the victorin expression level. Interestingly, the antifungal activity of the

TABLE 1. Diameter of inhibition zone

Transformant	Diameter (mm)		
	24 h	48 h	72 h
A-9 ^a	20	15	13
A-T1	25	18	16
A-T2	24	17	15
A-T3	26	17	15
A-T4	28	18	17
A-T5	24	16	14
A-T6	26	17	16
A-T7	26	17	16
B-2 ^b	0	0	0
B-T1	14	10	6
B-T2	10	6	0
B-T3	17	11	6
B-T4	6	0	0
B-T5	6	0	0
B-T6	19	12	11
B-T12	20	12	10
B-T18	20	15	12
B-T20	16	10	9

^a Wild-type virus-infected *Helminthosporium victoriae* isolate A-9 and its *vin* transformants A-T1 to A-T7.

^b Wild-type virus free isolate B-2ss and its *vin* transformants B-T1 to B-T6 and B-T12, B-T18, and B-T20.

culture filtrates of the *vin* transformants also correlated well with the western and northern analyses (data not shown). Although the antifungal activity of some the *vin* transformants (transformed with construct pCB-ClaI) was as high as that of culture filtrates of the virus-infected strain A-9, many of the transformants showed lower activity (Table 1).

The colony morphology of the vector control (Fig. 4, vector) was indistinguishable from that of the wild-type B-2ss strain. On the other hand, the *vin* transformants, with the exception of the pCB-EcoRI transformants, showed reduced growth and sectoring (Fig. 4). Although the reduction in growth and sectoring represent features reminiscent of disease symptoms in virus-infected isolates, the latter usually exhibit more severe stunting, aerial mycelium collapse, and excessive sectoring (Fig. 4, isolate A-9).

Transformation of a virus-infected isolate with the *vin* constructs. It was of interest to determine whether overproduction of victorin in a virus-infected isolate would further enhance the antifungal activity of its culture filtrate because the antifungal activity of culture filtrates from the wild-type virus-infected isolate was still higher than most of virus-free transformants with the *vin* gene (Table 1). For this purpose, isolate A-9 was transformed with the pCB-ClaI construct and victorin production and antifungal activity were monitored using western blot analysis and the well assay. The results showed that victorin accumulated to higher levels in the A-9 transformants compared with the wild-type strain (Fig. 5). Interestingly, the antifungal activity of the A-9 *vin* transformants was markedly higher than that of the wild-type A-9 strain and the virus-free strain overexpressing the *vin* gene (Table 1).

Fig. 4. Colony morphology of representative *vin* and empty vector transformants and a virus-infected isolate of *Helminthosporium* (*Cochliobolus*) *victoriae*. Virus-free isolate (strain B2ss) transformed with empty vector, construct pCB-EcoRI, construct SnaBI/NotI, construct pCB-ClaBI, and construct pCB-ClaBI but grown on a medium containing hygromycin (50 µg/ml). A culture of virus-infected isolate is shown in panel strain A-9. Unless otherwise specified, all fungal isolates were grown on potato dextrose agar containing 0.5% (wt/vol) yeast extract medium for 7 days prior to photography.

DISCUSSION

Present evidence suggests that overproduction of the broad-spectrum antifungal protein victoriocin in culture filtrates of the virus-infected strain *H. victoriae* A-9 is correlated with virus infection because virus-free *H. victoriae* isolates secrete little or no victoriocin (2). We have previously presented evidence that victoriocin is encoded by a host gene, designated the *vin* gene, and predicted that it is expressed in vivo as a prepro toxin (preprovictoriocin). We envisioned, based on its structure, that the preprovictoriocin is processed by a signal peptidase and a kexin-like endopeptidase to generate the provictoriocin and the mature victoriocin, respectively (2). In the present study, we over-expressed the *vin* gene in a virus-free *H. victoriae* strain and clearly demonstrated the presence in the *vin* transformants of the preprovictoriocin and its post-translationally generated products, the provictoriocin and the mature victoriocin. Processing of the victoriocin prepro toxin requires eukaryotic host factors because no processing occurred in the in vitro translation system or in bacteria. Furthermore, culture filtrates of the *vin* transformants exhibited antifungal activity markedly higher than that revealed

by the nontransformed virus-free strains. Moreover, transformation of the wild-type virus-infected *H. victoriae* strain with the *vin* gene resulted in still higher production of victoriocin and higher antifungal activity in the culture filtrates of the *vin* transformants compared with the virus-infected wild-type strain. Taken together, these results suggest that victoriocin is the primary protein responsible for the antifungal activity in culture filtrates of virus-infected *H. victoriae* isolates and that virus infection upregulates the expression of victoriocin.

The virus-infected diseased strain A-9, used in our study, shows typical disease symptoms of stunted and highly sectorized colonies, aerial mycelial collapse, and swollen hyphae at the edge of colonies (7). Interestingly, some of the virus-free isolates transformed with the *vin* gene exhibited some features of disease symptoms, including moderate stunting and sectoring. It is not known, at present, whether victoriocin plays a direct or indirect role in disease development. Overproduction of victoriocin may give the slower-growing virus-infected fungal strains some competitive advantage by inhibiting the growth of aggressive fungi. The finding that victoriocin has little or no growth inhibition effects on *H. victoriae* isolates (2) rules out a direct role in disease

Fig. 5. Western blot analysis of virus-free and virus-infected *Helminthosporium victoriae* isolates transformed with the *vin* gene. **A**, Virus-infected strain A-9 and its *vin*-transformants, T1 through A-T7. **B**, Virus-free strain B-2 and its transformants, B-T1 through B-T6 and B-T-12, B-T18, and B-T20.

development. Victoriocin may play an indirect role in disease development through the overexpression of the cell wall protein P30 (2). P30, which is overproduced in culture filtrates of virus-infected isolates and co-purifies with victoriocin, shares sequence similarity with PhiA cell wall proteins from several species of *Aspergillus*. PhiA, a homologue of *H. victoriae* P30 (2), is known to be overexpressed in *Aspergillus nidulans* in response to treatments with antifungal and antibacterial agents and may contribute to the resultant abnormal swollen hyphal morphology. The latter is reminiscent of some aspects of *H. victoriae* disease symptoms (7,11).

Another host factor that may play a role in disease development is the host gene *Hv-p68* (13,14). It was previously demonstrated that *Hv-p68* is upregulated as a result of virus infection. The *Hv-p68* protein belongs to the large family of FAD-dependent GMC oxidoreductases with 67 to 70% sequence identity to the alcohol oxidases of methylotrophic yeasts. Although the natural substrate for *Hv-p68* is not known, the structurally similar alcohol oxidases are known to oxidize primary alcohols irreversibly to toxic aldehydes. Overexpression of *Hv-p68* and resultant putative accumulation of toxic intermediates was proposed as a possible mechanism underlying the disease phenotype of virus-infected *H. victoriae* isolates. However, overproduction of *Hv-p68* in virus-free fungal isolates resulted in a significant increase in colony growth and did not induce a disease phenotype (18). *Hv-p68*, which copurifies with viral dsRNA (mainly that of HvV145S), is a novel multifunctional protein with alcohol oxidase, protein kinase, and RNA-binding activities. The RNA-binding activity of *Hv-p68* has been demonstrated by gel mobility shift and north-western blot analysis (13,14). Because dsRNA-binding proteins are known to sequester dsRNA and suppress antiviral host defense mechanisms, it is feasible that overexpression of the dsRNA-binding protein *Hv-p68* may lead to the induction of the disease phenotype by suppressing host defense. This idea is consistent with the finding that overexpression of *Hv-p68* led to enhancement in the accumulation of HvV145S dsRNA (18).

Regarding the viral factors that function as symptom determinants, our results to date indicate that HvV190S-CP is a major player in this regard (6) (*unpublished data*). Transformation of a normal virus-free fungal isolate with a full-length cDNA of Hv190SV dsRNA conferred a disease phenotype in some transformants. Symptom severity varied among the transformants from symptomless to severely stunted and highly sectorized. Symptom severity correlated well with the level of viral capsid accumulation, with empty capsids accumulating to markedly higher levels in transformants exhibiting the disease phenotype. The role of the HvV145S in disease development, however, has yet to be elucidated (8).

In summary, the broad-spectrum antifungal protein victoriocin is encoded by the host *vin* gene, which is upregulated by virus infection. Like several other antifungal proteins, including the virally encoded killer proteins, victoriocin is expressed as a preprotoxin precursor. The marked similarities between some chromosomally encoded and virally encoded antifungal proteins suggest that the viral genes coding for these antifungal proteins were acquired by the virus from its eukaryotic host. We previously described the similarities between the chromosomally encoded victoriocin and the virally encoded zygocin (2). Victoriocin is believed to confer ecological advantage to its slow-growing host by inhibiting the growth of other fungi. Furthermore, up-

regulation of the *vin* gene in the virus-infected isolates may indirectly contribute to the disease phenotype.

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