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RESEARCH PAPERS

The role of the β-1,6-endoglucanase gene vegB in physiology and virulence of *Verticillium dahliae*

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Summary. The β-1,6-endoglucanase gene (*veg*B) of *Verticillium dahliae* was isolated using a genome walking technique. Nucleotide and deduced amino acid sequences of the gene showed high identity with the PAN1 sequence deposited at the Verticillium genome database (Broad Institute), but significant differences in intron numbers and sites of insertion. Detailed *in silico* analysis, accompanied by sequencing of both genomic and cDNA, as well as RT-PCR experiments, provided the correct size of the gene and the exact number, length and positions of introns. The putative protein of this gene was compared with corresponding β-1,6-endoglucanases from other fungi, and sequences were used to construct a phylogenetic tree. A clear differentiation between enzymes derived from plant pathogenic and mycoparasitic fungi was observed, fully supported by bootstrap data. An internal fragment (1.2kb) of *veg*B was used to disrupt the wild-type gene of a *V. dahliae* tomato race 2 strain, and the mutant strain, *veg*B-, was tested for pathogenicity on tomato plants. Results showed a small but constant reduction in disease symptoms only on eggplants for the *veg*B- strain in comparison with the wild type. Growth on minimal medium supplemented with different carbon sources showed reduced ability of the mutant to breakdown cellulose, whereas growth on glucose, pectin and sucrose was similar to the wild type.

Key words: gene disruption, cell wall degrading genes, pathogenicity, genome walking.

Introduction

Verticillium dahliae Kleb. is a cosmopolitan soil-borne fungus that causes vascular wilt diseases in a wide range of hosts, including economically important annual herbaceous plants, perennial plants and trees (Pegg and Brady, 2002). The fungus produces microsclerotia that can survive in the soil more than 10 years, thus increasing its capacity to infect a large number of hosts (Pegg and Brady, 2002; Klosterman *et al.*, 2009). Although host cultivars tolerant to Verticillium wilt have been identified for some plants, resistance to the fungus in most plants still remains rare or undiscovered, and the fungus poses chronic economic problems in crop production (Pegg and Brady, 2002; Fradin and Thomma, 2006; Vallad and Subbarao, 2008). For the few crops in which resistance exists, tomato is the best studied example. Resistant plants carry the *Ve1* resistance gene, which encodes for a cell surface receptor with extracellular leucine-rich repeats (LRRs) (Kawchuk *et al.*, 2001), which upon recognition of the pathogen activates plant defence responses (Fradin *et al.*, 2009; De Jonge *et al.*, 2012). Two *V. dahliae* races (race 1 and race 2) have been identified according to their virulence on resistant plants. Race 1 isolates are unable to cause disease in resistant plant varieties because they carry

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the avirulence gene *Ave1*, which encodes for an effector protein that is recognized by the *Ve1* gene. *Ave1* has been recently characterized, and shown to be very similar to plant natriuretic peptides and plant proteins with a conserved endoglucanase domain. It is hypothesized that this gene is implicated in water regulation and maintenance of ion state (De Jonge *et al*., 2012). Race 2 isolates lack the avirulence gene *Ave1*, are not recognized by host plants and, therefore, are pathogenic. So far, no source of resistance to race 2 has been detected (Kawchuk *et al.,* 2001; Fradin *et al*., 2009; Klosterman *et al*., 2009).

In a compatible interaction, *V. dahliae* infects the plant through the root and invades the vascular system (Schnathorst, 1981; Vallad and Subbarao, 2008). Invading hyphae produce conidia that spread systemically through the xylem vessels and are trapped at vessel end walls. The fungus produces hydrolytic enzymes that erode the pit membrane enabling hyphae to grow toward adjacent vessel walls (Cooper and Wood 1975; Bishop and Cooper 1983a, b; Fradin and Thomma, 2006). Since hydrolytic enzymes of fungal plant pathogens are known to facilitate release of nutrients and penetration of plant cell walls, they are generally considered important for pathogenesis (Pegg, 1981; Puhalla and Bell, 1981; Walton, 1994; Pegg and Brady, 2002). However, due to functional redundancy, it is difficult to assess the role of single cell wall degrading enzymes (CWDE) in pathogenesis (García-Maceira *et al.,* 2000; Idnurm and Howlett, 2001; Dobinson *et al.,* 2004). In *Verticillium,* the synergistic role of several CWDEs in virulence has been demonstrated through different approaches (Pegg, 1981; Puhalla and Bell, 1981; Durrands and Cooper 1988a; Durrands and Cooper, 1988b; Cooper and Durrands, 1989; Pegg and Brady, 2002; Tzima *et al*., 2011). In a recent study on insertional mutagenesis of *V. dahliae*, a single endoglucanase gene was found to be important for virulence (Maruthachalam *et al.,* 2011).

β-glucans are the most abundant naturally occurring polysaccharides, found mainly in plants due to the presence of cellulose, but also in microorganisms and some invertebrates (Pitson *et al.,* 1993). Other β-glucans, like β-1,3- or β-1,6-glucan, although less common, are synthesized by many fungi as components of their cell walls or as extracellular metabolites, and enzymes cleaving such bonds have been isolated from many fungi, including *Gibberella fujikuroi*, *Acremonium persicinum*, *Neurospora crassa*, *Rhizopus chinensis*, *Penicillium* spp., and *Saccharomycopsis fibuligera* (Shibata and Fukimbara, 1972; Shibata and Fukimbara, 1973; Shibata, 1974; Yamamoto *et al.,* 1974; Santos *et al.,* 1979; Schep *et al.,* 1984; Hiura *et al.,* 1987; Mulenga and Berry, 1994; Pitson *et al.,* 1996; Moy *et al*., 2002).

During parasitic growth, extracellular β-glucanases secreted by plant pathogenic fungi are presumed to degrade the β-1,3-glucan plant wall callose that surrounds the invading hyphae. Various roles have been attributed to the β-glucans liberated from hosts or pathogens, such as acting as inducers or suppressors of defense responses in the host, protecting the fungus from adverse host responses and entrapping hydrolytic enzymes within the fungal hyphae (Pitson *et al.,* 1993; Moy *et al.,* 2002; Shinya *et al.,* 2006; Martin *et al*., 2007). Fungal derived glucanases and/or host defense enzymes contribute to the release of the entrapped hydrolytic enzymes, increasing the ability of the fungus to attack host cell walls (Gil-ad *et al.,* 2001; Martin *et al.,* 2007). At early stages of infection, β-glucanases were shown to provide plasticity to β-1,3-1,6-D glucan sheaths facilitating attachment of *Phanerochaete chrysosporium* and transport of fungal enzymes to infected cell walls of *Populus tremula*, thus inducing wilting symptoms (Ruel and Joseleau, 1991). In response to conditions of carbon and energy source exhaustion, β-1,3- and β-1,6-endoglucanases probably play a role in degradation and mobilization of β-glucans produced by the fungal pathogen (Zevenhuizen and Bartnicki-Garcia, 1970; Pitson *et al.,* 1993). Similar functions have been suggested for β-glucanases from several other β-glucan-producing fungi, including *Phanaerochaete chrysosporium* (Bes *et al.,* 1987), *Sclerotium glucanicum* (Rapp, 1989, 1992), *B. cinerea* (Stahmann *et al.,* 1992, 1993) and *A. persicinum* (Stasinopoulos and Seviour, 1989; Pitson *et al.,* 1991; 1997; Martin *et al.,* 2007).

In endophytic and mycoparasitic fungi, the role of β-1,6-glucanases has been assessed through several biochemical approaches, as well as through gene disruption (Pitson *et al.,* 1993; de la Cruz *et al.,* 1995b; Moy *et al.,* 2002; Martin *et al.,* 2007). In particular, in the mycopathogen *Verticillium fungicola*, disruption of a β-1,6-glucanase gene resulted in reduced virulence on the cultivated mushroom *Agaricus bisporus* (Amey *et al.,* 2003). In contrast, the role of β-1,6-glucanases in plant pathogenic fungi is almost unknown, and has not been studied through gene

inactivation or disruption. Thus, the aim of the present study was to increase understanding of the role of β-1,6-endoglucanases in the plant pathogenic fungus *V. dahliae*. For that purpose, the full length cDNA of the β-1,6-endoglucanase gene, *veg*B, was isolated from *V. dahliae* and was phylogenetically and functionally analyzed. The phylogenetic relationship of β-1,6-endoglucanases between plant pathogenic and mycoparasitic fungi, as well as the probable role of *veg*B in physiology and virulence of the fungus, are presented and discussed in this paper.

Materials and methods

Fungal strain and growth conditions

A tomato race 2 *V. dahliae* isolate (123wt-r2) from Greece was used in this study. For long term storage, the wild type and mutant strains were stored as microconidial suspensions, at -80°C in 25% glycerol. Cultures were reactivated on freshly made potato dextrose agar (PDA).

Growth experiments on different carbon sources were performed using a basal medium containing mineral salts and trace elements, as in minimal medium (Puhalla and Mayfield 1974). Carbon sources were added individually to the basal medium (at final concentrations of 2% w/v), and the media were adjusted to pH 6.5. Radial growth assays were performed on basal medium containing 2% agar, supplemented with 2% cellulose, pectin, glucose and sucrose (all from Sigma). After placing a 10 μl conidial suspension (10^7 conidia mL^{-1}) at the center of each plate, the plates were incubated at 23°C. Colony diameter was recorded at intervals after 6 and 11 d.

Nucleic acid manipulations

Mycelium preparation and fungal DNA extraction were performed according to previously described methods (Typas *et al.*, 1992). Polymerase chain reactions (PCR) were performed in a thermocycler (MJ Research) using Taq DNA polymerase from Promega. Primers V6F and V6R were used to amplify an internal 1.2 Kb fragment of the β-1,6-endoglucanase gene (*veg*B), which was cloned and sequenced. PCR amplification was performed under the following conditions: Initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, polymerization at 72°C for 1 min and a final extension at 72°C for 7 min. The PCR product was cloned into the *Eco*RV site of pBluescript II KS (Stratagene, La Jolla, CA), creating pBvegB1200. DNA sequencing was conducted using an automated sequencer (Licor IR^2), and the sequenced data were analyzed with Lasergene software (DNASTAR). All primers used are listed on Table 1.

Total RNA was extracted from lyophilized mycelia using TRI reagent (Molecular Research Center) according to the manufacturer's instructions. One microgram of total RNA treated with RNase-free DNase (Invitrogen) was transcribed into cDNA with RevertAid™ M-MuLV Reverse Transcriptase (Fermentas) using oligo dT as primers.

For Southern analysis, about 20 μg of genomic DNA from individual strains was digested with *BamH*I, transferred to Hybond membrane by capillary transfer and fixed with UV light. Hybridization was performed using a digoxigenin (DIG)-labeled 1200 bp probe, which was generated by PCR using DIG-dUTPs (DIG Labeling and Detection kit, Roche), with primers V6F / V6R and the pBvegB1200 vector as template.

Isolation of *veg*B by genome walking

Following DNA hybridization against a genomic library of *V. dahliae*, a 3.5 kb genomic fragment that hybridized strongly with a probe containing part of the *veg*B gene (Pantou and Typas, 2005) was detected. The 3′ and 5′ ends of *veg*B gene were traced on this fragment using the Takara genome walking *LA* PCR *in vitro* cloning kit (code num. RR01) according to the manufacturer's instructions. In brief, genomic DNA from the wild-type *V. dahliae* strain was fully digested with *Hind* III, the DNA was precipitated and re-suspended in 10 μL sterilized distilled water in an eppendorf tube, to which 5 μL *Hind*III cassette, 30 μL ligation solution I and 15 μL ligation solution II were added and mixed. The reaction sample was incubated at 16°C for 1h. Immediately after the reaction, the ligated DNA was precipitated and dissolved in 5 μL sterilized distilled water. The first PCR step was carried out for 30 cycles, at 94°C for 30 sec, at annealing temperature of 55°C for 30sec, and extension at 72°C for 4 min, using 1 μL DNA sample from the previous step, the cassette primer C1 and primer VdgInS (designed from a part of the target gene; see Table 1), according to the manufacturer's instructions. The

second PCR was performed by diluting 1 μL of the above PCR product in 33 μL of sterilized water. One μL of the diluted PCR product was used in a PCR reaction with primers C2 and VdgInS2, following the standard protocol described above, with 30 cycles of denaturation at 94°C for 20 sec and extension at 65°C for 15 min.

Phylogenic analysis of fungal β-1,6-glucanases

DNA sequence alignments were made using CLUSTALW (Thompson *et al.*, 1994) with the multiple alignment parameters set to default, and then edited by visual inspection. Neighbour-Joining (NJ) analysis was used to create the phylogenetic tree of fungal β-1,6-glucanases. The PAUP* programme ver. 4.0b10 (Swofford, 2002) was used (using the Kimura-2 parameter model), with 10,000 replicates, and with random addition of taxa and tree-bisection reconnection branch swapping. Reliability of nodes was assessed using 1,000 bootstrap iterations. The amino acid sequences of β-1,6-glucanases were retrieved from Protein – GenBank, and are depicted on Figure 2 with Accession Numbers.

Protoplast preparation

Protoplasts were prepared as described by Couteaudier *et al.*, (1996) with some modifications. *Verticillium dahliae* conidia (10^6) were inoculated into 100 mL of a complete medium (Cazpek-Dox supplemented with 2% of each of: peptone, malt, yeast and tryptone extracts; Oxoid) and grown under shaking (180 rpm) for 24 h at 23°C in the dark. Fungal mycelia were harvested by filtration through Whatman filter paper No.1, and resuspended (≈ 50 mg mL^{-1}) in an isotonic buffer (0.6 M KCl; 0.1 M sodium phosphate, pH 5.8) for 2 h, at 30°C, with constant shaking (100 rpm). The suspension was centrifuged (10 min, 6,000 rpm) and the resulting pellet was re-suspended in the buffer, treated with sterile (filtering through 0.22 μm) Glucanex (Novo Nordisk Ferment) and incubated for 4 h, at 30°C, with gentle shaking (40 rpm). The suspension was filtered through sterilised sintered glass and the filtrate containing the protoplasts was centrifuged for 10 min at 4°C at 5,000 rpm. The pellet was dissolved in GMB (0.9M sorbitol, 125 mM EDTA, pH 7.5) and centrifuged again in the above conditions. After two washes with ice-cold STC (0.8M sorbitol, 125 mM EDTA, pH 7.5), the protoplasts were finally re-suspended in STC at a final concentration of approximately 1×10^7 protoplast mL^{-1}. The protoplasts were kept on ice and used immediately for transformation.

Disruption of *vegB* and analysis of transformants

The transformation vector used for the disruption of *veg*B gene was pUCATPH, which contained the *hph* gene, conferring hygromycin resistance, driven by the *trp*C *Aspergillus nidulans* promoter (Lu *et al.*, 1994). A 1.2kb *Kpn*I-*Sac*I fragment of *veg*B was subcloned into the *Kpn*I-*Sac*I sites of the vector resulting in construct pUC-Vdg6 (see Figure 1), which was subsequently used for PEG mediated transformation of the *V. dahliae* wild-type strain according to Amey *et al.* (2002). Sixteen transformants were selected, genomic DNA was isolated from each of these and they were screened by PCR to check: (i) for the insertion of the transforming DNA at the correct locus, using primer p*trp*CR that binds to the promoter of the hygromycin resistance gene and primer Vdbng-6bR that binds on the native *veg*B gene, outside the region used for disruption (Table 1); and (ii) for absence of the intact *veg*B gene, with primers that bind both within (primers V6F-V6R) and outside (primer Vdbng6bR) the 1.2 kb *veg*B region used for transformation.

Pathogenicity assays on tomato and eggplant

Virulence of the wild type and mutant *veg*B^- was evaluated using tomato (susceptible NILs of cv. Ailsa Craig) and eggplant (cv. Black Beauty). To prepare inocula, fungal cultures were grown for 5–7 d in SSN medium (Sinha and Wood, 1968) and passed through several layers of cheesecloth (to remove mycelia). The conidial concentration was adjusted to approximately 1×10^7 conidia mL^{-1}. For the virulence assay on tomato, 12 plants were inoculated for each isolate at the third true leaf stage, by immersing their roots in the conidial suspension for 20 min. For the eggplant assay, six plants at the third true leaf stage were inoculated by root dipping (as described above), and 10 plants were inoculated by drenching the roots with 10 mL conidial suspension of each isolate. Virulence assays were performed once in the tomato and eggplant root dipping assay and twice in the eggplant root drenching assay.

Disease severity at each observation was expressed by the percent of leaves that showed wilting

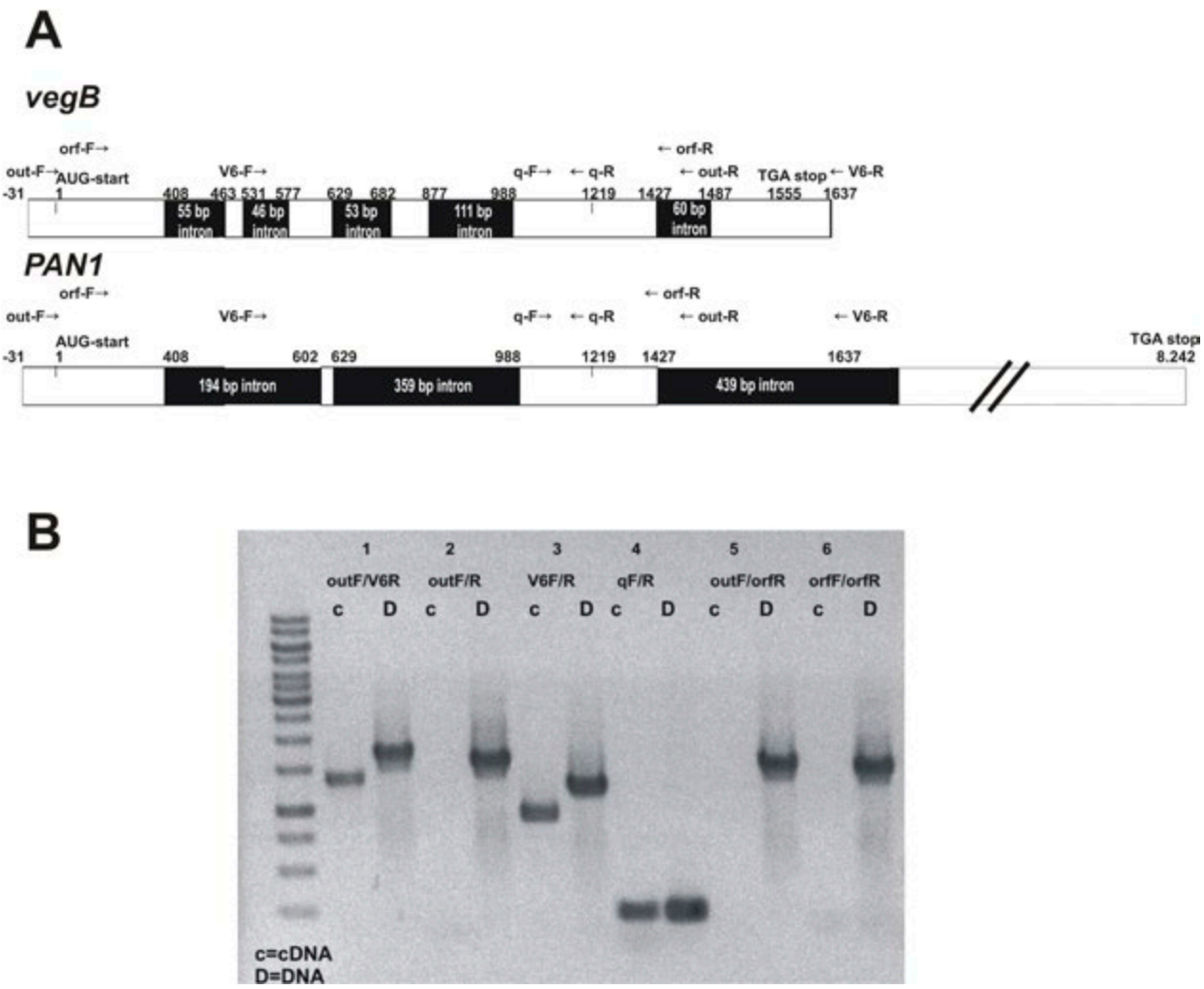

Figure 1. Determination of *veg*B open reading frame and intron locations. A) A schematic presentation of primer location and the presence of introns (black boxes) on the *veg*B nucleotide sequence (upper line) compared to the identical *PAN1* sequence, as it appears in the Verticillium genome database (lower line), B) Verification of the presence of introns on *veg*B by PCR with different primer combinations using both genomic *V. dahliae* DNA and cDNA from the wild type strain as template (c = cDNA, D = genomic DNA; primers as in Table 1).

symptoms. Disease ratings were plotted over time to generate disease progress curves and the area under the disease progress curve (AUDPC) was calculated using the trapezoidal integration method (Campbell and Madden, 1990). Disease severity was expressed as a percentage of the maximum possible AUDPC for the whole period of the experiments, and it is referred as relative AUDPC (Korolev *et al.*, 2001). Relative AUDPC values calculated for each treatment were subjected to ANOVA, and means were separated by Duncan's multiple range test.

Results

Isolation and characterization of the *V. dahliae* β-1,6-endoglucanase gene

The 568 bp clone that was previously found to contain part of the endo-1,6-β-glucanase gene (Pantou and Typas, 2005) was used as a probe for DNA hybridizations to detect the entire gene from a genomic library of *V. dahliae*. A 3.5kb genomic fragment that hybridized strongly with the above probe was isolated and through restriction analysis, hy-

bridizations, use of the genome walking technique and sequencing, a fragment of 1,830 bp containing most of the gene but lacking its 5'-end was obtained. The nucleotide sequence of this fragment showed high identity with the PAN1 gene sequence (VDAG_05935.1). Using primers based on the above sequence, helped to obtain a 1,230 bp cDNA sequence (named *veg*B), coding for a putative protein of 409 amino acids. A comparison of the genomic DNA (Broad Institute, Verticillium group database, http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/MultiHome.html) with the cDNA sequence revealed the presence of introns. The use of appropriate primers verified the complete *veg*B open reading frame (ORF) and the exact intron sizes (Supplementary Figure 1). The genomic sequence of *veg*B was concluded to be 1,555 bp, containing an ORF of 1,230 bp, interrupted by five introns (respectively, 55 bp, 46 bp, 53 bp, 111 bp and 60 bp in length, located at positions 408, 531, 629, 877 and 1427). The *veg*B sequence analysis differed in intron numbers and sizes with the corresponding introns of the PAN1 sequence provided by the Verticillium genome database (Broad Institute). More particularly, the VDAG_05935.1 PAN1 sequence was 8,272 bp long, and the first 1,680 bp were identical to our *veg*B sequence. In contrast with our analysis, the 1,680 bp coding sequence according to the Verticillium genome database is interrupted by three introns of, respectively, 194 bp, 359 bp and 210 bp in length, located at positions 408, 629 and 1427 (Figure 1A). To clarify this disagreement in results we expanded our *in silico* analysis with putative coded proteins (see below), and combined this with RT-PCR experiments in order to locate the exact positions and sizes of introns, as well as the true ORF of *veg*B. Amplicons obtained both from genomic DNA and cDNA templates using different combinations of primer pairs (Table 1; Figure 1) were sequenced, and verified the intron positions that we determined, as well as the exact length of *veg*B (the sequence is deposited in GenBank under Acc.No. KF395362). Therefore, we propose that the PAN1 sequence should be re-annotated to include two ORFs, the first of which is *veg*B.

Blast searches of the VegB putative protein indicated high levels of identity (from 35% *Metarhizium anisopliae* EFZ03924 up to 86% *Verticillium albo-atrum* XP003003310.1) and similarity (from 50% EFZ03924 *Metarhizium anisopliae* to 89% *Verticillium albo-atrum* XP003003310.1) with β-1,6-glucanase orthologs. The searches also revealed putative conserved domains of the glyco-hydro-2-c superfamily pfam00150. Protein alignment of VegB with β-1,6-glucanases, of superfamily pfam00150 and other plant pathogenic and mycoparasitic fungi, revealed the active sites of these model enzymes matching conserved glutamic acid residues of VegB and the other fungal endoglucanases, in full agreement with VegB probable active sites at 222 and 328, according to the Pfam software (http://pfam.sanger.ac.uk/family/pf00150) (Supplementary Figure 2). In addition, the putative PAN1 protein sequence showed high identity for approximately the first 400 amino acids of its N-terminal sequence with many other fungal endo-β-1,6-glucanase orthologs (e.g. *Verticillium albo-atrum* endo-β-1,6-glucanase, XP_003003310) but no sequence similarity to PAN1 orthologs (data not shown). This further confirms our conclusions about the size of *veg*B. Phylogenetic analysis using all publically available β-1,6-glucanases from ascomycetes revealed the formation of two separate groups, with strong bootstrap support (Figure 2). All β-1,6-glucanases from plant pathogenic fungi, including VegB, clustered in one branch, together with the corresponding proteins of various *Aspergillus* species. The second branch contained β-1,6-glucanases only from endophytic and mycoparasitic fungi (Figure 2). VegB displayed high identity (99%) to the *Verticillium albo–atrum* β-1,6-glucanase protein (VDBG_06872) and an identical structural organization of the corresponding chromosomal region. The *veg*B gene was therefore flanked by the gene coding for the pantetheine-phosphate adenylyltransferase family protein of *V. albo-atrum* (VDBG 06871) and the hypothetical PAN1 gene. This further strengthens our argument that the region designated as PAN1 on the *V. dahliae* genome (VDAG 05935.1) is not a single gene but a composite region that includes *veg*B and other putative ORFs.

Disruption of vegB gene in *Verticillium dahliae* strain 123v race 2

The involvement of β-1,6-endoglucanase gene in pathogenicity of *V. dahliae*, was studied by inactivating the gene and constructing a mutant *veg*B$^-$ strain. For this, a 1.2 kb internal fragment of the *veg*B gene was amplified by PCR, cloned into pBluescript (pBS), doubly digested with *Kpn*I and *Sac*I to release the insert, and further ligated to a compatibly di-

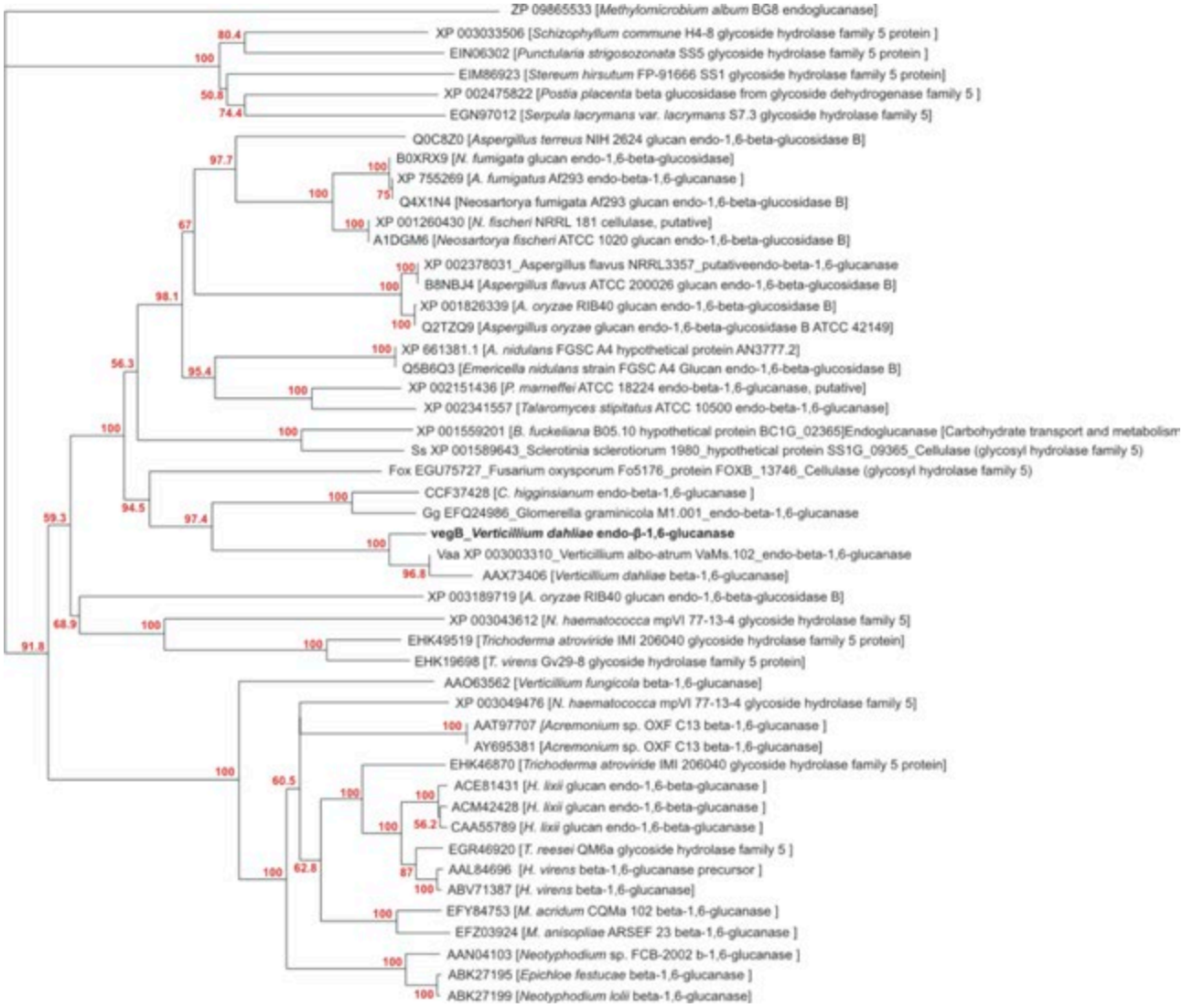

Figure 2. Phylogenetic tree constructed from unambiguously aligned amino acid sequences of the nuclear VegB and other fungal β-(1,6)-glucanases, as produced by Neighbour Joining analysis, using the PAUP software (Swofford, 2002). The aa sequence obtained during this study is presented in bold and all others retrieved from Protein - GenBank are shown with their Accession Numbers and names of fungal species. Clade credibility using NJ-bootstrap calculated from 10,000 replicates is shown.

gested disruption vector (pUCATPH) containing the selective marker hygromycin phosphotransferase gene (*hph*) (Figure 3). The recombinant vector (6.3 kb) was used to transform 1×10^7 protoplasts of the wild-type race 2 *V. dahliae* strain (Figure 3A). Sixteen stable transformants were isolated and tested for successful homologous integration at the target gene by PCR, using primers that bind to the hygromycin gene and to the chromosome outside the region of the 1.2 kb fragment (primer PtrpCR and Vdbng6bR, respectively; Figure 3A), which results in a 740 bp PCR product only when homologous recombination has taken place. In the first screening, when using primers V6F and V6R amplifying the 1.2 kb *veg*B fragment, the tranformants with a homologous integration gave no fragment (Figure 3B, lane 3), where-

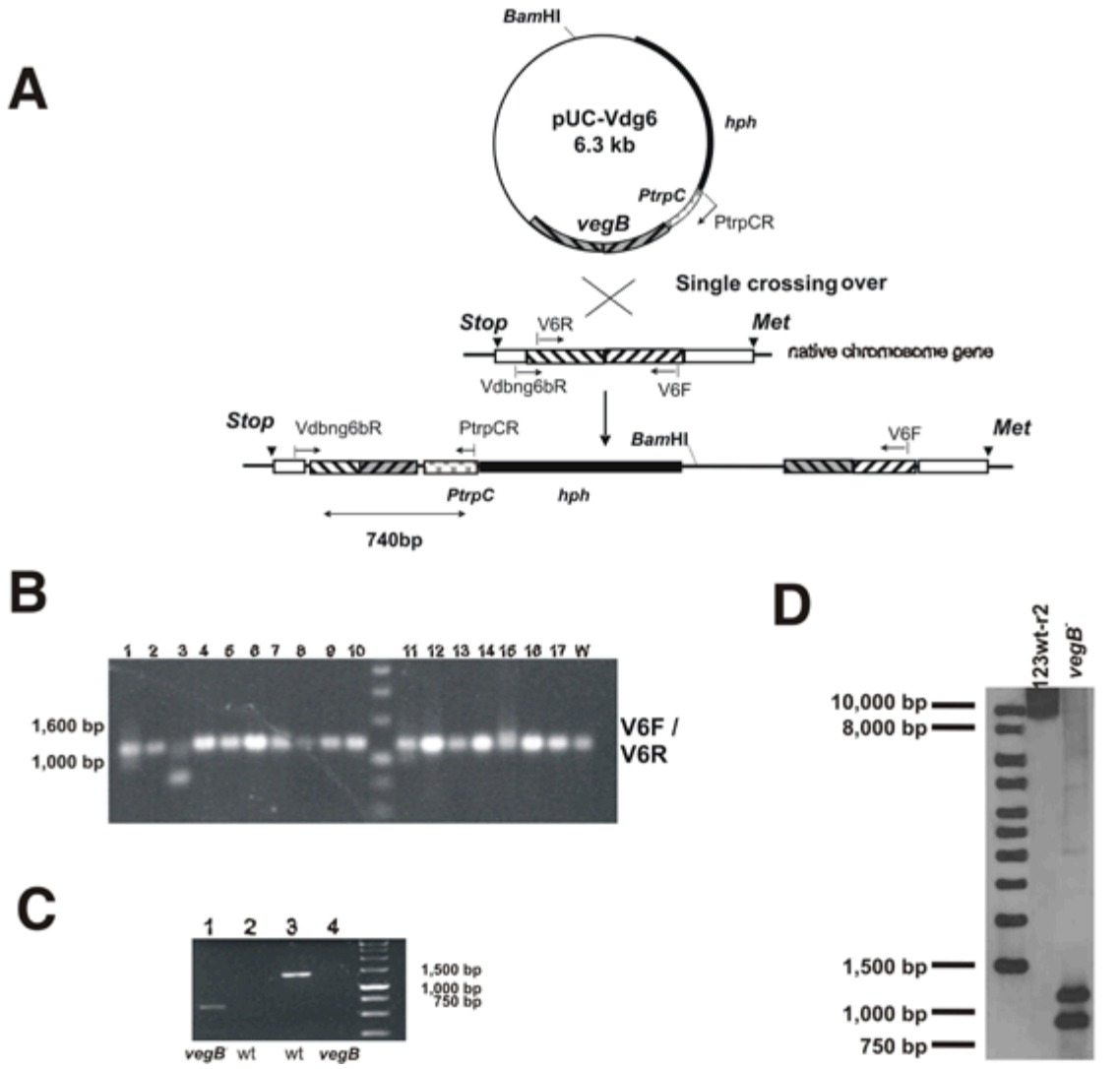

Figure 3. A) Schematic presentation of *veg*B disruption using vector pUC-Vdg6 (6.3 kb). The vector is incorporated by a single crossing over event disrupting the native chromosomal *veg*B gene. Positions of primers used for screening of transformants and verification of disruption of *veg*B are indicated (arrows). B) Selection procedure of *veg*B disruptans by PCR with primers V6F/V6R amplifying the 1.2 kb *veg*B fragment. C) Verification of *veg*B disruption by applying PCR with primers PtrpCR and Vdbng6bR on mutant *veg*B$^-$ (lane 1) and the wild strain (lane 2) and PCR with primers V6F and Vdbng6bR on the wild type (lane 3) and on transformant *veg*B (lane 4). D) Confirmation of gene disruption by Southern blot analysis. Genomic DNA from wild type (123wt-r2) and mutant *veg*B$^-$ was digested with *Bam*HI. The 1200 bp fragment of *veg*B was used as a probe. Molecular weight markers are indicated on the left side.

as a fragment of about 1.2 kb was amplified in the wild type strain and ectopic transformants (Figure 3B). In the second screening, a primer based on the region of the *Pst*I site of the *trpC*-promoter and the hygromycin phosphotransferase gene (*hgh*) (PtrpCR), and a second primer placed about 200 bp outside the 1.2 kb fragment (primer Vdbng6bR), were used. One of the transformants (Figure 3C, lane 1) gave an amplicon of about 740 bp, suggesting that a homologous integration event by single crossing over had taken place at the correct locus. As expected, no fragment was amplified in the wild type strain

(Figure 3C, lane 2). To further confirm this, transformants were screened by PCR with primers V6F and Vdbng6bR. The wild-type *V. dahliae* strain gave the expected product of about 1,400 bp, while the transformant gave no amplicon (Figure 3 C, lanes 3 and 4, respectively). Disruption of the native *veg*B gene was further confirmed by Southern Blot analysis (Figure 3D). Using the 1.2 Kb *veg*B fragment as a probe, the native *veg*B gene was detected in the wild type on a band of about 15 kb, which was further verified by *in silico* analysis of the *Verticillium* genome database. In the *veg*B$^-$ mutant strain, two smaller bands were detected indicating insertion of the entire plasmid within the 1200 bp of *veg*B, through a single homologous recombination event.

To investigate the role of *veg*B in physiology and development of *V. dahliae*, the wild-type and the mutant *veg*B$^-$ strains were characterized for conidia production and their ability to degrade different carbon sources. The mutant *veg*B$^-$ strain produced significantly fewer conidia (about a 50% reduction) than the wild type strain (Supplementary Figure 3), but showed no significant differences in radial growth rates on minimal medium containing glucose, xylose, sucrose, pectin or sucrose, used as the sole carbon sources. In cellulose, however, there was a reduction in the radial growth of *veg*B$^-$ mutant compared with the wild type strain, suggesting that the *veg*B$^-$ mutant strain had reduced ability to utilize complex nutrients (Supplementary Figure 4).

Virulence assays on tomato and eggplant

Infection assays on tomato and eggplant were performed to determine the effect of *veg*B$^-$ on pathogenicity of *V. dahliae* in comparison with the wild type strain. Disease severity caused by the *V. dahliae* strains following root dipping and root drenching was recorded over time during the course of the experiment and was expressed as relative AUDPC (Figure 4). There was a slight reduction of pathogenicity of *veg*B$^-$, on both hosts. In the tomato assay, the reduction in virulence (about 27% reduction in amount of disease) was not statistically significant. Since race 2 is known to cause milder symptoms (De Jonge *et al.*, 2012), tomato plants were infected only through root dipping. Infection through root drenching would further increase variation in symptom appearance. On eggplant, which is a more sensitive host than tomato, plants were infected both through root drenching and root dipping. When eggplants were infected by root dipping, the mutant *veg*B$^-$ caused about 20% less disease, in comparison with the wild type. When plants were infected by root drenching, however, the reduction in virulence (43% reduction in amount of disease) was not statistically significant due to fluctuation in symptom development (Figure 4). During root dipping, conidia are brought into immediate contact with host root surfaces, which facilitates uniformity in symptom development. Root drenching, on the other hand, resembles natural infection of the host. Nevertheless, conidia need to recognize and be

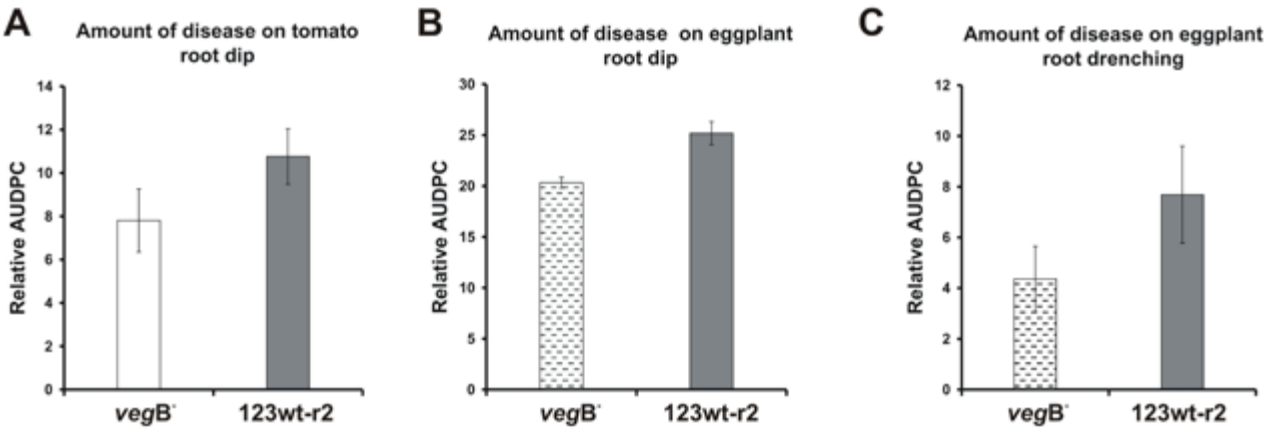

Figure 4. Disease severity caused by the *Verticillium dahliae* disruption mutant *veg*B$^-$ and wild type 123wt-r2 on tomato plants (A) and on eggplants infected by root dipping (B) and root drenching (C). Disease severity was expressed as relative AUDPC. Error bars indicate standard errors calculated for 12 replicates in graph A, six replicates in graph B and 20 replicates in graph C.

attracted by plant root exudates, before establishing infections, which increases variation in symptom appearance between the different inoculation methods.

Discussion

Fungi are known as the main producers of β-glucan-degrading enzymes, which are hydrolytic enzymes classified on the basis of the β-glucosidic linkage types they cleave and the modes of substrate hydrolysis. The most abundant of these, cellulases, cause endo-hydrolysis of 1,4-linkages in cellulose and in β-D-glucans containing 1,3- and 1,4-linkages. However, other hydrolytic enzymes such as endo- or exo-β-1,3-glucanases and endo-β-1,6-glucanases, which respectively cleave 1,3- and 1,6-linkages in β-1,3-D and β-1,6-glucans, and release α-glucose, are also hydrolysis facets of fungi (Pitson *et al.*, 1993). In spite of their principal specificity for β-1,6-glucosidic linkages, β-1,6-glucanases isolated from several fungi were found to also be capable of efficiently hydrolyzing β-1,3-linkages (Shibata and Fukimbara, 1972; 1973; Shibata, 1974; Yamamoto *et al.*, 1974; Pitson *et al.*, 1996; Moy *et al.*, 2002). Thus, although plants lack β-1,6-glucans, the implication of β-1,6-endoglucanases in the virulence of fungi, like the mycoparasitic fungus *Verticillium fungicola* against *Agaricus bisporus* or *Trichoderma harzianum* against many fungi, and their capability to hydrolyze β-1,3-linkages that are abundant in plants (Amey *et al.*, 2003), has triggered our study to investigate the role of such an enzyme in the plant pathogenic fungus *V. dahliae*.

The approx. 1,800 bp fragment, which contained most of the *V. dahliae* β-1,6-endoglucanase gene (*veg*B) but lacking approx. 120 bp from its 5'-end, was obtained by genome walking. When the sequence of this gene was analyzed, it was found as part of the PAN1 gene (VDAG_05935T0) of the Verticillium genome database (Broad Institute). Detailed *in silico* analysis showed that while the C-terminus of the annotated PAN1 protein showed high similarity with other PAN1 orthologs, the N-terminus exhibited similarity with several fungal β-1,6- endoglucanase genes. Sequencing of both the genomic and expressed (cDNA) putative *veg*B gene from our *V. dahliae* strain showed several differences in intron sizes/positions compared with those proposed for the PAN1 sequence. These were all resolved by RT-PCR experiments that fully verified the positions and lengths of introns and the length of β-1,6-endoglucanase gene (Figure 1). The most likely explanation for this annotation discrepancy is the base pair threshold used in the computational analysis (Schnoes *et al*., 2009). Protein blast searches of the deduced *veg*B amino acid sequence indicated high levels of similarity/identity with other β-1,6-endoglucanase fungal genes, found mainly in plant pathogenic fungi.

The construction of a phylogenetic tree composed of all fungal β-1,6-endoglucanase protein sequences (Figure 2) demonstrated clear differentiation between enzymes derived from pathogenic and mycoparasitic fungi, fully supported by bootstrap values. This is important, because it allows an easy grouping of fungal β-1,6-endoglucanases according to their hosts, Further, it suggests that the specificity of these enzymes is an evolutionary adaptation that has taken place at an early stage of diversification of these fungi. Chitin, which consists of N-acetylglucosamine units linked by β-1,4-glycosidic bonds is the main component of fungal cell walls. It therefore becomes evident that the mycoparasitic β-1,6-endoglucanases which have been implicated in virulence against basidiomycetes (e.g. *Verticillium fungicola* on *Agaricus bisporus* or *Trichoderma harzianum* on many fungi) have a dual action, primarily on β-1,6- and secondarily on β-1,4-glycosidic bonds (Moy *et al.*, 2002; Amey *et al.*, 2003). Moreover, the synergistic action of β-1,6-glucanases with other hydrolytic enzymes demonstrated previously *T. harzianum* (de la Cruz *et al.*, 1995a), and the contribution of these enzymes to *T. harzianum* and *T. virens* nutrition by degrading β-glucan polymers in their environments (Lora *et al.*, 1995; Kim *et al.*, 2002), may be another possible role for these enzymes in plant pathogenic fungi.

In previous studies, hydrolytic enzymes produced by *V. dahliae*, particularly pectinases and cellulases, have been considered to be important for expression of disease symptoms and pathogenesis (Pegg, 1981; Puhalla and Bell, 1981; Pegg and Brady, 2002). Similar to other plant pathogenic fungi, in *Verticillium* several cell wall degrading enzymes act synergistically during host colonization (Durrands and Cooper, 1988b; Fradin and Thomma, 2006). It has been suggested that during infection, when easily fermented carbon sources become limited, the sucrose non-fermenting 1 gene (*SNF1*) de-represses genes, including CWDEs, to enable the fungus to metabolize alternative carbon sources, such as sucrose, galactose, pectin, xylose and initiate infection

(Tonukari *et al.*, 2000). In *V. dahliae*, cell wall degrading enzymes were shown to be under catabolite repression controlled by *VdSNF1* (Tzima *et al.*, 2011). Similar to *V. dahliae* cell wall degrading enzymes, expression of *veg*B was also found to be reduced in *VdSNF* mutants, compared to the wild type strain, indicating that *veg*B might be controlled by an analogous mechanism (Tzima *et al.*, unpublished results).

Agrobacterium tumefaciens mediated transformation (ATMT) has become instrumental for insertional mutagenesis of *V. dahliae* strains, gene inactivation and production of mutant strains, to study the possible functions of interrupted genes (Maruthachalam *et al.*, 2011; Tzima *et al.*, 2011; De Jonge *et al.*, 2012). Among the genes identified through ATMT insertional mutagenesis, endoglucanase 1 (EG-1) was important for the depolymerisation of plant cellulose (Gough *et al.*, 1988; Roberts *et al.*, 1988). This indicates that EG-1 in *V. dahliae* plays an important role in plant penetration or early stages of colonization. Similarly, Maruthachalam *et al.* (2011) have shown that another endoglucanase gene (VDAG 07825.1) was important for virulence in *V. dahliae*. Pathogenicity tests in the present study showed that the *veg*B$^-$ mutant strain, constructed by insertional mutagenesis on a tomato race-2 strain of *V. dahliae*, was able to infect host plants through the roots and caused typical disease symptoms. However, only a slight reduction in the amount of disease was observed in *veg*B$^-$ when compared with the wild type, and this occurred only for eggplants. These results indicate that the involvement of *veg*B in plant pathogenesis is of minor importance, and is likely to be indirect. Several hypotheses can be made for this indirect involvement, like *veg*B's possible contribution to the degradation of other plant cell wall components, such as the β-1,3-glucan plant wall callose that is produced upon pathogen invasion (Pitson *et al.*, 1993; Agrios, 2005; Aro *et al.*, 2005; Martin *et al.*, 2007). The reduced growth ability of the *veg*B$^-$ mutant on solid media containing cellulose as the sole carbon source in comparison with the wild type strain may support of this suggestion. Similarly, the small reduction in pathogenicity observed in *veg*B$^-$ mutants may be due to the absence of the β-1,6-glucanase necessary for the modification and plasticity of fungal cell walls during parasitic growth. Since β-1,6-glucanases are implicated in branching and sporulation (Wessels, 1994; 1999; Sietsma *et al.*, 1995; Moy *et al.*, 2002), the lack of this enzyme in *veg*B$^-$ mutants may also negatively affect conidium production, as shown in the present study. Furthermore, non-cellulolytic glucanases have been postulated to degrade glucan sheaths that hold hydrolytic enzymes within fungal hyphae (Gil-ad *et al.*, 2001). Thus, another explanation could be that the absence of β-1,6-glucanase in *V. dahliae*, and this might reduce the infection of the mutant by inhibiting β-glucan degradation and release of hydrolytic enzymes. Finally, *veg*B might be implicated in attachment of *V. dahliae* to plant cell walls, as has been reported in other cases of fungal/host interactions (Ruel and Joseleau, 1991). All of these hypotheses equire additional study for verification. However, what remains clear from the present study is that the targeted disruption of β-1,6-endoglucanase showed a contributory role in pathogenicity of *V. dahliae*. As plants lack the *β*-1,6-glucan substrate, *β*-1,6-glucanase genes would be good candidates for engineering plants in order to confer resistance to fungi.

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