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Cloning and targeted disruption, via *Agrobacterium tumefaciens*-mediated transformation, of a trypsin protease gene from the vascular wilt fungus *Verticillium dahliae*

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Abstract A gene encoding a trypsin protease was isolated from a tomato isolate of *Verticillium dahliae*. The gene, designated *VTP1*, contains two introns and is predicted to encode a protein of 256 amino acids. The gene is present in *V. dahliae* isolates from different host plants and in *V. albo-atrum*; weakly hybridizing sequences are present in *V. tricorpus*. *VTP1* cDNA sequences were identified in a sequence tag analysis of genes expressed under growth conditions that promote microsclerotia development. Replacement of the gene, by *Agrobacterium tumefaciens*-mediated transformation (ATMT), with a mutant allele construct did not noticeably alter either pathogenicity or growth in culture. Searches of expressed sequence tag databases showed that, in addition to the *VTP1* gene, *V. dahliae* contains two genes encoding subtilisin-like proteases similar to those produced by pathogenic *Aspergillus* spp. This is the first description of the application of ATMT to the molecular analysis of phytopathogenic *Verticillium* spp.

Keywords *Verticillium* wilt · Extracellular hydrolase · Subtilase · Transposon mutagenesis

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Introduction

Verticillium dahliae Kleb. causes vascular wilt in a wide variety of dicotyledonous plant species, and produces a variety of extracellular hydrolases, most notably pectinases and cellulases, which have long been considered important for the induction of disease symptoms (for a review, see Pegg 1981; Pegg and Brady 2002; Puhalla and Bell 1981). Protease activity has been reported in *V. dahliae* (Dobinson et al. 1997; Lambert and Pujarniscle 1984; St. Leger et al. 1997), which is able to hydrolyze a broad range of complex proteinaceous substrates.

Proteases have been proposed to facilitate penetration of the plant cell walls by fungal pathogens, degrade host defense-related proteins, and/or to allow the fungus to utilize complex plant cell wall proteins as a nutrient source during colonization of the plant (Dobinson et al. 1997; Murphy and Walton 1996). However, studies of the foliar pathogens *Cochliobolus carbonum* and *Stagonospora nodorum* and the vascular wilt fungus *Fusarium oxysporum* f. sp. *lycopersici* have demonstrated that targeted mutation of individual protease genes in these species does not affect overall growth or pathogenicity (Bindschedler et al. 2003; DiPietro et al. 2001; Murphy and Walton 1996). Moreover, Murphy and Walton (1996) identified in *C. carbonum* both a trypsin and a subtilisin and demonstrated that mutation of the trypsin gene resulted in only a 35–45% reduction in total protease activity, while in *F. oxysporum*, mutation of a subtilase gene had no significant effect on overall levels of protease activity (DiPietro et al. 2001). Similarly, Bindschedler et al. (2003) found that the SNP1 trypsin accounted for only approximately 50% of the protease activity in a wild-type strain of *S. nodorum* and that, in the absence of that enzyme, a subtilisin-like protease could be detected.

It has been reported that the phytopathogenic *Verticillium* species lack subtilisins (Bidochka et al. 1999; Segers et al. 1999). However, St. Leger et al. (1997)

described low levels of subtilisin activity in both *V. dahliae* and *V. albo-atrum* isolates for which trypsin-like enzymes were the primary proteases. Given that these species are poor saprophytes in field soil (Schnathorst 1981) and grow more slowly in culture than the more aggressive wilt pathogen *F. oxysporum* f. sp. *lycopersici*, we were interested in determining whether a previously identified extracellular trypsin protease from *V. dahliae* (Dobinson et al. 1997) is functionally redundant, as appears to be the case with the *F. oxysporum* f. sp. *lycopersici* extracellular proteases (DiPietro et al. 2001). We report here the cloning and mutation, by *Agrobacterium tumefaciens*-mediated transformation (ATMT), of the gene encoding the *V. dahliae* trypsin, and a preliminary analysis of gene sequences corresponding to two subtilisin-like proteases that were identified during the course of an expressed sequence tag (EST) analysis of *V. dahliae*.

Despite the abundance of molecular methods that have been developed for the study of fungal plant pathogens and the worldwide economic importance of verticillium wilt, this disease is poorly represented in the arena of molecular plant pathology. The results of our experiments demonstrate that targeted gene disruptions may be readily produced in *V. dahliae* by transposon mutagenesis of a cloned gene of interest, followed by transfer of the mutant gene into *V. dahliae* via ATMT. These tools, together with the ability to search recently generated EST databases (Neumann and Dobinson 2003) for genes of interest, thus provide us with valuable resources for the molecular analysis of phytopathogenic *Verticillium* spp.

Materials and methods

Fungal strains and growth conditions

All isolates of *V. dahliae* and other *Verticillium* spp used in this study are derived from single-spore cultures and maintained as described by Dobinson (1995). Cultures were grown on complete medium (CM; Dobinson et al. 1997) agar and in liquid CM unless otherwise specified. Hygromycin B-resistant strains were grown on CM agar amended with 25 µg hygromycin B/ml. Spore inoculum was prepared from cultures grown in liquid CM, as described by Dobinson et al. (1997).

Nucleic acid manipulations

Genomic DNA used for DNA blot hybridizations was purified from *V. dahliae* spores using a glass bead breakage method (Dobinson 1995). Genomic DNA used for library construction was purified from nuclei of *V. dahliae* isolate Dvd-T5 (ATCC accession 201177), as described by Timberlake (1986). Plasmids were purified from bacterial cultures by an alkaline lysis method using the QIAprep 8 miniprep kit (Qiagen, Mississauga, Canada).

A lambda genomic DNA library was constructed from Dvd-T5 DNA partially digested with *Sau3AI* and cloned into λGEM11 *XhoI* half-site arms (Promega Corp., Madison, Wis.), using procedures recommended by the supplier. The library was screened by plaque hybridization with the 41-base oligonucleotide 5'-ACT TTGAAGGGAAAGTCGCCGCGCTGGCAGCAACACCAC C-3', which was designed based on the N-terminal amino acid

sequence of the *V. dahliae* trypsin VDP30 (Dobinson et al. 1997) and the codon usage of a *F. oxysporum* trypsin gene (Rypniewski et al. 1993). The oligonucleotide was 5' end-labeled with [γ - 32 P]-ATP, using Ready-To-Go T4 polynucleotide kinase (Amersham Pharmacia Biotech, Baie d'Urfé, Canada). Hybridizations and washes were done at 50 °C, as described by Dobinson et al. (1993).

A plasmid library of *HindIII* fragments (1.8–2.3 kb) from Dvd-T5 nuclear DNA was constructed in pBluescript II KS (+); the *HindIII*-digested DNA was fractionated by electrophoresis through 0.8% low-melting-point agarose gels and recovered from the agarose by treatment with β -agarase (New England Biolabs, Mississauga, Canada), using conditions recommended by the supplier. The library was screened by colony hybridization with a digoxigenin (DIG)-labeled PCR product (probe II, see Fig. 2), amplified as described below. These and all other hybridizations using DIG-labelled probes were done as described by Dobinson et al. (1998), except that salmon sperm DNA (final concentration 50 µg/ml) was added to the prehybridization and hybridization buffers.

For DNA blot hybridizations, genomic DNA (approx. 500 ng) was digested with the restriction enzymes specified, fractionated through 0.8% agarose gels, and transferred by capillary blotting to Hybond N+ membranes (Amersham Pharmacia Biotech).

DNA sequence analysis was done at the John P. Robarts Research Institute Sequencing Facility (London, Canada) or at the Southern Crop Protection and Food Research Centre sequencing facility at Agriculture and Agri-Food Canada (London, Canada), as described by Neumann and Dobinson (2003), using custom primers and primers complementary to cloning site sequences in the plasmid vectors. Protein sequences were aligned using the ClustalW algorithm of MegaAlign (DNASar, Madison, Wis.), and shaded using the publicly available program Boxshade ver. 3.21.

To construct the *VTP1* gene-replacement vector, a 2-kb *HindIII* fragment containing the full-length gene and flanking sequences was subcloned into the binary vector pDHT (Mullins et al. 2001). The cloned gene was subjected to in vitro transposon mutagenesis, using the EZ::TN system (Epicentre Technologies, Madison, Wis.) and custom vector pSK846, which is derived from pMOD-2 (Epicentre Technologies) and contains, between the EZ::TN transposase recognition sequences, the chloramphenicol resistance gene amplified from pBCSK (Stratagene, La Jolla, Calif.) and the hygromycin B resistance gene cassette from plasmid pCB1004 (Carroll et al. 1994). The transposon was released from pSK846 by digestion with *XmnI*. Mutagenized plasmids were propagated in *Escherichia coli* strain DH5 α MRF' and chloramphenicol-resistant clones that contained the transposon inserted into the *VTP1* gene coding region were identified by PCR amplification, as described below, using primers complementary to sequences at the 5' and 3' ends of the *VTP1* coding sequence. Transposon insertion sites within the coding region were determined by sequence analysis, as described above, using the pMOD-2 forward and reverse sequencing primers (Epicentre Technologies).

PCR amplification reactions contained 1× PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 2.5 mM MgCl₂, 200 µM of each dNTP, 5–7 pmol of each primer, 0.5 units of *Taq* or Platinum *Taq* DNA polymerase (Invitrogen, Burlington, Canada), and template (1–5 ng of plasmid, 10–15 ng of genomic DNA, or approx. 8×10⁵ plaque-forming units of phage stock from a developing microsclerotia cDNA library; Neumann and Dobinson 2003). A denaturation step (2 min at 94 °C) was followed by 30 cycles of denaturation (45 s at 94 °C), annealing (45 s at the specified temperatures), and elongation (1 min at 72 °C), followed by a final elongation step (5–7 min at 72 °C). DIG-labelled amplicons used for DNA blot hybridizations were synthesized from plasmid templates, using the PCR-DIG labeling kit (Roche Diagnostics, Laval, Canada), primers complementary to vector sequences flanking the cloning site, and an annealing temperature of 65 °C.

For RT-PCR analyses, fungal cultures were grown on basal medium agar and total RNA was extracted with TRIzol reagent (Invitrogen), as described by Neumann and Dobinson (2003). Prior to use, RNA (0.5 µg in sterile distilled water) was treated with amplification grade DNase I (Invitrogen) and reverse transcribed from an oligo(dT)_{12–18} primer with SuperScript II Rnase

H⁻ reverse transcriptase (Invitrogen), according to the supplier's protocols. *VTP1* and actin gene sequences were amplified from the cDNA and genomic DNA as described above, using an annealing temperature of 60 °C. Reactions contained both *VTP1* gene-specific primers (5'-GCGGTGGCTGGTTCCTATCAAC-3' and 5'-CAACGACTTCGCCATCTGGAAG-3') and actin gene-specific primers (5'-GCCCTCTCCAGCCCTCCGTTCTC-3' and 5'-TCGGCGTGGTTTTGTGGTGAG-3'); both primer sets flanked introns in the respective genes.

Transformation-mediated mutagenesis of *V. dahliae* *VTP1* gene

ATMT of *V. dahliae* was done as described by Mullins et al. (2001), with several modifications as follows: (1) prior to co-cultivation, *V. dahliae* was grown in liquid CM and conidia were harvested as described above, (2) *A. tumefaciens* AGL-1 cells containing the gene-replacement vector were grown in induction medium without acetosyringone, and (3) *A. tumefaciens* cells and *V. dahliae* spores were co-cultivated for 2 days on a nitrocellulose membrane, before the membrane was transferred to selective medium (CM containing 50 µg hygromycin B/ml, 100 µg moxalactam/ml, 200 µM cefotaxime). Hygromycin B-resistant transformants were single-spore purified as described by Dobinson (1995) and screened by PCR amplification and DNA blot hybridization, as described above, to identify gene-replacement mutants.

Protease activity and pathogenicity assays

Protease activity was assessed by growing cultures on a skim-milk agar medium (Dobinson et al. 1997) containing 2% or 1% skim-milk powder and 0.5% glucose. Cultures were incubated at 24 °C or 28 °C for 12 days.

Pathogenicity was assessed using 14-day-old tomato seedlings (cv. Bonny Best) and a root dip inoculation method, as described by Dobinson et al. (1996).

Results

Characterization of the *V. dahliae* *VTP1* gene

DNA sequences corresponding to an extracellular trypsin-like protease (previously designated VDP30) produced by the *V. dahliae* tomato isolate Dvd-T5 (Dobinson et al. 1997) were isolated by screening the lambda Dvd-T5 genomic DNA library with an oligonucleotide probe, as described in the Materials and methods. Under the low stringency conditions of the hybridization, the probe hybridized to six families of clones. DNA sequence analysis indicated that only one of these families contained trypsin gene sequences. The full-length gene (designated *VTP1*) (GenBank accession AY354459), located on a 2-kb *Hind*III restriction fragments and sequenced in both directions, as described in the Materials and methods.

The *VTP1* sequence encodes a predicted amino acid sequence of 256 amino acids (28.2 kDa); the nucleotide sequence surrounding the putative translation initiation codon (CATCATGGTT) is consistent with the fungal translation initiation consensus sequence (Edelmann and Staben 1994). Comparison of the genomic DNA sequence with that of the cDNA (amplified from

recombinant phage of a *V. dahliae* cDNA library, as described in the Materials and methods) confirmed the presence of two small introns (56 bp, 66 bp) with 5' and 3' donor sites and splice branch sites similar to those in previously characterized fungal introns (Edelmann and Staben 1994). A polyadenylation site 307 bp downstream of the translation stop codon was identified by sequence analysis of a clone from the same cDNA library.

DNA blot hybridization analysis showed that *VTP1* gene sequences are highly conserved in the genomes of *V. dahliae* strains isolated from a variety of host plants, including tomato, potato, and Japanese maple, and in isolates of the closely related plant pathogen *V. albo-atrum* (Fig. 1). Weakly hybridizing sequences were also detected in isolates of another plant-associated species, *V. tricorpus*, suggesting that this species contains a *VTP1* homologue, but none were detected in *V. nigrescens* or in the mushroom pathogen *V. fungicola* (Fig. 1).

Transformation-mediated disruption of the *VTP1* gene

For gene disruption analysis, the *Hind*III fragment carrying the *VTP1* gene was subcloned into the binary vector pDHt and subjected to in vitro transposon mutagenesis. A mutant plasmid (pEATn30) was selected in which the transposon was inserted after the 13th base of the second intron (Fig. 2A). A *V. dahliae* *VTP1* gene-replacement mutant was generated by ATMT of the mutant gene into strain Dvd-T5. Among the first few transformants analyzed, one (VDAT6-6) was identified

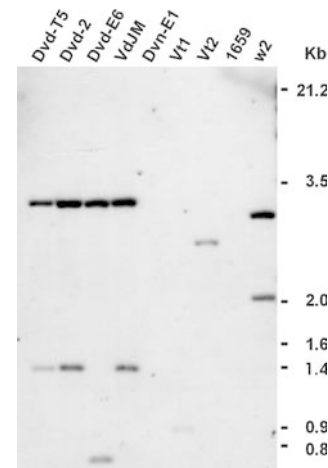


Fig. 1 Southern hybridization analysis of the *VTP1* gene in *Verticillium* spp. Genomic DNA was digested with *Xho*I and hybridized at 65 °C with the digoxigenin (DIG)-labeled *VTP1* gene probe I (shown in Fig. 2), as described in the Materials and methods. *V. dahliae* strains from tomato (*Dvd-T5*, *Dvd-2*), eggplant (*Dvd-E6*), and Japanese maple (*VdJM*), *V. nigrescens* isolate from eggplant (*Dvn-E1*), *V. tricorpus* (*Vt1*, *Vt2*), *V. fungicola* (1659), *V. albo-atrum* (*w2*). Sizes of DIG-labeled molecular size markers (Roche Molecular Biochemicals) are indicated to the right of the figure. Equivalent amounts of genomic DNA from each *Verticillium* isolate were in each lane, as indicated by ethidium bromide staining of the gel (data not shown)

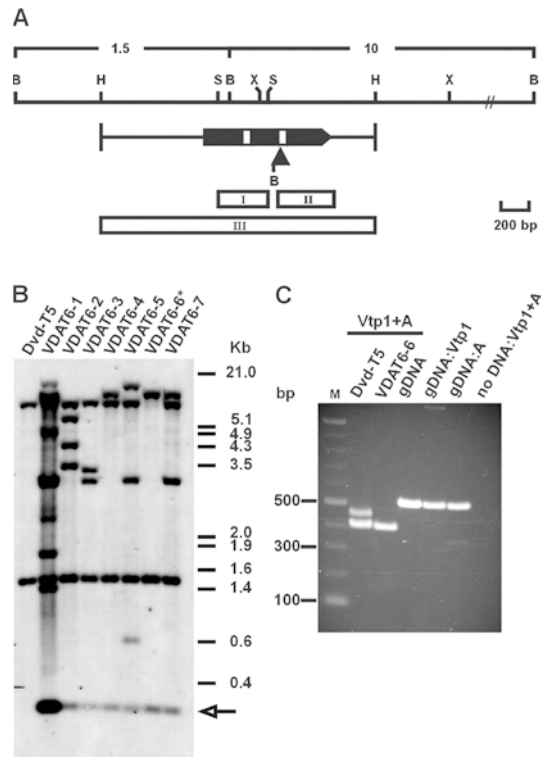


Fig. 2A–C Targeted mutation of the *V. dahliae* *VTP1* gene. **A** *VTP1* gene disruption vector pEATn30. Black boxes *VTP1* gene coding region, white boxes introns. The insertion site of the approx. 2.5-kb transposon containing the hygromycin B resistance gene cassette is indicated by an arrowhead beneath the second intron. Restriction sites: B *Bam*HI, H *Hind*III, S *Sac*I, X *Xho*I. The locations of hybridization probes used in this study are indicated by the open boxes (I, II, III) below the restriction map. **B** Southern blot analysis of wild-type strain Dvd-T5 and hygromycin B-resistant transformants (VDAT6-1 to VDAT6-7) containing the *VTP1* gene disruption construct. Genomic DNA was digested with *Bam*HI and hybridized with DIG-labeled *VTP1* gene probe III, as described in the Materials and methods. The asterisk denotes the gene-replacement mutant, and the arrow indicates the restriction fragment (approx. 360 bp) that spans the *Bam*HI sites in the transposon and the *VTP1* open reading frame. Sizes of selected DIG-labeled molecular weight markers are indicated to the right of the photograph. **C** RT-PCR analysis of Dvd-T5 and VDAT6-6, grown for 7 days on basal agar medium. RT-PCR was done as described in the Materials and methods, using *VTP1* gene-specific primers (*Vtp1*) and actin gene-specific primers (*A*). The *VTP1* and actin gene amplicons generated from the cDNA are approx. 450 bp and 400 bp, respectively. PCR amplification from Dvd-T5 genomic DNA (gDNA) yielded a product of approx. 500 bp for each primer set. Sizes of molecular size markers in lane M are indicated to the left of the photograph

in which the wild-type gene had been replaced by the mutant allele (Fig. 2B). Although transcription of *VTP1* was abolished in this transformant (Fig. 2C), its growth rate on a variety of media was not significantly different from that of the wild-type strain, nor were microsclerotia production or pathogenicity noticeably affected (data not shown).

When *V. dahliae* is grown on a skim-milk agar medium, protease production is detected as a zone of clearing around the colonies. At 28 °C, growth of all

strains on medium containing either 1% or 2% skim-milk was much slower than at 24 °C. Under all growth conditions, the zone of clearing initially appeared to be somewhat reduced in VDAT6-6, in comparison with that of Dvd-T5 and the ectopic transformants (data not shown). However, within 12 days post-inoculation, no consistent differences were seen between the strains (Fig. 3, data not shown).

Identification of two subtilase family protease genes in *V. dahliae*

During the course of an EST analysis of *V. dahliae* (Neumann and Dobinson 2003), cDNA clones were identified that corresponded to *VTP1* and to two genes encoding putative extracellular proteases not previously identified in *V. dahliae*. Sequence comparisons showed that the encoded proteins (VSP1, VSP2) were similar to members of the peptidase S8 family of subtilases, including the *Aspergillus fumigatus* oryzin (54% identity with VSP1, 51% with VSP2), and *A. oryzae* oryzin (50% identity with VSP1, 49% with VSP2) and contained the conserved aspartate, histidine, and serine residues of the subtilase catalytic triad (Fig. 4). Although the *VSP1* gene sequence was first identified in the cDNA library generated from *V. dahliae* cells grown under conditions that promote near-synchronous development of microsclerotia, and the *VSP2* sequence was identified in the library generated from cells grown in a simulated xylem fluid medium (Neumann and Dobinson 2003), the two sequences could be amplified from both cDNA libraries (data not shown).

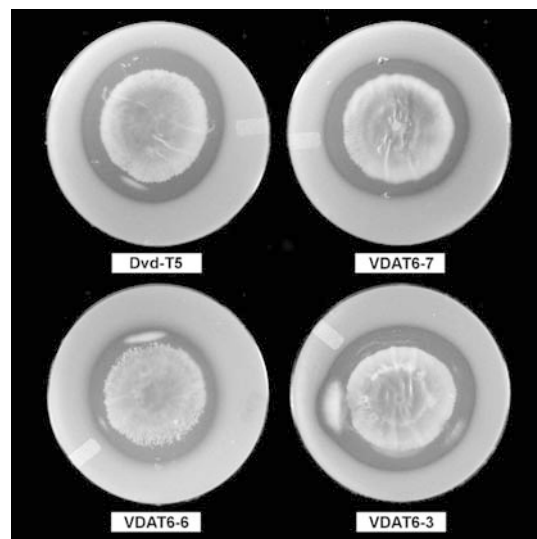


Fig. 3 Growth and protease production of the wild-type strain Dvd-T5, gene disruption mutant VDAT6-6, and ectopic transformants VDAT6-3 and VDAT6-7. Cultures were grown for 12 days at 24 °C on an agar medium containing 2% skim-milk, as described in the Materials and methods

Fig. 4 Alignment of translated cDNA sequences from *VSP1* and *VSP2* (GenBank accessions AY356397 and AY356398, respectively) with subtilase family enzymes *Aspergillus oryzae* (*Ao*) oryzin (accession P12547), and *A. fumigatus* (*Af*) oryzin (accession P28296). Alignment was done as described in the Materials and methods. Identical amino acids are highlighted in black and indicated in the consensus line with asterisks. Conserved amino acids are highlighted in gray and indicated in the consensus line with dots. The amino acids of the catalytic triad are indicated with asterisks above the sequences

VSP2	1	MVNNFQRLSVLVAALLPVGLAAPVQSEERDAESFGVVVGASAKDSYITLTKDDL EARDVES
VSP1	1	MVNNFQRLSVLVAALLPVGLAAPVQSEERDAESFGVVVGASAKDSYITLTKDDL EARDVES
Ao oryzin	1	M-OSTKRTLLLLGALLPAVLGAPVQETRRAAEKI-----PGKYIVTFKPGIDEAKIQE
Af oryzin	1	M-LSIKRTLLLLGAVLPAVFGAPVQETRRAAQKI-----PGKYIVTFKPGTDTATLES
consensus	1*
VSP2	1SDDDMILQIMNEPEVAWV
VSP1	61	HLDWLNEVQARALNKRDFP-----GVKKHYDI GAYHGYSGKFDEETLEAL KASPEVAAV
Ao oryzin	53	HTLWATNIFORSLERRGATGGDL PVGIERNYKINKFAAYAGSFDDATI EELRKNE DVAVV
Af oryzin	53	HTLWATDLHKNLERRDTTSGEP PVGIEKSYKIKDFAAYAGSFDDATI EELRKRGDVAHV
consensus	61*
VSP2	19	EANARVKLSASVAQVNAPPGLNRLSNAQAGTGNVVFDTTGGEGVTYVVVDLIGRTHDSEF
VSP1	115	EKDQIWLLEAVTSSQSGAPYGLGSLSSRTGASTIYRYDSSAGOGTYAYVVDGVOVGHGQF
Ao oryzin	113	EEDQIYYLDGLTTQKAPWGLGSI SHKGOQSTDYI YDTSAGAGTYAYVVDG VNVVDHEEF
Af oryzin	113	EEDQI WYLDALTTQKAPWGLGSI SHKGOASTDYI YDTSAGAGTYAYVVDG VNVVDHEEF
consensus	121*
VSP2	79	QGRATFQVNTVDNVNDQNGHGS HVAGTICGOTIEGVAKSANVVAVKVLDGTGAGSNAGVL
VSP1	175	GGRATLGSNPAGGATIDTSSGHGT HVAGTIGSTYGVAKRTNLSVKVVFVGNATA-STSVIL
Ao oryzin	173	EGRASKAYNAAGGQHVDSI GHGT HVSGTI AGKTYGIAKAKASLSVKVVFQGES- STSVIL
Af oryzin	173	ESRASLAYNAAGGS HVDSI GHGT HVAGTI GGKTYGVAKKINLLSVKVVFQGES- STSVIL
consensus	181*
VSP2	139	DGLQFIINDVQQKRLRGKAVMNMSLGGPQSAAVNRAVQALFDAGVVPVVAAGNENQDAAN
VSP1	234	SGFNWAANDIRTKARTITTSVVNLSLGGGFSSAFNNAVNAASQGVVSVI AAGNENQNVAN
Ao oryzin	232	DGFNWAANDI VSKKRTSKAAI NMSLGGGYSKAFNDAVENAFEQGLSVVAAGNENS DAGG
Af oryzin	232	DGFNWAANDI VSKGRTKKAAI NMSLGGGYSYAFNNAVEAFDEGLSVVAAGNENS DASIN
consensus	241*
VSP2	199	TSPASAPKAITVGAIDASNDQKASFSNFADVDIFAPGVVLSVGI RSNTATDTLSGTSM
VSP1	294	VSPASAAAI AVGAVDSAWA- IASYSNMGAGVALFAPGSNVL SVAWGSNTATRSI SGTSM
Ao oryzin	292	TSPASAPDAITVAALQKSNR-RASFSNFGKVVDFAPGQDLSVAWGSNTATRSI SGTSM
Af oryzin	292	TSPASAPNALTVAALNKSNA-RASFSNFGSVVDIFAPGQDLSVAWGSNTATNTI SGTSM
consensus	301*
VSP2	259	ASPHVAGLAAYLI ALENINTPBAVASRI TELATASGAQ-VLPGA-----
VSP1	353	ASPHVAGLVVYLQRLLEGLASPAAKARLEI ALATTGRVTGNLRGSPNRL LAYNGVA
Ao oryzin	351	ATPHIVGLSVYLALENLDGPAAVTKRIKELATKDDVKV-DVKGSPNLL LAYNGNA
Af oryzin	351	ATPHIVGLSVYLVLENLSGPAAVTARIKELATNGVVT-NVKGSPNKL LAYNGNA
consensus	361*

Discussion

We cloned and characterized the *VTP1* gene corresponding to the previously identified VDP30 trypsin-like protease from *V. dahliae* (Dobinson et al. 1997). Comparison of the experimentally determined amino-terminal sequence from purified VDP30 with that of the predicted *VTP1* protein sequence showed that the purified protein lacked the 29 N-terminal amino acids of the predicted protein. These data indicate that, as with other trypsins, the primary translation product of the *VTP1* gene undergoes proteolytic cleavage to release the mature protein. Although amino acid 1 and amino acid 15 of VDP30 (Dobinson et al. 1997) differed from those in the predicted sequence encoded by *VTP1*, such discrepancies are not uncommon and have been attributed to protein sequencing errors (Görlach et al. 1998; Scott-Craig et al. 1992).

The results of the gene knockout (KO) experiment demonstrated that the trypsin encoded by *VTP1* is not essential for overall growth and pathogenicity. The gene disruption data also indicated that *V. dahliae* produces at least one other protease and, in our expressed sequence tag databases (Neumann and Dobinson 2003), we identified genes corresponding to two proteases in the S8 subtilase family. These data are thus consistent with the results of similar studies on the proteases of other foliar or root-invading phytopathogenic fungi (Bindschedler et al. 2003; DiPietro et al. 2001; Murphy and Walton 1996) and with the overall level of redundancy among extracellular

hydrolases produced by plant pathogenic *Verticillium* spp (Bidochka et al. 1999; St. Leger et al. 1997). Bindschedler et al. (2003) suggested that the loss of a single protease in *S. nodorum* could be compensated for by the production of additional proteases. That this may also occur with *V. dahliae* is supported by our finding that, although subtilisin activity has either not been detected in trypsin-producing isolates of *V. dahliae* and *V. albo-atrum* (Bidochka et al. 1999; Segers et al. 1999), or been detected at only low levels (St. Leger et al. 1997), *V. dahliae* strain Dvd-T5 expresses at least two subtilisin-like genes when grown in vitro under conditions designed to mimic in planta nutrient conditions.

Trypsin proteases may be particularly well suited to the degradation of plant proteins and may indeed have been co-opted by plant-pathogenic *Verticillium* species for that purpose, as has been suggested by St. Leger et al. (1997). However, although the limited saprophytic capacity of these species (Schnathorst 1981) suggested to us that the previously identified *V. dahliae* trypsin (Dobinson et al. 1997) might be important for in planta growth, it appears that, as with the more aggressive wilt pathogen *F. oxysporum* (DiPietro et al. 2001), *V. dahliae* maintains an array of proteases for the degradation of complex, proteinaceous nutrient sources.

Since the first report of DNA transfer into *Saccharomyces cerevisiae* by *Agrobacterium tumefaciens* (Bundock et al. 1995), ATMT has been successfully applied to a diverse array of fungi (Abuodeh et al. 2000; Chen et al. 2000; Combier et al. 2003; Covert et al. 2001; de

Groot et al. 1998; Gouka et al. 1999; Hanif et al. 2002; Malonek and Meinhardt 2001; Mullins et al. 2001; Rho et al. 2001; Sullivan et al. 2002; Zhang et al. 2003; Zwiers and De Waard 2001). ATMT exhibits a number of advantages as a method for transforming fungi, including: (1) high efficiency transformation (de Groot et al. 1998; Mullins et al. 2001; Rho et al. 2001), (2) increased frequency of homologous recombination (Bundock et al. 1999; Mullins et al. 2001; Zwiers and De Waard 2001), (3) the ability to transform spores, hyphae, and even mushroom fruiting body tissue, thus circumventing the problems (low yield, viability) typically associated with protoplast isolation (Chen et al. 2000; de Groot et al. 1998; Mullins et al. 2001), and (4) low copy number of inserted T-DNA per genome (de Groot et al. 1998; Mullins et al. 2001; Rho et al. 2001; Sullivan et al. 2002). To our knowledge, this is the first report of the use of ATMT for transformation and gene disruption in *V. dahliae*. To facilitate the rapid generation of mutant alleles for gene KO via ATMT, in this study we also tested an in vitro transposon-mediated mutagenesis technique. A conventional method for constructing a mutant allele is to replace parts of a target gene with an antibiotic resistance gene using unique restriction enzyme sites, which is time-consuming and often limits the size of gene fragments that can be used. Based on the data in this report and our ability to use a combination of transposon tagging and ATMT for the mutagenesis of several other genes (Klimes and Dobinson 2003; Kang et al., unpublished data; Dobinson et al., unpublished data), this gene KO method provides us with a valuable tool for the molecular analysis of verticillium wilt.

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